Regulation of the mitochondrial permeability transition in kidney proximal tubules and its alteration during hypoxia-reoxygenation

Thorsten Feldkamp,1,2* Jeong Soon Park,1* Ratna Pasupulati,1* Daniela Amora,1 Nancy F. Roesser,1 M. A. Venkatachalam,3 and Joel M. Weinberg1

1Division of Nephrology, Department of Internal Medicine, Veterans Affairs Ann Arbor Healthcare System and University of Michigan, Ann Arbor, Michigan; 2Department of Nephrology, University Duisburg-Essen, Essen, Germany; and 3Department of Pathology, University of Texas Health Science Center, San Antonio, Texas

Submitted 24 July 2009; accepted in final form 3 September 2009

Feldkamp T, Park JS, Pasupulati R, Amora D, Roesser NF, Venkatachalam MA, Weinberg JM. Regulation of the mitochondrial permeability transition in kidney proximal tubules and its alteration during hypoxia-reoxygenation. Am J Physiol Renal Physiol 297:F1632–F1646, 2009. First published September 9, 2009; doi:10.1152/ajprenal.00422.2009.—Development of the mitochondrial permeability transition (MPT) can importantly contribute to lethal cell injury from both necrosis and apoptosis, but its role to mitochondria is incompletely defined. In particular, the MPT can be strongly sensitizing factor for the MPT that overcomes the antagonistic effect of endogenous metabolites and cyclophilin D inhibition, particularly in the presence of complex I-dependent substrates, which predominate in vivo.

A VARIETY OF INSULTS can produce a sustained increase of the permeability of the inner mitochondrial membrane that prevents it from maintaining the transmembrane ion gradients necessary for energy conservation. The process, now most commonly called the mitochondrial permeability transition (MPT), is characterized by loss of inorganic and small organic matrix solutes and mitochondrial swelling. Considerable evidence supports the concept that the MPT results from reversible opening of an ~3-nm-diameter pore with a size exclusion limit of ~1,500 Da that is frequently termed the permeability transition pore (PTP) (5, 7, 32, 34, 39, 40, 48, 69, 75, 82). There also is evidence for more selective substates of the MPT (10, 13, 40, 46, 51) and for its transient opening under physiological conditions (42, 70). In isolated mitochondria, the development of the PTP is regulated by matrix divalent cations, mitochondrial membrane potential ($\Delta \Psi_m$), matrix pH, ADP, and NAD(P)H redox state (5, 32, 34, 39, 48, 82).

Opening of the pore can be prevented and reversed by cyclosporine A (CsA), via inhibition of CsA of the binding of mitochondrial matrix cyclophilin D with inner mitochondrial membrane components of the pore that remain incompletely defined (7, 9, 15, 26, 34, 45, 69). “Inducing factors” that promote development of the MPT include Ca$^{2+}$, phosphate, decreased $\Delta \Psi_m$, oxidizing conditions, a variety of lipid metabolites including nonesterified fatty acids (NEFA) and their CoA and carnitine esters, and the ADP/ATP carrier (AAC) inhibitor atracyloside. Conditions or agents other than CsA that inhibit the MPT include decreased pH, ADP, Mg$^{2+}$, antioxidants, carnitine, local anesthetics, several calmodulin and Ca$^{2+}$ channel antagonists, and the AAC inhibitor bongkrekic acid (5, 7, 32, 34, 39, 40, 48, 69, 75, 82).

Many of the inducing factors and inhibitory conditions that are normally present in cells (Ca$^{2+}$, phosphate, reactive oxygen species, lipid metabolites, pH, Mg$^{2+}$, and ADP) undergo major changes during ischemia and postischemic reoxygenation that could variably serve to promote or retard development of the MPT. Although the MPT predictably occurs at a late stage of lethal necrotic cell death when extracellular Ca$^{2+}$ floods the cytosol and cytosolic metabolites that limit the MPT are lost (71), there is now substantial evidence from studies of mice with targeted deletion of cyclophilin D for prelethal involvement of the MPT in mitochondrial damage in vivo during heart and brain ischemia (2, 3, 53, 64), and beneficial effects of CsA for cardiac reperfusion injury in humans have been reported (61). Recent studies also have suggested involvement of the MPT in the pathogenesis of ischemia-reperfusion injury to the kidney (16, 62). The MPT can contribute to the development of apoptosis but is not required for it (2, 53). Despite this recent progress, regulation of the MPT within the intact cell remains incompletely defined. In particular, the MPT can be strongly suppressed by several endogenous metabolites, including ADP and Mg$^{2+}$ (13, 14, 32, 32, 36, 39, 40, 54, 57, 75). These metabolites are abundant in cells and should oppose sustained opening of the PTP before catastrophic plasma membrane damage. There is little information on expression of the MPT and its regulation by endogenous metabolites in kidney proximal tubules, where mitochondria are especially critical for the cell energy supply given that glycolysis is limited or absent, depending on the segment, which will increase their susceptibility to ischemia-induced ATP depletion (76). Recent studies...
using freshly isolated proximal tubules and approaches optimized to dynamically follow changes of mitochondrial energetics in both intact and acutely permeabilized tubules have defined persistent partial mitochondrial deenergization mediated in a nondisruptive fashion by accumulated NEFA as the mechanism for the energetic failure that is a major early determinant of cellular recovery and survival after hypoxic insults relevant to understanding ischemia in vivo (20, 22, 24). NEFA and deenergization are promoters of PTP opening in isolated mitochondria (6, 32, 18a, 36, 39–41). In the present studies we describe and validate versatile approaches to follow the MPT in fully differentiated proximal tubules and use them to assess its regulation by ADP, Mg2+, and NEFA, and we assess the changes in sensitivity to expression of the MPT that occur as a result of H/R.

**MATERIALS AND METHODS**

**Materials.** Female New Zealand White rabbits (1.5–2.0 kg) were obtained from Harlan, (Indianapolis, IN). Type I collagenase was obtained from Worthington Biochemical (Lakewood, NJ). Percoll was purchased from Amersham Biosciences (Piscataway, NJ). Isolation of tubules. Proximal tubules were prepared from kidney cortices by collagenase digestion and centrifugation on self-forming Percoll gradients as described previously (20–23, 77–79).

**Isolation of tubules.** Proximal tubules were prepared from kidney cortices by collagenase digestion and centrifugation on self-forming Percoll gradients as described previously (20–23, 77–79). Incubation conditions were similar to those described previously (20–23, 77–79). Tubules were suspended at 3.0–5.0 mg tubule protein/ml in a 95% air-5% CO2-gassed medium containing 110 mM NaCl, 2.6 mM KCl, 25 mM NaHCO3, 2.4 mM KH2PO4, 1.25 mM CaCl2, 1.2 mM MgCl2, 1.2 mM MgSO4, 5 mM glucose, 4 mM sodium lactate, 0.3 mM alanine, 5 mM sodium butyrate, 2 mM glycine, and 1.0 mg/ml bovine gelatin (75 bloom) (solution A). For studies limited to normoxic conditions, tubules were preincubated for 15 min at 37°C and then resuspended in fresh solution A containing 2 mM heptanoic acid instead of sodium butyrate and 250 μM AMP (77, 79) for an additional 60 min before sampling for experiments. For studies comparing normoxia with H/R, at the end of the 15 min, preincubation tubules were resuspended in fresh solution A and reoxygenated with either 95% air-5% CO2 (normoxic controls) or 95% N2-5% CO2 (hypoxia). During hypoxia, solution A was kept at pH 6.9 to simulate tissue acidosis during ischemia in vivo, and the usual substrates, glucose, lactate, alanine, and butyrate, were omitted. These incubation conditions result in near anoxic conditions. It is not possible to confirm the presence of complete anoxia in the flask, so we use the term hypoxia to describe the oxygen deprivation. After 67.5 min, samples were removed for analysis. The remaining tubules were pelleted and then resuspended in fresh 95% air-5% CO2-gassed, pH 7.4 solution A with experimental agents as needed. Sodium butyrate in solution A was replaced with 2 mM heptanoic acid during reoxygenation, and to assure availability of purine precursors for ATP resynthesis, 250 μM AMP was included. After 60 min of reoxygenation, tubules were sampled for MPT studies.

**Assessment of the MPT by following changes of ΔΨm with safranin O.** At the end of either normoxic control incubation or H/R, samples of tubule suspension were immediately diluted into an ice-cold holding solution containing 110 mM NaCl, 25 mM Na-HEPES, pH 7.2, 1.25 mM CaCl2, 1.0 mM MgCl2, 1.0 mM KH2PO4, 3.5 mM KCl, 5.0 mM glycine, 5% polyethylene glycol (average mol wt 8,000), and 2.0 mg/ml bovine gelatin, washed once in the same solution, and then held in it at 4°C until use. For the safranin O uptake measurements (20–23), the tubules in the holding solution were pelleted and resuspended at a final concentration of 0.10–0.15 mg/ml in an intracellular-buffer-type solution containing 120 mM KCl, 1 mM KH2PO4, 5 μM safranin O, 100–150 μg digitonin/mg protein, and 10 mM K-HEPES, pH 7.2 at 37°C (solution B) supplemented with 4 mM concentrations of potassium salts of the substrates that are described with the data and 40 μM EGTA to provide a small excess above the Ca2+ carried over from the holding medium (20–30 μM). Fluorescence was measured once every second at 485-nm excitation/586-nm emission using Photon Technology International Deltascan and Alphascan fluorometers (Lawrenceville, NJ) equipped with temperature-controlled (37°C), magnetically stirred cuvette holders to follow safranin O uptake by the mitochondria. When uptake was maximal, the MPT was induced by addition of CaCl2 using a constant concentration for all experiments each day based on a preliminary titration to establish the minimum amount needed to initiate rapid deenergization and light scattering with a single addition (see RESULTS). The concentration of Ca2+ required ranged from 35 to 45 μM. Additional conditions and maneuvers for specific experiments are as described in RESULTS. Fluorescence values are given relative to the value at the start of uptake when safranin O was entirely in the medium. Uptake of safranin O into the matrix of energized mitochondria results in quenching of its fluorescence, so the measured signal decreases. To make it easier to follow the tracings with respect to high and low ΔΨm graphs are inverted.

**Measurement of medium Ca2+ with calcium orange-5N or calcium green-5N.** For these studies, tubules were washed quickly with ice-cold solution B containing 40 μM EGTA just before being placed into the cuvette for the experiment to prevent carryover of Ca2+ from the holding medium and allow greater consistency of Ca2+ for induction of the MPT. Otherwise, experiments were run exactly as for measurements of ΔΨm with safranin O, except that safranin O was replaced with one of two low-affinity Ca2+ indicators. Calcium orange-5N (0.75 μM, 549-nm excitation/582-nm emission) was used for initial studies but is no longer commercially available, so calcium green-5N (0.15 μM, 506-nm excitation/536-nm emission) was used for subsequent work with equivalent results. To allow calculation of medium Ca2+ from the measured fluorescence values, each experiment was ended by the addition of 10 μM of the uncoupler carboxylcyanide 3-chlorophenyl-hydrazone (CCCP) followed by 400 μM EGTA, to be in excess of total Ca2+ added during the study to determine the minimum fluorescence intensity (Fmin) value, followed by 2 mM CaCl2 to determine the maximum fluorescence intensity (Fmax) value. Ca2+ values for each experimental point were then calculated from its fluorescence (Fx) as Ca2+ = Kq × (Fx – Fmin)/(Fmax – Fmin), Fx values determined in the experimental buffer (solution B) without tubules were 30 μM for calcium orange-5N and 22.5 μM for calcium green-5N. Ca2+ uptake and retention was further quantified by factoring the maximal change in Ca2+ concentration by milligram of tubule protein measured using the Lowry assay.

**Assessment of changes of matrix volume by light scattering.** Fluorescence was followed at 520-nm excitation/520-nm emission (33, 39) simultaneously with either the safranin O uptake measurements of ΔΨm or the calcium green-5N measurements of medium Ca2+.

**Ultrastructural studies.** Tubules were incubated under exactly the same conditions as for the corresponding studies of ΔΨm and light scattering, except larger volumes were used to provide enough material for processing. At desired points during the experiments, samples...
of the suspension were fixed in 2% glutaraldehyde with 50 mM lysine, 50 meq Na⁺, and 100 meq cacodylate acid, pH 7.4, and then further processed for electron microscopy as previously described (28).

ATP production by permeabilized tubules. Tubules were suspended in solution B as for safranin O uptake measurements, with further addition to solution B of 10 mM glucose, 10 U/ml hexokinase, 0.2 mM NADP, 5 U/ml glucose-6-phosphate dehydrogenase, and 30 μM diadenosine-5’-pentaphosphate to inhibit adenylate kinase (56). ATP production was followed as formation of NADPH at 360-nm excitation/450-nm emission. Safranin O fluorescence was followed simultaneously at 485-nm excitation/586-nm emission. Ouabain, used in our earlier studies of this type (22), was omitted because it was not found to affect ATP production under the high-K⁺ conditions of solution B. The ADP concentration studied in these experiments was limited to 100 μM to allow the 30 μM adenosine-5’-pentaphosphate to inhibit its conversion to ATP by adenylate kinase.

Measurement of ATP levels. Tubule samples were immediately deproteinized in trichloroacetic acid, neutralized with trietylamine: CFC 113, and stored at −20°C as previously described (20–23). Purine nucleotides and their metabolites in 20-μl aliquots of the neutralized extracts were separated and quantified using a reversed-phase, ion-pairing gradient HPLC method as previously described (20–23).

Statistics. Paired and unpaired t-tests were used as appropriate. Where experiments consisted of multiple groups, they were analyzed statistically by analysis of variance for repeated measures or independent group designs as needed. Individual group comparisons for the multigroup studies were then made using the Holm-Sidak test for multiple comparisons (SigmaStat 3; SPSS, Chicago, IL). P < 0.05 was considered to be statistically significant. Data shown are either means ± SE of no less than three to five experiments on separate tubule preparations or tracings representative of the behavior in that number of experiments.

RESULTS

Induction of the MPT by Ca²⁺ and its reversal as assessed by changes of ∆Ψₘ. Figure 1 illustrates the basic approach for inducing the MPT with Ca²⁺ in permeabilized tubules, using changes of ∆Ψₘ to follow the process. In Fig. 1A, tubules were energized with succinate until uptake of safranin O was complete, followed by a single addition of Ca²⁺ to induce deenergization, followed by dBSA to bind excess NEFA at either 1,000 s just after safranin O release was complete or 45 min later at 2,200 s. dBSA did not detectably affect energization. However, subsequent addition in both experiments of a combination of reagents known to promote the closed state of the PTP, i.e., ADP, Mg²⁺, and CsA (ADP/Mg/CsA), at concentrations where maximal effects would be expected (54), led to a rapid and complete reenergization. Final addition of the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) produced rapid deenergization again, indicating that the safranin O movements were indicative of dynamic, reversible changes of ∆Ψₘ.

If the changes of ∆Ψₘ in Fig. 1A are secondary to opening and closing of the PTP, then uptake of the Ca²⁺ added to the medium should trigger the MPT, inducing release of the Ca²⁺ and closing of the pore, whereas reenergization by ADP/Mg/CsA should lead to reaccumulation of the Ca²⁺. Figure 1B displays the results of parallel measurements of ∆Ψₘ using safranin O uptake (top tracing) and medium Ca²⁺ levels followed with calcium orange-5N (bottom tracing) to directly assess whether these events occurred. Addition of Ca²⁺ to bring medium Ca²⁺ up to 20 μM was followed by Ca²⁺ uptake, but it was incomplete and release ensued. Addition of dBSA after Ca²⁺-induced collapse of ∆Ψₘ was without effect on either medium Ca²⁺ or ∆Ψₘ during 30 min. However, subsequent addition of ADP/Mg/CsA induced rapid and complete recovery of ∆Ψₘ, which was accompanied by reuptake of Ca²⁺ from the medium that was then retained in the mitochondria. Final addition of the uncoupler FCCP collapsed ∆Ψₘ and released the Ca²⁺ again.

Mitochondrial matrix volume changes. Opening of the PTP is classically associated with mitochondrial matrix swelling, which can be followed as decreases of 90° light scattering in both isolated mitochondrial preparations (58, 68) and whole cells (33, 35, 38). Figure 2 shows simultaneous measurements of ∆Ψₘ with safranin and light scattering measured at 520-nm excitation/520-nm emission. The safranin O uptake tracing displays behavior similar to that shown in Fig. 1 with dissipa-
tion of $\Delta \Psi_m$ after addition of Ca$^{2+}$ and strong recovery with subsequent ADP/Mg/CsA. There was no further benefit from late addition of EGTA. The experiment ended with alamethicin, a pore-forming peptide (30) that rapidly collapse densities (arrows) typical of those expected after Ca$^{2+}$ uptake. C: condensed mitochondria with matrix densities (arrows) typical of those expected after Ca$^{2+}$ uptake. D: swollen mitochondria with more disruption of matrix architecture. By the experimental additions indicate alterations of mitochondrial volume is shown by the ultrastructural results from a parallel set of experiments. Before Ca$^{2+}$ addition (Fig. 2A), most mitochondria had an orthodox configuration. After Ca$^{2+}$ addition (Fig. 2B), mitochondria were swollen and had matrix densities (arrows) typical of those expected after Ca$^{2+}$ uptake. After ADP/Mg/CsA, the mitochondria were strikingly condensed (Fig. 2C). After alamethicin addition, they were swollen with more disruption of matrix architecture (Fig. 2D).

Changes of light scattering such as those shown in Fig. 2 did not occur without Ca$^{2+}$ addition (Supplemental Fig. 1A) and were not reproduced when deenergization resulted from inhibition of electron transport (Supplemental Fig. 1C), uncoupler (Supplemental Fig. 1C), or NEFA in the absence of Ca$^{2+}$ (Supplemental Fig. 1D). (Supplemental data are available online at the American Journal of Physiology-Renal Physiology website.)

Thus the results shown in Figs. 1 and 2 illustrate classic features of Ca$^{2+}$-induced MPT in permeabilized tubules, including deenergization, transient Ca$^{2+}$ uptake followed by release, large-amplitude mitochondrial matrix swelling characteristic of the high-permeability state of the PTP, and reversal by the pore-closing agents ADP, Mg$^{2+}$, and CsA. The results are also notable for the ability to close the PTP in this system after remarkably long durations, i.e., $>$ 30 min.

**Contribution of NEFA to the PTP.** Long-chain NEFA are considered to promote the MPT by lowering $\Delta \Psi_m$, which increases sensitivity to PTP opening (6, 58), changing surface potential (6, 11) and directly interacting with pore components (6, 18a). dBSA effectively reverses NEFA-induced deenergization in both intact and permeabilized proximal tubules (20, 22, 24) but had no effect after Ca$^{2+}$ in studies shown in Fig. 1, indicating that the decreased $\Delta \Psi_m$ in that setting was being driven by more than simple accumulation of NEFA and that removing NEFA alone was insufficient to close the PTP. Figure 3 provides additional information about the contribution of NEFA. Including dBSA in the medium to bind NEFA before the addition of Ca$^{2+}$ to induce the MPT slightly slowed deenergization and the increase of matrix volume but did not prevent them or change their magnitudes (Fig. 3, A and B). Once the PTP was opened by Ca$^{2+}$, omitting the dBSA before ADP/Mg/CsA resulted in incomplete recovery of $\Delta \Psi_m$ (Fig. 3, C and D, tracing 4), but subsequent addition of BSA then completely restored energization. An alternative explanation for the incomplete recovery of $\Delta \Psi_m$ in the experiment represented by tracing 4 is that the high-permeability state of the PTP had fully closed but, in the absence of dBSA, NEFA generated during the MPT limited maximal $\Delta \Psi_m$ recovery, either by their uncoupling effects (20, 22) or by incompletely reverting the PTP to a low-permeability state (10, 13, 40, 46, 51). However, the light scattering studies in Fig. 3D show that recovery from matrix swelling completely paralleled increases in $\Delta \Psi_m$ (note the benefit of late dBSA for both $\Delta \Psi_m$ and light scattering in tracing 4), supporting the conclusion that facilitation of closure of the high-permeability state of the PTP accounts for the effects of the dBSA. In addition to

![Fig. 2. Simultaneous measurements of $\Delta \Psi_m$ with safranin O and light scattering measured at 520-nm excitation/520-nm emission. The top tracing of each set was done with tubules present (+tubules); bottom tracings were done without tubules (−tubules) and document the absence of artifacts from reagent addition. The experimental additions were the same as in Fig. 1 except for the use of alamethicin (10 µg/ml) instead of FCCP at the end. Samples for ultrastructure were collected in parallel experiments sampled at the indicated points. A: electron micrograph showing mitochondria with orthodox configuration before Ca$^{2+}$ addition. B: swollen mitochondria with matrix densities (arrows) typical of those expected after Ca$^{2+}$ uptake. C: condensed mitochondria after ADP/Mg/CsA addition. D: swollen mitochondria with more disruption of matrix architecture.](image-url)
binding NEFA, it is possible that dBSA could have opposed the MPT by lowering medium Ca$^{2+}$.

However, direct measurements of dBSA-induced changes of medium Ca$^{2+}$ done as part of studies in which it was monitored as a main end point (see below) indicated that dBSA as used did not lower Ca$^{2+}$ concentration or limit its mitochondrial uptake.

**Interactions of ADP, Mg$^{2+}$, and CsA to modify the MPT.** ADP, Mg$^{2+}$, and CsA were used in combination for the studies represented in Figs. 1–3 based on extensive prior observations that they act synergistically to modify the MPT (13, 14, 32, 40, 54, 55, 57, 75). Moreover, these interactions show tissue-specific differences (54, 57). Figure 4 further addresses the effect of ADP and its interactions with Mg$^{2+}$ and CsA in the kidney tubules. The MPT was induced by Ca$^{2+}$ addition as shown in Figs. 1–3, followed by dBSA, and then the test additions indicated were made at 1,500 s. The light scattering tracings in B and D are offset from each other to make it easier to see the changes in each.

*Fig. 3. Effect of dBSA on development and reversal of the mitochondrial permeability transition (MPT). Conditions for the studies and additions were identical to those of Figs. 1 and 2 except as noted. $\Delta \Psi_m$ and light scattering were measured during the induction of MPT and its reversal by ADP/Mg/CsA in studies testing the effects of including dBSA before Ca$^{2+}$ addition (A and B) and omitting dBSA before addition of ADP/Mg/CsA (C and D). A and B: 0.5 mg/ml dBSA was included in the medium in the tracing 2 experiment from time 0. C and D: the tracing 5 experiment used sham Ca$^{2+}$ followed by dBSA, the tracing 3 experiment used the standard protocol of Ca$^{2+}$ addition followed by ADP/Mg/CsA, and the tracing 4 experiment had dBSA added after ADP/Mg/CsA at 1,500 s. The light scattering tracings in B and D are offset from each other to make it easier to see the changes in each.*
which was studied under each condition, is circled to facilitate comparison. When ADP was added alone at either 100 or 200 μM (Fig. 4A), reversal of the MPT indicated by the magnitude of reenergization was weak. Addition of 1 mM Mg\(^{2+}\) alone (Fig. 4B, “No ADP” tracing) had no effect, but it strongly enhanced the effect of ADP so that complete reenergization was seen at 100 μM ADP. Addition of 1 μM CsA alone (Fig. 4C, “No ADP” tracing) or with 50 μM ADP was without effect, but it strongly synergized with 100 and 200 μM ADP. Addition of Mg\(^{2+}\) and CsA (Mg + CsA) without added ADP (Fig. 4D, “NO ADP” tracing) had a weak effect that was strongly potentiated by as little as 1 μM ADP and gave a near-maximal effect with 10 μM ADP. The sensitivity of the MPT in the tubules to ADP concentrations in the 1–10 μM range in the presence of Mg + CsA was similarly evident in studies where ADP was generated by addition of ATP and its steady-state concentration was directly measured (Supplemental Fig. 2).

Added ADP can be converted to ATP either by adenylate kinase or, after closing of the PTP, by the mitochondrial F\(_{1}\)F\(_{0}\)-ATPase (Supplemental Fig. 3). Both processes were observed under the conditions studied (Supplemental Fig. 4). However, conversion to ATP was not required for the benefit of ADP. Oligomycin, which inhibits the F\(_{1}\)F\(_{0}\)-ATPase (17) (Supplemental Figs. 3 and 4) did not block the effect of ADP when used alone or in combination with the adenylate kinase inhibitor adenosine-5’-pentaphosphate (56) (Fig. 5). Carboxyatractyslide, which blocks movement of ADP on the AAC (44) (Supplemental Figs. 3 and 4) and can independently promote the MPT by its direct interaction with the carrier (32, 39, 47, 54, 55) slowed the effect of ADP to close the PTP but did not prevent it (Fig. 5).

Although the studies represented in Fig. 5 show that conversion of ADP to ATP was not necessary for its effect on the PTP, closing of the PTP with ADP/Mg/CsA and the concomitant restoration of mitochondrial proton motive force, as indicated by the strong recovery of ΔΨ\(_{m}\), should theoretically allow resumption of ATP production from the added ADP. Supplemental Fig. 5 shows that this occurred and documents retention after PTP closing of functional integrity of other inner membrane components required for oxidative phosphorylation.

**Increased susceptibility to the MPT during energization supported by complex I-dependent substrates.** The experiments represented in Figs. 1–5 used succinate as the substrate. Figure 6 illustrates expression and reversal of the MPT using the complex I-dependent substrates α-ketoglutarate + malate or glutamate + malate compared with succinate. Other than the complex I-dependent substrates, conditions were the same as for the studies in Fig. 1–3, except for the use of 100 μM rather than 500 μM ADP. With succinate as the substrate, ADP/Mg/CsA quickly restored ΔΨ\(_{m}\) when added after opening of the PTP (Fig. 6A), but there was little benefit of ADP/Mg/CsA with either α-ketoglutarate + malate or glutamate + malate as the substrate until succinate was added subsequently. Light scattering measurements (not shown) such as those in Figs. 2 and 3 indicated that the increases of ΔΨ\(_{m}\) were due to PTP closing. When ADP/Mg/CsA was present before addition of Ca\(^{2+}\) (Fig. 6B), pore opening and deenergization were substantially more delayed when succinate was substrate than with α-ketoglutarate + malate or glutamate + malate. Addition of dBSA after pore opening in the experiments shown in Fig. 6B was of only minimal benefit with α-ketoglutarate + malate and glutamate + malate, but subsequent addition of succinate fully reenergized. The effects of succinate were detectable at 0.5 mM and were maximal at 3–4 mM (data not shown). In the studies represented in Fig. 6, C and D, all test additions were present before Ca\(^{2+}\). With no test additions, typical deenergization occurred when pore opening was induced by Ca\(^{2+}\). With ADP/Mg/CsA, deenergization after Ca\(^{2+}\) was substantially delayed in the presence of succinate compared with glutamate + malate as in the similar study in Fig. 6B. dBSA alone minimally delayed deenergization by Ca\(^{2+}\), but the combination of dBSA with ADP/Mg/CsA prevented deenergization with both types of substrates. Light scattering studies (not shown) confirmed that the deenergization was due to pore opening. These results suggest substantially stronger NEFA dependence of MPT development in the presence of the complex I substrates.

**Fig. 4.** Concentration dependence of effect of ADP to reverse the MPT as a function of Mg\(^{2+}\) and CsA availability. MPT induction by Ca\(^{2+}\) addition at 600 s was as described in Figs. 1–3, followed by dBSA at 750 s (not marked) and then the test additions at 850 s. Numbers next to each tracing are the tested micromolar concentrations of ADP. Tracings are truncated to start at the point of Ca\(^{2+}\) addition. A: studies with ADP alone. B: studies with ADP + 1 mM Mg\(^{2+}\). C: studies with ADP + 1 μM CsA. D: studies with ADP + 1 mM Mg\(^{2+}\) + 1 μM CsA. All tracings shown are from the same tubule preparation and are directly comparable to each other.
The measurements of $\Delta \Psi_m$ changes with safranin O and matrix volume changes by light scattering in Figs. 1–6, although highly informative, do not lend themselves to straightforward quantitation of absolute changes of the underlying process being measured. However, this can be done by following medium Ca$^{2+}$ levels as shown in Fig. 1B, because the capacity for uptake and retention of Ca$^{2+}$ is a direct function of sensitivity to the MPT. Higher Ca$^{2+}$ uptake and retention indicate decreased sensitivity to the pore opening that releases the accumulated Ca$^{2+}$ down its concentration gradient and collapses the membrane potential necessary to drive further uptake (3, 57). Measurements of Ca$^{2+}$ levels can be made simultaneously with light scattering, providing information about both parameters. This general behavior and effects of MPT modifiers and substrates on it are shown in Figs. 7 and 8.

Figure 7, top, shows tracings from representative experiments with permeabilized tubules where, after a baseline period of 500 s, Ca$^{2+}$ was added to increase the medium concentration to either 10 (left) or 40 μM (right) in the presence of either succinate or glutamate+malate as substrates either with or without ADP/Mg/CsA and using calcium green-5N fluorescence to follow Ca$^{2+}$. As in the similar study in Fig. 1B, transient uptake of Ca$^{2+}$ was followed by release. Ca$^{2+}$ uptake was less and release quicker with glutamate+malate as the substrate than with succinate in the absence of ADP/Mg/CsA. In contrast, with ADP/Mg/CsA, Ca$^{2+}$ uptake was increased and release delayed with both types of substrate. At 1,000 s, addition of an uncoupler, CCCP, released the retained Ca$^{2+}$.

Figure 7, bottom, shows the light scattering tracings that were obtained simultaneously with the Ca$^{2+}$ uptakes. In every case, the speed and extent of matrix volume increase corresponded to opening of the PTP as indicated by Ca$^{2+}$ release. It is also notable in the Fig. 7 light scattering tracings that the late addition of CCCP in the Ca$^{2+}$-loaded, ADP/Mg/CsA-protected groups induced an increase of matrix volume that is not seen in the absence of Ca$^{2+}$ loading (Supplemental Fig. 1C). This behavior is consistent with the uncoupler-induced opening of the PTP that follows Ca$^{2+}$ loading in isolated mitochondria (6, 58).

Averages of calcium retention capacity (i.e., the amount Ca$^{2+}$ uptake before spontaneous release due to induction of the MPT) for a series of studies such as these are summarized in Fig. 8A. Calcium retention capacity with glutamate+malate was significantly lower than with succinate both with and without ADP/Mg/CsA. The data also show that use of the lower concentration of Ca$^{2+}$ (10 μM) underestimated the maximal uptake possible and obscured the difference between glutamate+malate and succinate in the presence of ADP/Mg/CsA.

Figure 8B summarizes the results of studies testing modification of susceptibility to the MPT by NEFA binding with dBSA as a function of substrate conditions using the approach illustrated in Figs. 7 and 8A. Ca$^{2+}$ retention capacity after increasing medium Ca$^{2+}$ to 40 μM was measured with no further additions to the indicated substrates or with dBSA, ADP/Mg/CsA, or dBSA+ADP/Mg/CsA. Substrate conditions tested were succinate, glutamate+malate, and glutamate+malate+NAD. NAD helps maintain matrix NAD, which can be lost as the PTP opens (1, 14, 18, 54), thereby lowering the efficacy of complex I-dependent substrates. Under all substrate conditions, resistance to development of the MPT indicated by magnitude of Ca$^{2+}$ reten-
tion was significantly increased in the dBSA groups relative to the groups without it except for glutamate/malate without ADP/Mg/CsA, and groups with ADP/Mg/CsA were significantly increased relative to groups without them. Glutamate/malate-supported uptake was significantly less than succinate-supported uptake in all corresponding groups, although the difference was less than that in the Fig. 8A studies. NAD significantly increased glutamate/malate-supported uptake only in the absence of ADP/Mg/CsA, but the effect was small in magnitude.

These data confirm the large difference in resistance to the MPT with succinate as substrate compared with glutamate/malate as substrate and the contribution of NEFA to MPT development shown in the Fig. 6 studies. Importantly, the data show that under conditions that allow maximal Ca\(^{2+}\) retention capacity to be quantitated, a large difference between succinate and glutamate/malate persists even during maximal suppression of the MPT with dBSA/ADP/Mg/CsA.

Effects of H/R on expression of the MPT. Figure 9 compares expression of the MPT by using changes of \(\Delta \Psi_m\) to follow the process in tubules subjected to normoxic control incubation with tubules at the end of 67.5 min of hypoxia followed by 60 min of reoxygenation. Effects of ADP/Mg/CsA to reverse and prevent the MPT in both types of tubules were tested using addition of dBSA either after induction of the MPT (Fig. 9A) or before it (Fig. 9B). Prior to Ca\(^{2+}\) addition, H/R tubules had weaker energization than normoxic control tubules, which was not modified by the ADP/Mg/CsA (Fig. 9A, compare tracings 1 and 2).

**Fig. 6.** MPT expression and reversibility with complex I-dependent substrates. Efficacy of ADP (100 \(\mu\)M), MgCl\(_2\) (1 mM), and CsA (1 \(\mu\)M) to reverse (A) or prevent (B–D) the MPT using succinate as the substrate compared with the complex I-dependent substrate combinations \(\alpha\)-ketoglutarate/malate (4 mM each) or glutamate/malate (4 mM each) was determined. A and B: each experiment started with the substrates indicated for the tracing; succinate was added at the indicated points in the complex I substrate studies. C and D: substrates for all tracings were either succinate (C) or glutamate/malate (D). \(\alpha\)KG, \(\alpha\)-ketoglutarate; GLUT, glutamate; MAL, malate.
Inclusion of dBSA restored energization of the H/R tubules to the same level as the normoxic controls (Fig. 9B), consistent with prior studies implicating NEFA as the main mediators of the impaired energization under this condition (20, 22). Ca\textsuperscript{2+}-induced deenergization indicative of the MPT was more rapid in the H/R tubules than in the normoxic controls (Fig. 9, compare tracings 1 and 2), even when dBSA was present before the Ca\textsuperscript{2+} (Fig. 9B, compare tracings 5 and 6). In the absence of dBSA, addition of ADP/Mg/CsA before Ca\textsuperscript{2+} substantially slowed Ca\textsuperscript{2+}-induced deenergization with roughly parallel behavior of normoxic controls (Fig. 9A, tracing 3) and H/R tubules (Fig. 9A, tracing 4). When dBSA was present along with ADP/Mg/CsA before Ca\textsuperscript{2+} addition, deenergization was completely blocked in both the normoxic control and H/R tubules (Fig. 9B, tracings 7 and 8). When the MPT was induced without prior dBSA or ADP/Mg/CsA (Fig. 9A, tracings 1 and 2), subsequent addition of dBSA followed by ADP/Mg/CsA fully restored energization consistent with closing of the PTP in both normoxic and H/R tubules. When the MPT was induced in the presence of ADP/Mg/CsA before Ca\textsuperscript{2+} addition, subsequent addition of excess EGTA and Ca\textsuperscript{2+} (not shown) were used to calibrate the signal. The corresponding light scattering tracings are shown at bottom, CCCP rather than FCCP was used for these studies with calcium green-5N because FCCP produces an artifactual change in the signal when used along with dBSA in this type of experiment.

The effects of H/R are further addressed in the studies presented in Fig. 10, which used Ca\textsuperscript{2+} movements to follow the MPT and its modification. In these studies, succinate was compared with glutamate and ADP/Mg/CsA strongly restored (Fig. 9B, normoxic control tracing 5 and H/R tracing 6). Together, these data indicate that, with succinate as substrate, the higher levels of NEFA present in the H/R tubules account for the major differences in MPT expression between them and the normoxic controls, and the remarkable capacity to close the PTP observed in normoxic tubules (Figs. 1 and 2) is preserved after H/R.

Fig. 7. Ca\textsuperscript{2+} movements during the MPT in the presence of succinate or complex I-dependent substrates. After 500 s of incubation with either succinate or glutamate+malate as substrate and calcium green-5N to follow Ca\textsuperscript{2+}, medium Ca\textsuperscript{2+} was increased to 10 or 40 \(\mu\text{M}\) either with or without ADP (100 \(\mu\text{M}\)), MgCl\textsubscript{2} (1 mM), and CsA (1 \(\mu\text{M}\)). At 1,000 s, the uncoupler CCCP (10 \(\mu\text{M}\)) was added to release the retained Ca\textsuperscript{2+}. Final additions of excess EGTA and Ca\textsuperscript{2+} (not shown) were used to calibrate the signal. The corresponding light scattering tracings are shown at bottom, CCCP rather than FCCP was used for these studies with calcium green-5N because FCCP produces an artifactual change in the signal when used along with dBSA in this type of experiment.
Fig. 8. A: quantitative Ca\(^{2+}\) uptake supported by succinate vs. glutamate+malate with and without ADP/Mg/CsA. Ca\(^{2+}\) retention capacity for a series of experiments such as those depicted in Fig. 7 was calculated as the amount of Ca\(^{2+}\) removed from the medium at the point of maximal Ca\(^{2+}\) uptake just before spontaneous release factored for tubule protein. If there was no Ca\(^{2+}\) release, the value just before addition of CCCP was used. The total area under each curve during the entire Ca\(^{2+}\) uptake period was also measured with similar results (not shown). Values are means ± SE for n = 5. Maximal uptake with ADP/CsA/Mg was only evident with the addition of 40 μM Ca\(^{2+}\), which provided enough Ca\(^{2+}\) to exceed the mitochondrial capacity under every condition. *P < 0.001 vs. corresponding 10 μM group. #P < 0.03 vs. same substrate without ADP/CsA/Mg. @P < 0.005 vs. corresponding succinate group. B: modification of susceptibility to the MPT by nonesterified fatty acid binding with dBSA as a function of substrate conditions. Ca\(^{2+}\) uptake was measured as described in Fig. 7 and in A with no further additions (NFA), 0.5 mg/ml dBSA, 100 μM ADP + 1 mM MgCl\(_2\) + 1 μM CsA (ADP/Mg/CsA), or dBSA + ADP/Mg/CsA in the presence of either succinate (S), glutamate+malate (G/M), or G/M + 3 mM NAD. Values are means ± SE for n = 4–7. *P < 0.001 vs. succinate. #P < 0.006 vs. corresponding group without dBSA. @P < 0.001 vs. G/M without NAD. All groups with ADP/Mg/CsA were significantly different (P < 0.001) from the corresponding groups without those agents.

Concentrations were modified to assess additional conditions. ADP was tested at two lower concentrations because its level decreases during prolonged hypoxia (see DISCUSSION). Because free Mg\(^{2+}\) increases during ATP depletion (29, 50, 52), its concentration was increased to 2 mM. CsA was not studied in this set of experiments because comparing the two concentrations of ADP fully occupied the time available to run the studies. Under normoxic conditions with succinate as substrate, equivalently strong Ca\(^{2+}\) retention capacity (i.e., suppression of the PTP) was seen with 100 μM ADP + 2 mM Mg\(^{2+}\) irrespective of the presence of dBSA to remove NEFA. Similarly strong Ca\(^{2+}\) retention was seen with 10 μM ADP + 2 mM Mg\(^{2+}\) in the presence of dBSA, but 10 μM ADP + 2 mM Mg\(^{2+}\) in the absence of dBSA was weak. Compared with the succinate-supported tubules, Ca\(^{2+}\) retention capacity of the normoxic tubules with glutamate+malate as substrate in these studies was weaker under all corresponding conditions except the groups with no further addition, despite the presence of NAD.

H/R notably altered tubule behavior and the differences between substrates. It lowered Ca\(^{2+}\) retention capacity compared with normoxic tubules with both substrates under all conditions, with particularly large effects in the ADP+Mg groups without dBSA. H/R also eliminated the differences in Ca\(^{2+}\) retention capacity between succinate and glutamate+malate in the groups without dBSA+ADP+Mg. In the H/R groups with succinate+ADP+Mg, dBSA strongly increased Ca\(^{2+}\) retention capacity, allowing it to reach nearly normoxic levels with 100 μM ADP + 2 mM Mg\(^{2+}\). This is similar to the effect of dBSA+ADP+Mg on ΔΨ\(_{m}\) after H/R in the studies shown in Fig. 9. However, with glutamate+malate, the benefit of dBSA in the presence of 100 μM ADP + 2 mM Mg\(^{2+}\) was less.

In an additional series of experiments on tubules subjected to H/R, we tested the effect of adding CsA under the 10 μM ADP + 2 mM Mg\(^{2+}\) condition. In normoxic tubules, CsA moderately increased Ca\(^{2+}\) retention capacity only with succinate in the absence of dBSA and with glutamate+malate in the presence of dBSA. After H/R, CsA increased Ca\(^{2+}\) retention capacity in the presence of dBSA when succinate was the substrate but had no effect when glutamate+malate was the substrate (Supplemental Fig. 6).

DISCUSSION

In these studies we have taken advantage of permeabilized, freshly isolated proximal tubules to investigate regulation of the MPT in these cells and the impact on the MPT of H/R. The use of whole tubules rather than isolated mitochondria avoids selection bias and alterations during isolation that can be a particularly complicating issue in the injury setting while still providing direct access to the mitochondria, which is of importance in the context of the present work insofar as it allowed precise delivery of Ca\(^{2+}\) to elicit the MPT and manipulation of the modulating factors of interest. It was possible with the approaches employed to observe typical MPT-related changes in several separate major parameters reflecting the process, including ΔΨ\(_{m}\), Ca\(^{2+}\) movements, and matrix swelling, to allow its assessment with a high degree of confidence from independent measurements and to use differences of Ca\(^{2+}\) retention capacity to quantitatively compare sensitivity to the MPT.
Additions at the indicated points were 500 experiment before Ca\textsuperscript{2+} tracings 3, 4, 7, and CsA (ADP/Mg/CsA), 0.5 mg/ml dBSA, 200 by glycine or low pH develop lethal cell injury with the isolated mitochondria (3, 57). When tubules not protecteding maneuvers are quite comparable to those reported for increases measured in the present studies with MPT-modify-
total cell protein or 150 nmol/mg mitochondrial protein control (NC; tracings 1, 3, 5, and 7) or H/R (tracings 2, 4, 6, and 8) tubules. Additions at the indicated points were 500 \mu M ADP, 1 mM Mg\textsuperscript{2+}, and 1 \mu M CsA (ADP/Mg/CsA), 0.5 mg/ml dBSA, 200 \mu M EGTA, or 5 \mu M FCCP. For tracings 5, 4, 7, and 8 ADP/Mg/CsA was present from the start of the experiment before Ca\textsuperscript{2+} addition.

between conditions. Ca\textsuperscript{2+} retention capacity in the absence of modifying agents in normoxic tubules was ~50 nmol/mg total cell protein or 150 nmol/mg mitochondrial protein when adjusted for a mitochondrial contribution of 30% of total tubule cell protein (59, 74). This value and the increases measured in the present studies with MPT-modifying maneuvers are quite comparable to those reported for isolated mitochondria (3, 57). When tubules not protected by glycine or low pH develop lethal cell injury with the MPT, total Ca\textsuperscript{2+} in the remaining more intact population that is likely just entering the process increases from a baseline of 15 nmol/mg protein to 40–60 nmol/mg total cell protein (72), i.e., similar to the Ca\textsuperscript{2+} retention capacity in the present studies. The system was also remarkably stable relative to expectations from studies with isolated mitochondria insofar as it was possible to reverse the PTP with complete recovery of mitochondrial energization after more than 30 min at 37°C and also to resume high rates of ATP production by oxidative phosphorylation after closure of the PTP.

Despite a large amount of work over the past two decades since the discovery of its CsA sensitivity suggested that the MPT was likely to be a protein-mediated process, the specific protein(s) that form the channel have not been defined. The matrix cyclophilin D targeted by CsA plays a modulatory role by increasing Ca\textsuperscript{2+} sensitivity (2, 3, 53, 64). Although the AAC has been implicated in the MPT (34), studies with AAC-deficient cells have indicated that the MPT can occur in its absence (45), so it also may play primarily a modulatory role. Very recent studies have implicated phosphate and the phosphate carrier in the PTP (4, 49).

ADP and Mg\textsuperscript{2+} suppress the MPT (14, 36, 54, 55, 57), ADP possibly by its interaction with the AAC (34, 54) or the voltage-dependent anion carrier, another putative PTP component (63), and Mg\textsuperscript{2+} by competing with Ca\textsuperscript{2+} (8). Loss of ADP and Mg\textsuperscript{2+} from the mitochondrial matrix as the PTP starts to open (32, 54, 75) will accelerate the process. Therefore, consideration of their contribution is important for assessing the likelihood of PTP opening preceding lethal plasma membrane damage in intact cells, where these metabolites are abundant, and their mitochondrial matrix concentrations will be better maintained during the MPT because they are not moving down a large concentration gradient into a very dilute medium, as is the case during in vitro studies without them. The PTP in the tubules was highly sensitive to medium ADP and Mg\textsuperscript{2+} with strong synergism between the two metabolites. Also important in this regard, inhibition of cyclophilin D with CsA did not oppose the MPT in the absence of ADP and Mg\textsuperscript{2+}, but CsA synergized strongly with ADP, even more strongly with ADP+Mg, and moderately with Mg\textsuperscript{2+} alone, as described in other systems (14, 54, 55, 57). The effects of ADP to oppose PTP opening are considered to be direct, rather then via its conversion to ATP (37, 55), and we confirmed this for the tubules by showing that blocking ATP production by inhibiting the F\textsubscript{1}F\textsubscript{0}-ATPase and adenylate kinase did not prevent PTP closure by ADP. Most data favor a matrix site of ADP action to oppose PTP opening (37, 39, 54). Even though the AAC inhibitor carboxyatractyloside promotes MPT opening (32, 47, 55) and its efficacy to block the AAC in this system was confirmed (Supplemental Fig. 4), carboxyatractyloside did not prevent the effect of ADP to close the PTP in the tubules (Fig. 5). Under this condition, ADP can reach the matrix by moving through the PTP (37).

The ADP content of normoxic control tubules factored for total cell water is 400–500 \mu M and typically decreases by ~50% during 60 min of hypoxia as a result of conversion to AMP by adenylate kinase. In the present studies, ADP measured in the normoxic time control tubules was 1.03 \pm 0.04 nmol/mg protein [420 \pm 20 \mu M accounting for proximal tubule cell water content of 2.5 \mu l/mg protein (73)] and at the end of hypoxia was 0.55 \pm 0.02 nmol/mg protein (220 \pm 6 \mu M based on normal cell water, but conceivably less with increased cell water during hypoxia). Although the mitochondrial matrix concentration of ADP is higher than that of the cytosol (65), opening of the PTP will equilibrate the two compartments, so the averaged total cell level is a reasonable approximation of the amount available to oppose the MPT. The two higher medium ADP concentrations tested during the H/R studies, 100 and 500 \mu M, therefore span a range that covers expected cytosolic levels in normoxic and hypoxic tubules, including changes from any increases of cell water during hypoxia. The
ADP was present for these experiments under energized conditions before the addition of Ca\(^{2+}/H_11001\), so matrix uptake via the ADP:ATP carrier would allow matrix ADP to also be in a range relevant to that present in situ prior to pore opening. Taken together, the present data show that in situ ADP levels in both normoxic and hypoxic tubules before their loss during lethal plasma membrane injury are sufficient to strongly suppress development of the MPT. The experiments that used 10/\(H_9262\)M ADP were done to provide an additional comparison of normoxic and H/R tubules at a more limiting ADP concentration that might better reveal an incremental benefit from CsA addition.

Recent studies have shown that accumulation of long-chain NEFA in hypoxic tubules to levels similar to those seen after ischemia in vivo play a major role in persistent, reversible mitochondrial dysfunction after H/R, before development of the MPT (20, 22, 24). Long-chain NEFA are generated by mitochondria during the MPT and promote it (6, 11, 27, 32, 43, 46, 60, 67). Arachidonic acid may be particularly potent in this regard (67, 18a), but the property is shared by saturated and unsaturated NEFA (11, 32, 46, 67). Although MPT-associated NEFA generation was originally considered to be mediated by Ca\(^{2+}/H_11001\)-dependent mitochondrial phospholipases, it was recently recognized for heart (80), liver (11, 27), and kidney (43) mitochondria that mitochondrial matrix Ca\(^{2+}/H_11001\)-independent phospholipase-2\(\gamma\) (iPLA\(_{2\gamma}\)) is largely responsible. In liver mitochondria it appears that activation of iPLA\(_{2\gamma}\) occurs as a result of deenergization by any means and is not specific for PTP opening (11, 27), but this was not the case in isolated kidney mitochondria where it required the PTP (43). NEFA are thought to promote the MPT by effects to either deenergize (6, 58) or alter surface potential on the inner mitochondrial membrane (6, 11) or via direct interaction with PTP pore components (6, 18a).

The efficacy of dBSA to prevent and reverse the MPT in the present studies indicates that NEFA contributed to the MPT in both normoxic control tubules and even more so after H/R. NEFA have been considered relatively weak inducers of the MPT (32), and, consistent with this, removing them with dBSA had only a small amount of benefit in the absence of other modifying agents (i.e., ADP, Mg\(^{2+}/H_11001\), or CsA). In the presence of the other modifiers, dBSA had much larger effects to improve both energization and Ca\(^{2+}/H_11001\) retention capacity. The benefit of dBSA for these parameters in the presence of other pore closers may be partly explained by the effect of NEFA to decrease energization and, therefore, energy-driven Ca\(^{2+}/H_11001\) uptake and retention by their uncoupling effects even if the PTP is closed. However, our studies showing that decreases of matrix volume produced by ADP/Mg/CsA are not maximal in the absence of dBSA (Fig. 3) indicate that binding of NEFA with dBSA is necessary for full closure of the PTP.

Both complex I-dependent substrates and succinate have been used in prior studies of the MPT, but usually not with systematic comparisons between them. Limited data (14, 25), including one study with isolated kidney mitochondria (14), have suggested increased sensitivity to development of the
MPT in the presence of complex I substrates, and complex I itself has been suggested to be a pore component (25). The present work documents large differences in susceptibility to the MPT as a function of substrate type with greater sensitivity in the presence of complex I substrates. A simple explanation would be loss of NAD, necessary for the initial electron transfer of reducing equivalents to the complex, as the PTP opens (1, 14, 18, 54). This would be exaggerated in vitro as the metabolite moved into a large volume of NAD-free medium, similar to the behavior of matrix ADP and Mg\(^{2+}\). However, addition of NAD had relatively little benefit, so other factors must be involved. These were not further assessed in the present studies but could include damage to complex I itself from reactive oxygen species generated during pore opening (62), which would then limit electron transport and the ability to maintain a higher ΔΨ\(_m\) to oppose the MPT.

Immediately after reoxygenation, ischemic cardiac myocytes are very susceptible to development of the MPT, which leads to necrosis that can be prevented by CsA and by the absence of cyclophilin D in experimental models (2, 16, 19, 31, 53), and there is evidence that CsA can decrease cardiac reperfusion injury clinically (61). The role of the MPT as a prelethal pathogenic process for renal proximal tubules, major sites of damage during acute kidney injury, has been less clear. In prior studies of H/R injury to isolated proximal tubules, we found that they developed a sustained impairment of mitochondrial energetics that could be modified by agents with effects on development of the MPT (77), but further work showed that this energetic deficit, although predominantly NEFA mediated (20, 22, 24), did not involve the classic high-permeability form of the MPT, because mitochondria remained partially energized and did not develop increases of matrix volume (78, 79). The present studies provide additional information about these processes and about modification by H/R of sensitivity to the MPT. After H/R, tubules had increased sensitivity to the MPT as elicited under the permeabilized conditions. With succinate as substrate and in the presence of a range of concentrations of ADP and Mg\(^{2+}\) spanning those likely to be present in the intact cell, the increased sensitivity was almost entirely NEFA dependent in that it was corrected with dBSA. With complex I-dependent substrates, there was still substantial NEFA dependence, but, in contrast to the behavior with succinate, sensitivity remained increased despite the presence of dBSA, Mg\(^{2+}\), and ADP. Depletion of NAD occurs during hypoxia (21), and additional loss of matrix NAD during opening of the PTP (1, 14, 18, 54) could further contribute to impairment of complex I-dependent respiration; however, the increased sensitivity to the MPT after H/R was seen despite supplementation with NAD. It merits emphasis that when NEFA were not removed with dBSA (the condition present during injury to the intact cell), sensitivity to the MPT was similar with both succinate and glutamate + malate as substrates and only minimally benefited from the presence of ADP + Mg\(^{2+}\) (Fig. 10). Inhibition of cyclophilin D with CsA did not overcome the effect of NEFA despite the presence of ADP + Mg\(^{2+}\).

The MPT is not necessary for cytochrome c release during apoptosis (2, 53) but can facilitate it (66). In previous studies, cytochrome c release did not contribute to the NEFA-induced energetic deficit preceding development of the MPT (22, 79). We did not directly address cytochrome c release in the present studies, which focused on events subsequent to induction of the MPT by Ca\(^{2+}\). However, the strong recovery of substrate and electron transport-dependent energization after PTP closing (Figs. 1–6 and 9) plus measurements of respiration with both succinate and complex I-dependent substrates (not shown) indicate that large amounts of cytochrome c release sufficient to impair electron transport did not occur under the conditions studied, probably because the extent of matrix swelling was moderate (Fig. 2) and did not lead to outer mitochondrial membrane rupture. It is notable in this regard that cytochrome c release without detectable changes of total mitochondrial cytochrome c has been described during tubule injury both in vitro and in vivo (81). The present model could be of use for further studying this process. The model is also potentially relevant for assessing the effect on the MPT of mitochondrial fission, which a recent report has implicated in the promotion of apoptosis during nephrotic acute kidney injury (12).

In conclusion, the MPT in proximal tubules is highly sensitive to ADP and Mg\(^{2+}\), with strong synergism between the two at physiological levels. It also is sensitive to inhibition of cyclophilin D by CsA, but only in the presence of ADP and/or Mg\(^{2+}\). NEFA that are endogenously generated by hypoxia before the MPT or produced during the MPT promote it. Sensitivity to the MPT is substantially greater when electron transport complex I-dependent substrates are used to support energization rather than the complex II-dependent substrate succinate, and this is not simply due to loss of NAD from the matrix during PTP opening. Accumulated NEFA play a large role in the decreased resistance to the MPT seen after H/R irrespective of the available substrate and the presence of ADP + Mg\(^{2+}\). CsA does not have much benefit after H/R. Removal of NEFA after H/R almost fully restores the normal resistance to the MPT in the presence of ADP+Mg with succinate as the substrate but is less effective with complex I-dependent substrates, which predominate in vivo. The higher sensitivity to the MPT in the presence of complex I-dependent substrates will increase the likelihood of developing the MPT during acute kidney injury despite the presence of ADP and Mg\(^{2+}\). However, NEFA are the predominant sensitizing factors, and inhibition of cyclophilin D does not substantially overcome their effect.

ACKNOWLEDGMENTS

We thank Tiffany Ostrowski, Sheila Madipelli, Srinivas Gullapalli, and Ruth Senter for assistance with several aspects of this work.


GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-54275 (to J. M. Weinberg), the Research Service of the Department of Veteran’s Affairs (to J. M. Weinberg), and German Research Foundation Grant DFG Fe 929 (to T. Feldkamp).

REFERENCES


2. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osniska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J,
F1645

REGULATION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION


25. Le Quoc K, Le Quoc D. Involvement of the ADP/ATP carrier in calcium-induced perturbations of the mitochondrial inner membrane per-


