

## Permeability transition in rat liver mitochondria is modulated by the ATP-Mg/P<sub>i</sub> carrier

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**Hagen, Thilo, Christopher J. Lagace, Josephine S. Modica-Napolitano, and June R. Aprille.** Permeability transition in rat liver mitochondria is modulated by the ATP-Mg/P<sub>i</sub> carrier. *Am J Physiol Gastrointest Liver Physiol* 285: G274–G281, 2003; 10.1152/ajpgi.00052.2003.—Mitochondrial permeability transition, due to opening of the permeability transition pore (PTP), is triggered by Ca<sup>2+</sup> in conjunction with an inducing agent such as phosphate. However, incubation of rat liver mitochondria in the presence of low micromolar concentrations of Ca<sup>2+</sup> and millimolar concentrations of phosphate is known to also cause net efflux of matrix adenine nucleotides via the ATP-Mg/P<sub>i</sub> carrier. This raises the possibility that adenine nucleotide depletion through this mechanism contributes to mitochondrial permeability transition. Results of this study show that phosphate-induced opening of the mitochondrial PTP is, at least in part, secondary to depletion of the intramitochondrial adenine nucleotide content via the ATP-Mg/P<sub>i</sub> carrier. Delaying net adenine nucleotide efflux from mitochondria also delays the onset of phosphate-induced PTP opening. Moreover, mitochondria that are depleted of matrix adenine nucleotides via the ATP-Mg/P<sub>i</sub> carrier show highly increased susceptibility to swelling induced by high Ca<sup>2+</sup> concentration, atractyloside, and the prooxidant tert-butylhydroperoxide. Thus the ATP-Mg/P<sub>i</sub> carrier, by regulating the matrix adenine nucleotide content, can modulate the sensitivity of rat liver mitochondria to undergo permeability transition. This has important implications for hepatocytes under cellular conditions in which the intramitochondrial adenine nucleotide pool size is depleted, such as in hypoxia or ischemia, or during reperfusion when the mitochondria are exposed to increased oxidative stress.

adenine nucleotides; calcium; phosphate; hypoxia; transport

THE PERMEABILITY TRANSITION pore (PTP) in liver mitochondria is a cyclosporin A-sensitive, high-conductance channel formed at contact sites between the mitochondrial inner and outer membranes. The PTP is a multi-protein complex that includes the voltage-dependent anion channel (outer membrane), the adenine nucleotide translocase (ANT; inner membrane), and cyclophilin-D (mitochondrial matrix). In vitro, PTP opening causes membrane depolarization as a primary conse-

quence and swelling due to equilibration of ionic gradients as a secondary consequence. In vivo, the PTP participates in the regulation of matrix Ca<sup>2+</sup>, pH, transmembrane potential, and volume, and there is evidence to suggest that its opening is a key event in a variety of toxic, hypoxic, and oxidative forms of cell injury (7). Mitochondrial permeability transition has been implicated in both apoptotic and necrotic cell death (8, 14).

In vitro, liver mitochondria undergo permeability transition in response to various inducers, including Ca<sup>2+</sup>, P<sub>i</sub>, the ANT inhibitor atractyloside, and prooxidants such as tert-butylhydroperoxide (tBH) (8). A fact that is often overlooked, however, is that standard conditions under which mitochondrial permeability transition is measured (i.e., the presence of a respiratory substrate, Ca<sup>2+</sup> at micromolar concentrations, and P<sub>i</sub> at millimolar concentrations) can cause net efflux of intramitochondrial adenine nucleotides (1, 2). This occurs via the ATP-Mg/P<sub>i</sub> carrier (1, 2), a Ca<sup>2+</sup> activated, atractyloside-insensitive transporter that catalyzes the divalent electroneutral exchange of ATP-Mg for P<sub>i</sub> (Fig. 1). The ATP-Mg/P<sub>i</sub> carrier in liver mitochondria is distinct from ANT, which mediates a membrane potential-dependent one-for-one exchange of cytosolic ADP for matrix ATP (12) and therefore does not account for net changes in the matrix adenine nucleotide content. Experimentally, net uptake and loss of adenine nucleotides via the ATP-Mg/P<sub>i</sub> carrier can be controlled by varying the external concentration of ATP in the presence of Mg<sup>2+</sup> and P<sub>i</sub> (5, 6). Incubation of energized rat liver mitochondria at an external ATP concentration >1 mM results in a net uptake of ATP; incubation at an external concentration of ATP <1 mM results in a net loss of matrix adenine nucleotides; and, incubation at an external concentration of 1 mM ATP results in a near-equilibrium steady state with no net movement of adenine nucleotides across the inner mitochondrial membrane. In vivo, modulation of liver intramitochondrial adenine nucleotide content via the ATP-Mg/P<sub>i</sub>

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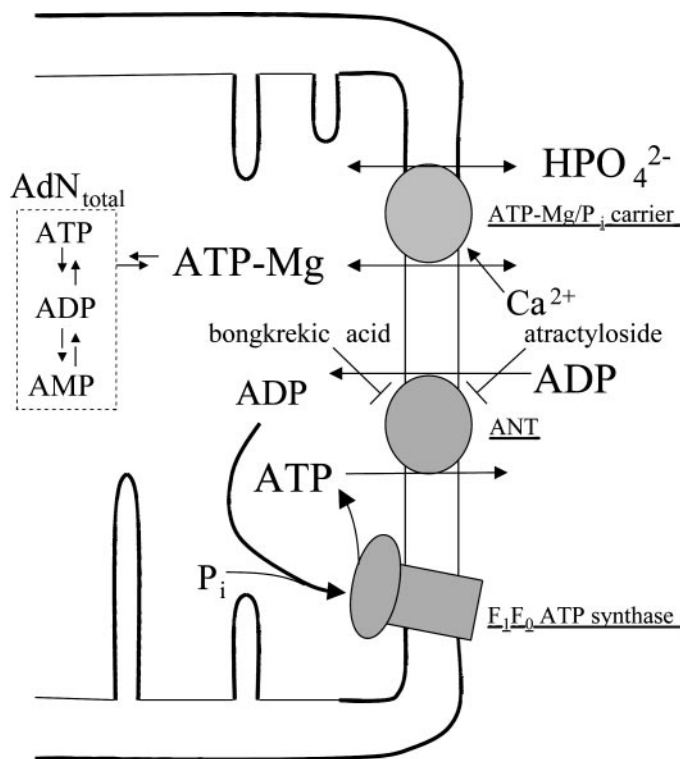


Fig. 1. Adenine nucleotide transport in liver mitochondria. The total matrix adenine nucleotide size ( $\text{AdN}_{\text{total}}$ ; sum of ATP + ADP + AMP) in liver mitochondria is regulated by the ATP-Mg/ $\text{P}_i$  carrier. This transporter catalyzes an electroneutral exchange of ATP-Mg carrying two negative charges for divalent phosphate. Because matrix ATP concentration ( $[\text{ATP}]$ ) is in equilibrium with ADP concentration and AMP concentration, changes in  $[\text{ATP}]$  due to net movement via the ATP-Mg/ $\text{P}_i$  carrier result in proportional changes in the concentrations of other adenine nucleotides. The ATP-Mg/ $\text{P}_i$  carrier is distinct from mitochondrial adenine nucleotide translocase (ANT), which mediates a membrane potential-dependent one-for-one exchange of cytosolic ADP for matrix ATP and therefore does not account for net changes in the matrix adenine nucleotide content. These adenine nucleotide carriers are further distinguished by different inhibitor sensitivities. Whereas the ANT is inhibited by atractyloside (binding from the cytosolic side) and bongkrekic acid (binding from the matrix side), the ATP-Mg/ $\text{P}_i$  carrier is insensitive to these inhibitors but is activated by micromolar  $\text{Ca}^{2+}$  concentrations.

carrier plays an important role in the regulating metabolic activities that have adenine nucleotide-dependent steps localized to the mitochondrial compartment (e.g., gluconeogenesis, urea synthesis, and oxidative phosphorylation) (1).

Changes in the matrix adenine nucleotide content (comprised of the sum of ATP, ADP, and AMP) are also likely to modulate the susceptibility of mitochondria to undergo permeability transition, because PTP opening has been shown to be regulated by molecules, including adenine nucleotides that can interact with ANT (8, 10). Atractyloside, which inhibits ANT by binding from the cytosolic side and thus brings the carrier into the so-called *c-state* (i.e., the binding center faces the cytosolic side of the inner membrane), induces PTP opening. In contrast, bongkrekic acid, which also inhibits ANT but binds from the matrix side and locks the carrier in the *m-state*, is a potent inhibitor of pore

opening. Moreover, externally added ANT substrates ATP and ADP also inhibit mitochondrial permeability transition (8). During periods of hypoxia and ischemia, mitochondrial permeability transition is believed to be one of the main factors contributing to cell damage and death (8). The decrease in cytosolic ATP that occurs under these conditions, along with a dramatic rise in the cytosolic  $\text{P}_i$  concentration due to phosphate hydrolysis of ATP to ADP and AMP and a rise in cytosolic  $\text{Ca}^{2+}$ , is assumed to trigger opening of the PTP (8). However, an important consideration often overlooked is that a decrease in cytosolic ATP and concomitant increases in cytosolic  $\text{P}_i$  and  $\text{Ca}^{2+}$  are also conditions that will favor adenine nucleotide loss from mitochondria via the  $\text{Ca}^{2+}$ -activated ATP-Mg/ $\text{P}_i$  carrier (1, 2). In fact, the mitochondrial adenine nucleotide content has been shown to be severely depleted under conditions of hypoxia and ischemia (9, 15, 18). A role for the ATP-Mg/ $\text{P}_i$  carrier in modulating sensitivity of mitochondria to undergo permeability transition could therefore have important implications under cellular conditions such as hypoxia and ischemia or during reperfusion when the mitochondria are exposed to increased oxidative stress.

The purpose of this study was to examine the role of ATP-Mg/ $\text{P}_i$  carrier-dependent changes in the matrix adenine nucleotide content in modulating PTP opening induced by  $\text{P}_i$ ,  $\text{Ca}^{2+}$ , atractyloside, and tBH. Results show that  $\text{P}_i$ -induced opening of the PTP in liver mitochondria is, at least in part, secondary to depletion of the intramitochondrial adenine nucleotide content via the ATP-Mg/ $\text{P}_i$  carrier. Furthermore, a decreased matrix adenine nucleotide content increases the susceptibility of mitochondria to PTP opening induced by  $\text{Ca}^{2+}$ , atractyloside, and tBH. In addition, changes in the cytosolic adenine nucleotide composition also appear to contribute to increased mitochondrial membrane permeability transition. These data suggest a role for the ATP-Mg/ $\text{P}_i$  carrier in modulating the sensitivity of mitochondria to undergo permeability transition under cellular conditions such as hypoxia, ischemia, or reperfusion.

## MATERIALS AND METHODS

**Isolation of liver mitochondria.** Rat liver mitochondria were isolated by differential centrifugation as described previously (6).

**Mitochondrial swelling measurements.** Mitochondrial swelling was measured as the decrease in light scattering by following optical density at 540 nm ( $A_{540}$ ) over time. Standard incubation conditions for the swelling assay were 250 mM sucrose, 10 mM Tris (pH 7.4), 5 mM succinate, and 5  $\mu\text{M}$   $\text{CaCl}_2$ . Qualitatively similar results were obtained if sucrose was replaced by 150 mM KCl in the swelling assay. Mitochondria were added at a final concentration of 0.5 mg protein/ml, and after equilibration for 1 min, swelling was induced by different agents as indicated in the figure legends.

**Manipulation of the matrix adenine nucleotide content.** To adjust the matrix adenine nucleotide content, mitochondria were incubated for 15 min at 30°C under the following incubation conditions (in mM): 225 sucrose, 10 Tris (pH 7.4), 10 KCl, 2  $\text{KPi}$ , 5  $\text{MgCl}_2$ , and 5 glutamate and malate. External

ATP was included at specific concentrations that result in predictable values of the matrix adenine nucleotide content: no ATP to completely deplete the mitochondria of adenine nucleotides, 0.15 mM ATP to deplete adenine nucleotides to intermediate levels, 1 mM ATP to maintain the adenine nucleotide content approximately at the initial level (12–15 nmol/mg protein in freshly isolated liver mitochondria), and 2 mM ATP to overload the mitochondria with adenine nucleotides (5, 6). After incubation, mitochondria were centrifuged, washed once in 250 mM sucrose and 10 mM Tris (pH 7.4), and then used for the swelling experiments.

**Adenine nucleotide measurements.** ATP, ADP, and AMP concentrations were determined enzymatically in neutralized PCA-extracts of mitochondria, as described previously (6).

**Measurements of state 3 respiration.** Mitochondrial oxygen consumption was assayed polarographically at 30°C by using a Clark electrode (Yellow Springs Instruments) as described previously (4). The medium used for respiration consisted of (in mM) 225 sucrose, 10 Tris (pH 7.4), 10 KCl, 1 EDTA, 10  $KP_i$ , 5  $MgCl_2$ , and 5 succinate. To measure state 3 respiration, mitochondria were incubated at a protein concentration of 0.5 mg/ml in the presence of 0.2 mM ADP.

## RESULTS

$P_i$ , in conjunction with  $Ca^{2+}$ , is a well-known inducing agent of PTP opening. Thus the addition of 10 mM  $P_i$  to isolated rat liver mitochondria in the presence of 5  $\mu M$   $Ca^{2+}$  and 5 mM succinate leads to mitochondrial swelling as detected by a decrease in light scattering (Fig. 2A). Results show also that  $P_i$ -induced swelling was inhibited in the presence of the calcium chelator

EGTA. Furthermore,  $P_i$ -induced swelling was sensitive to cyclosporin A (Fig. 2B), indicating that it was due to opening of the mitochondrial PTP. The initial small decrease in light scattering observed with cyclosporin A is likely to be caused by osmotic swelling as a result of  $P_i$  uptake by the mitochondria through the  $P_i/H^+$  carrier.

However, in addition to inducing PTP opening, millimolar concentrations of  $P_i$  are also known to induce a rapid net loss of adenine nucleotides in rat liver mitochondria via the atractyloside-insensitive,  $Ca^{2+}$  activated ATP-Mg/ $P_i$  carrier, which catalyzes exchange of  $P_i$  for ATP-Mg (1, 2). In the absence of external ATP, matrix ATP-Mg exchanges for external  $P_i$  leading to a decrease in the matrix adenine nucleotide pool. It may be hypothesized, therefore, that  $P_i$ -induced adenine nucleotide loss via the ATP-Mg/ $P_i$  carrier could account, at least in part, for the opening of the PTP on  $P_i$  addition and thus plays a role in increasing the susceptibility of mitochondria to undergo permeability transition.

Figure 3A demonstrates the rapid loss of matrix adenine nucleotides in rat liver mitochondria exposed to 10 mM  $P_i$ . No matrix adenine nucleotides were lost in the absence of  $P_i$  or in the presence of  $P_i$  and EGTA; under these conditions the  $Ca^{2+}$ -dependent ATP-Mg/ $P_i$  carrier is inactive (Fig. 3A). Furthermore,  $P_i$ -induced adenine nucleotide loss was delayed if the mitochondrial ATP synthase inhibitor oligomycin was added before the addition of the respiratory substrate (succinate). Under these conditions, no ATP can be synthesized, and therefore, the matrix ATP concentration is very low relative to the ADP concentration. Thus availability of matrix ATP for exchange against external  $P_i$  via the ATP-Mg/ $P_i$ -carrier is limited. The delay in  $P_i$ -induced adenine nucleotide loss in the presence of oligomycin (Fig. 3A) correlates well with a delay in the onset of  $P_i$ -induced swelling in the presence of oligomycin (Fig. 3B). This finding suggests that the initial swelling with  $P_i$  was related to a rapid loss of matrix adenine nucleotides rather than a direct consequence of  $P_i$  on the PTP.

To further test whether the matrix adenine nucleotide content affects PTP opening, mitochondrial adenine nucleotides were depleted by another method, i.e., by the addition of pyrophosphate. External pyrophosphate exchanges via the atractyloside-sensitive ANT for matrix ADP (13), resulting in a rapid decrease in the matrix adenine nucleotide content. Figure 4 shows that the onset of  $P_i$ -induced swelling in the presence of oligomycin was accelerated if 0.5 mM pyrophosphate was added. Pyrophosphate addition had no effect on the onset of swelling if carboxyatractyloside was present to inhibit the ADP/ATP carrier, ruling out any unspecific effect of pyrophosphate.

Thus the data presented so far indicate that  $P_i$ -induced opening of the PTP is, at least in part, secondary to depletion of mitochondrial adenine nucleotides. Results show that loss of adenine nucleotides via either the ATP-Mg/ $P_i$  carrier or the ANT will induce mitochondrial swelling. However, under physiological con-

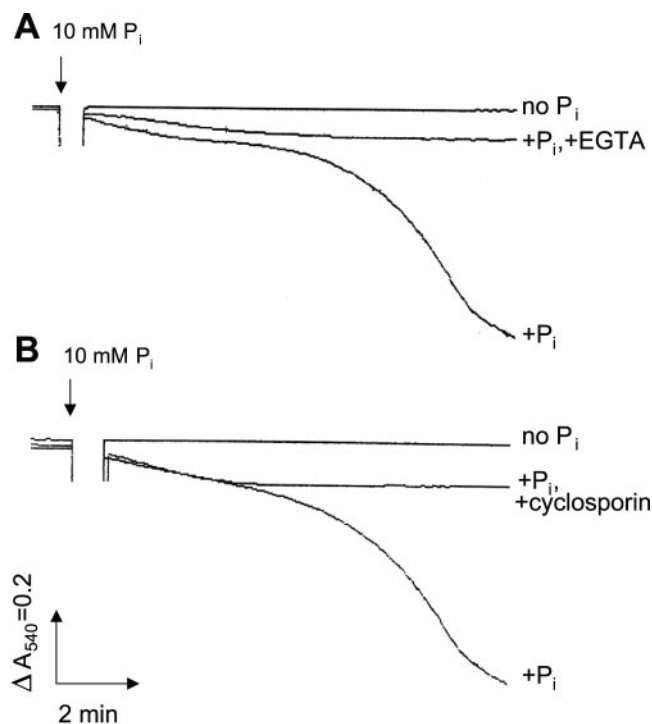


Fig. 2. Phosphate-induced swelling in isolated rat liver mitochondria.  $P_i$ -induced swelling was measured under standard incubation conditions as described under MATERIALS AND METHODS. EGTA (1 mM) or cyclosporin A (1  $\mu M$ ) were included as indicated. After obtaining the baseline for  $\sim 1$  min, mitochondrial swelling was induced by the addition of 10 mM  $P_i$ .  $\Delta A_{540}$ , change in optical density at 540 nm.

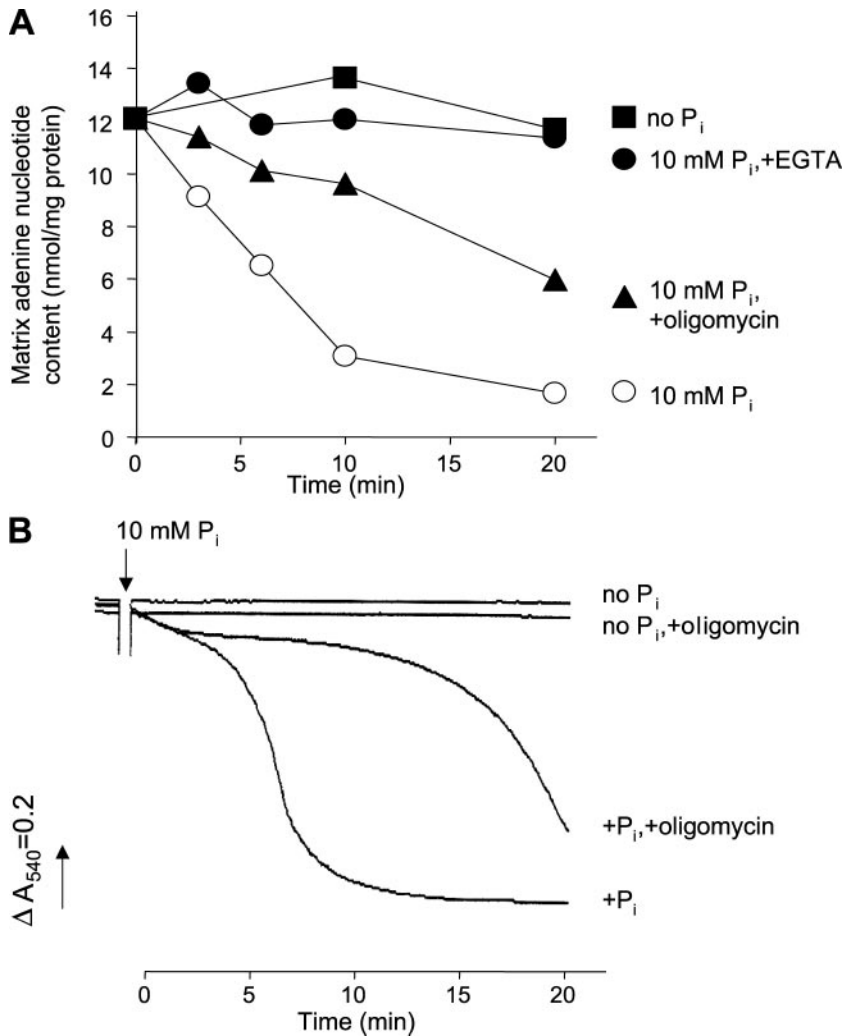


Fig. 3. Adenine nucleotide depletion is a contributing cause of phosphate-induced swelling. Mitochondria were incubated under standard conditions in the absence or presence of 10 mM  $P_i \pm 1$  mM EGTA or 1  $\mu$ g/ml oligomycin as indicated. A: at various time points between 0 and 20 min, 1 ml aliquots of mitochondria were removed from the incubation and centrifuged, and the adenine nucleotide content (ATP + ADP + AMP) was determined enzymatically in neutralized PCA extracts of the mitochondrial pellets. B: mitochondrial swelling was measured as  $\Delta A_{540}$  over the 20-min time course.

ditions, only the ATP-Mg/ $P_i$  carrier is likely to mediate net adenine nucleotide efflux, because mitochondrial adenine nucleotide loss has been demonstrated in intact hepatocytes on hormonal stimulation with glucagon or vasopressin, which increase the intracellular  $Ca^{2+}$  concentration leading to activation of the ATP-Mg/ $P_i$  carrier (9). In contrast, the ANT, which mediates a one-for-one exchange of ATP for ADP, does not contribute to adenine nucleotide net transport in liver mitochondria (6). The low intracellular pyrophosphate concentration and the low affinity of the ANT for pyrophosphate compared with ATP and ADP (13) make it unlikely that pyrophosphate-dependent adenine nucleotide net transport occurs under physiological conditions.

We then tested whether manipulation of the matrix adenine nucleotide content via the ATP-Mg/ $P_i$  carrier plays a general role in modulating the susceptibility of isolated mitochondria to undergo permeability transition. For these experiments, the intramitochondrial adenine nucleotide content (ATP + ADP + AMP) was adjusted to set values between 2.9 and 17.5 nmol/mg

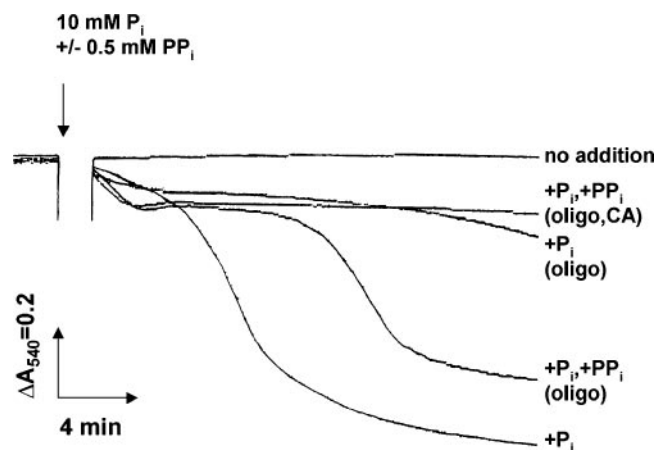


Fig. 4. Pyrophosphate-induced depletion of matrix adenine nucleotides enhances mitochondrial swelling. Mitochondrial swelling, measured under standard conditions, was induced by 10 mM  $P_i$ , in the presence or absence of 0.5 mM pyrophosphate ( $PP_i$ ). Oligomycin (1  $\mu$ g/ml) or carboxyatractyloside (CA; 5  $\mu$ M) was added as indicated. NaF (10 mM) was included in all assays to inhibit pyrophosphate hydrolysis.

mitochondrial protein by preincubating mitochondria with varying external ATP concentrations between 0 and 1.0 mM at 30°C for 15 min as described under MATERIALS AND METHODS. Depending on the specified external ATP concentration, ATP-Mg is released by mitochondria in exchange for  $P_i$  via the ATP-Mg/ $P_i$  carrier to a predictable steady-state level (5, 6). Note that the normal matrix adenine nucleotide content of freshly isolated rat liver mitochondria is between 12 and 15 nmol/mg protein.

Figure 5A shows that under standard incubation conditions (i.e., 5 mM succinate and 5  $\mu$ M  $Ca^{2+}$ ) mitochondria with the lowest adenine nucleotide content (2.9 nmol/mg protein) underwent modest swelling even in the absence of any added  $P_i$ . This swelling was completely inhibited by cyclosporin A. In the presence of 0.5 mM  $P_i$  and oligomycin to prevent further adenine nucleotide loss via the ATP-Mg/ $P_i$  carrier (oligomycin prevents phosphorylation of matrix ADP to ATP), both the rate of onset and magnitude of swelling of severely depleted mitochondria was enhanced (Fig. 5B). No swelling was observed in moderately depleted or nondepleted mitochondria in either the absence or presence of 0.5 mM  $P_i$  and oligomycin (Fig. 5, A and B).

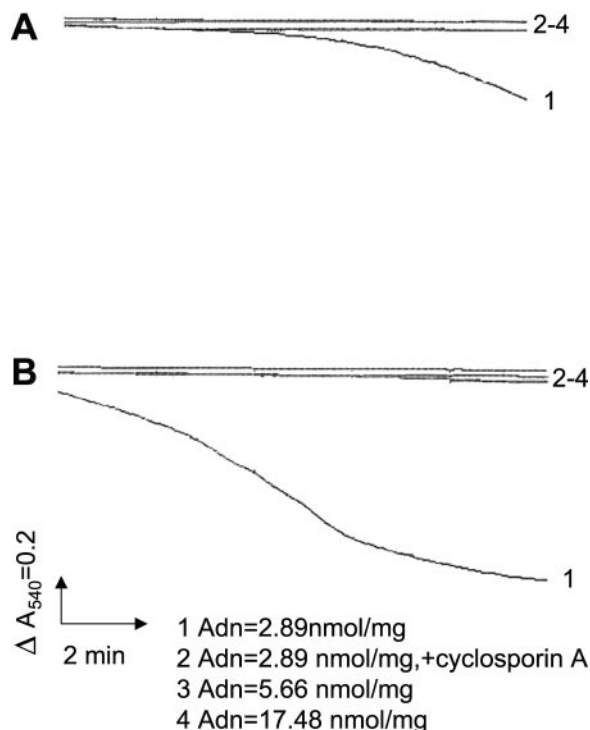


Fig. 5. Mitochondrial swelling is enhanced in mitochondria that have a low matrix adenine nucleotide content. For these experiments, the intramitochondrial adenine nucleotide content (ATP + ADP + AMP) was manipulated to set values between 2.9 and 17.5 nmol/mg mitochondrial protein by preincubating mitochondria with varying external ATP concentrations as described under MATERIALS AND METHODS. For comparison, the normal matrix adenine nucleotide content of freshly isolated rat liver mitochondria is between 12 and 15 nmol/mg protein. Mitochondrial swelling was measured under standard incubation conditions in the absence (A) or presence (B) of 0.5 mM  $P_i$  and 1  $\mu$ g/ml oligomycin. Cyclosporin A (1  $\mu$ M) was included as indicated.

Figure 6 compares the effects of  $Ca^{2+}$ , tBH or atractyloside on swelling in mitochondria that were moderately depleted of adenine nucleotides and in nondepleted mitochondria, which do not undergo rapid swelling in the absence of inducing agents. In the absence of  $P_i$ , 150  $\mu$ M  $Ca^{2+}$  induced rapid swelling in mitochondria that were depleted to an adenine nucleotide content of 5.48 nmol/mg protein, whereas in nondepleted mitochondria, almost no swelling was observed (Fig. 6A).  $Ca^{2+}$ -induced swelling in adenine nucleotide-depleted mitochondria was completely prevented with 1  $\mu$ M cyclosporin A or 2  $\mu$ M ruthenium red (not shown). Mitochondria moderately depleted of adenine nucleotides also showed increased sensitivity to swelling induced by 1 mM tBH (Fig. 6B) that was fully inhibited in the presence of 1  $\mu$ M cyclosporin A (not shown). Finally, increased susceptibility to undergo PTP opening was observed as well in adenine nucleotide-depleted mitochondria when swelling was induced with 50  $\mu$ M atractyloside (Fig. 6C). Atractyloside-induced swelling was completely prevented in the presence of 1  $\mu$ M cyclosporin A or 10  $\mu$ M bongkreikic acid (not shown).

To determine whether PTP opening in adenine nucleotide-depleted mitochondria was dependent on the intramitochondrial ATP/ADP ratio,  $Ca^{2+}$ -induced swelling and adenine nucleotide concentrations were measured in these mitochondria in the presence or absence of oligomycin. Figure 7 shows that even a 10-fold difference in ATP/ADP ratios produced no difference in  $Ca^{2+}$ -induced swelling. To be able to compare initial rates, the swelling was accelerated by including a low concentration of  $P_i$  (0.2 mM) in the incubation. At this concentration,  $P_i$  does not lead to further loss of matrix adenine nucleotides over 5 min (Fig. 7). The low ATP/ADP ratio with oligomycin present did not affect the rate of  $Ca^{2+}$ -induced swelling (Fig. 7), suggesting that in adenine nucleotide-depleted mitochondria either intramitochondrial ATP or ADP can inhibit PTP opening. Taken together, Figs. 5–7 show that it is the total sum of matrix ATP and ADP and not the relative ATP/ADP ratio that determines the susceptibility of mitochondria to undergo PTP opening.

Results indicate that mitochondria depleted of adenine nucleotides to levels of 5–6 nmols/mg protein or below are prone to undergo permeability transition, which could be of significance under conditions of hypoxia and ischemia when matrix adenine nucleotides are low. Conditions of prolonged hypoxia and anoxia have also been shown to lead to a marked decrease in cytosolic ATP and ADP and a concomitant increase in AMP in hepatocytes (9). Therefore, we were interested in measuring swelling in the presence of external ATP, ADP, or AMP in mitochondria moderately depleted of adenine nucleotides. Either ATP or ADP at an external concentration of 1 mM completely prevented swelling induced by  $Ca^{2+}$ , tBH, or atractyloside (Fig. 8, A–C). External 1 mM AMP was less effective in inhibiting  $Ca^{2+}$ -induced swelling (Fig. 8A). Interestingly, 1 mM AMP actually accelerated the onset of swelling induced

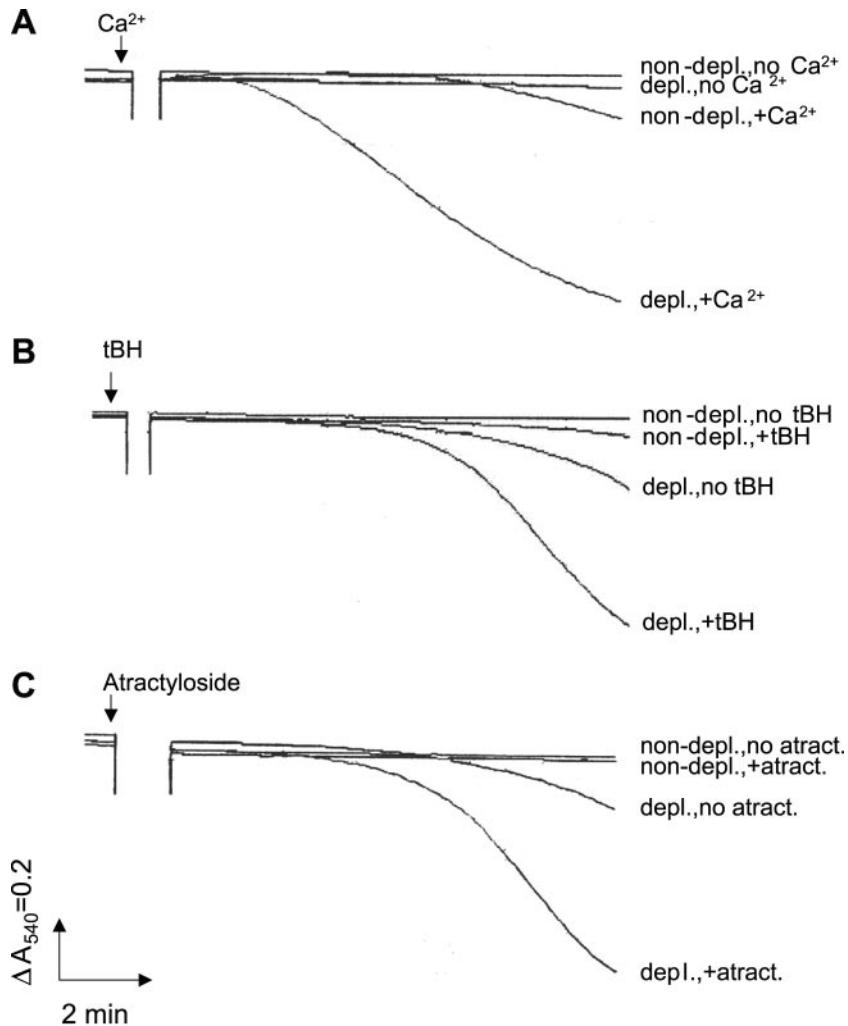


Fig. 6. Mitochondria moderately depleted of matrix adenine nucleotides show an increased susceptibility to swelling induced by  $\text{Ca}^{2+}$ , tert-butylhydroperoxide (tBH), and atractyloside. In the absence of  $\text{P}_i$ ,  $150 \mu\text{M}$   $\text{Ca}^{2+}$  induced rapid swelling in mitochondria moderately depleted of adenine nucleotides (to  $5.48 \text{ nmol/mg}$  protein), whereas in nondepleted mitochondria, almost no swelling was observed (A). Mitochondria moderately depleted of adenine nucleotides also showed increased sensitivity to swelling induced by  $1 \text{ mM}$  tBH ( $+20 \mu\text{M}$   $\text{Ca}^{2+}$ ) (B), and  $50 \mu\text{M}$  atractyloside ( $+20 \mu\text{M}$   $\text{Ca}^{2+}$ ) (C).

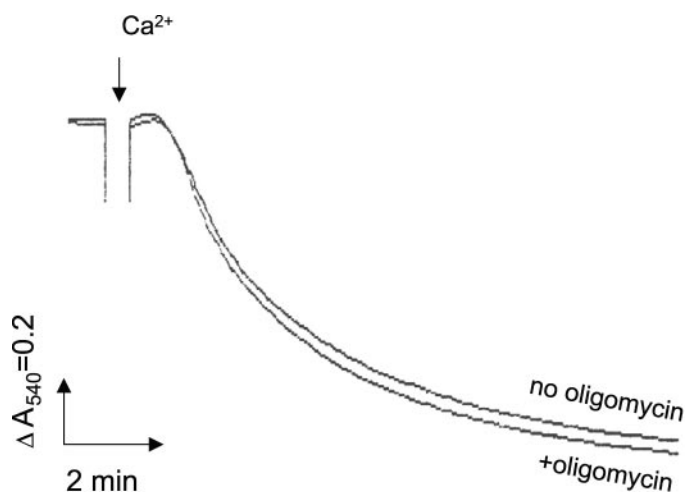
with tBH or atractyloside (Fig. 8, B and C). These results indicate that not only depletion of the matrix adenine nucleotide pool, but also changes in the composition of adenine nucleotides in the cytosol, may modulate the susceptibility of mitochondria to undergo permeability transition.

So far the results suggest a direct effect of matrix adenine nucleotide content on permeability transition, probably through occupancy of ANT binding sites at the PTP on the matrix face by either ATP or ADP. Additional experiments suggested that adenine nucleotide loss may also have an indirect effect on mitochondrial permeability transition. Figure 9 shows that mitochondrial *state 3* respiration varies as a function of matrix adenine nucleotide content, as has been reported previously (1, 4). Therefore, the low matrix adenine nucleotide content that occurs under conditions of tissue hypoxia and ischemia can be expected to lead to decreased oxidative phosphorylation. This contributes to a decrease in cytosolic ATP and ADP concentrations and an increase in AMP concentration and consequently leads to an increase in susceptibility for mitochondrial permeability transition, possibly through decreased binding of ATP and ADP to the cytosolic face of ANT in the PTP.

## DISCUSSION

Several important findings are revealed by this study. First, it is demonstrated that  $\text{P}_i$ -induced opening of the PTP in isolated mitochondria is due in some measure to depletion of the intramitochondrial adenine nucleotide pool via the ATP-Mg/ $\text{P}_i$  carrier. This result questions the general assumption that  $\text{P}_i$  exerts its effect on mitochondrial permeability transition solely through direct effects on the PTP complex, because we have shown that the effect of  $\text{P}_i$  may be largely secondary to the  $\text{P}_i$ -induced efflux of matrix ATP via the ATP-Mg/ $\text{P}_i$  carrier.

Second, it is shown that a decrease in the intramitochondrial adenine nucleotide pool leads to increased susceptibility for PTP opening induced by various inducing agents. These results should be taken into account when measuring the PTP opening under conditions that favor adenine nucleotide loss. These findings may also be of significance in intact cells under conditions of hypoxia and ischemia in which the matrix adenine nucleotide content is known to be decreased. In this situation, there is greater likelihood of the PTP opening being induced by elevated cytosolic  $\text{Ca}^{2+}$  concentration or increased oxidative stress during reper-



Adenine nucleotide concentrations  
(nmol/mg protein)

	ATP	ADP	AMP	Adn <sub>total</sub>	ATP/ADP
no oligom.	2.02	1.10	0.43	3.35	1.84
+ oligom.	0.33	1.84	1.21	3.58	0.18

Fig. 7. Effect of the intramitochondrial ATP/ADP ratio on the  $\text{Ca}^{2+}$ -induced swelling rate. Swelling of mitochondria partially depleted of adenine nucleotides was measured under standard incubation conditions in the presence of 0.2 mM  $\text{P}_i$  and in the absence or presence of 1  $\mu\text{g}/\text{ml}$  oligomycin. Swelling was induced by the addition of 50  $\mu\text{M}$   $\text{Ca}^{2+}$ . The matrix adenine nucleotide contents given in the table were measured in the same mitochondrial preparations under identical incubation conditions. To determine the actual ATP/ADP ratio under the incubation conditions, instantaneous separation of mitochondria from the incubation medium was necessary. This was done by rapid centrifugation of 1 ml aliquots layered over silicone oil and perchloric acid, as previously described (3). The perchloric acid extracts of the mitochondrial pellets were neutralized and adenine nucleotide concentrations were measured enzymatically.

fusion. The ATP-Mg/ $\text{P}_i$  carrier, by mediating a loss of mitochondrial adenine nucleotides to the cytosol under these conditions, thus plays an important role in the sequence of events leading to cellular dysfunction and apoptosis.

Third, our study confirms previous reports that external ATP or ADP is also protective against mitochondrial permeability transition (10, 16). The effects of intramitochondrial ATP and ADP as well as externally added ATP and ADP on permeability transition were observed are likely mediated via their binding to the ANT, a component of the PTP complex. This hypothesis is supported by the demonstration that carboxyatractyloside antagonizes the inhibitory effect of ADP on pore opening (10) and that reconstituted purified ANT exhibits  $\text{Ca}^{2+}$ -dependent channel activity inhibited by ADP (17). Our results agree with previous reports (10) that external AMP is unable to inhibit PTP opening, and we found that AMP even accelerates permeability transition induced by tBH and atractyloside. The lack of inhibition of the PTP by AMP may be due to its inability to interact with the ANT (11).

In conclusion, the results demonstrate that both the intramitochondrial and the cytosolic adenine nucleo-

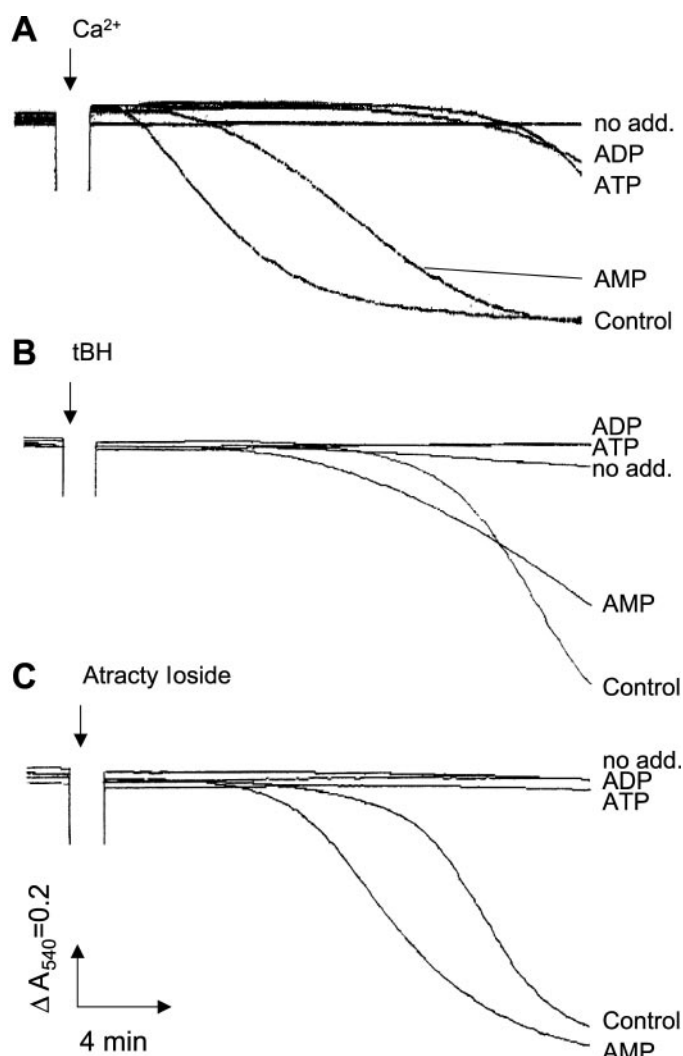


Fig. 8. Effect of external ATP, ADP, and AMP on mitochondrial swelling induced by  $\text{Ca}^{2+}$ , tBH, and atractyloside. Swelling in mitochondria depleted of adenine nucleotides to 5.85 nmol/mg protein was induced by 150  $\mu\text{M}$   $\text{Ca}^{2+}$  (A), 1 mM tBH (+20  $\mu\text{M}$   $\text{Ca}^{2+}$ ) (B), or 50  $\mu\text{M}$  atractyloside (+20  $\mu\text{M}$   $\text{Ca}^{2+}$ ) (C) in the presence and absence of externally added 1 mM ATP, ADP, or AMP as indicated.

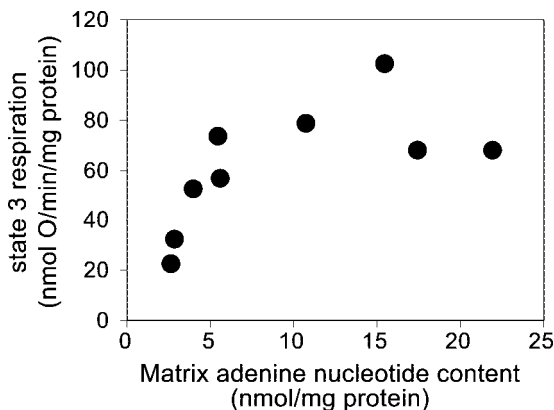


Fig. 9. Mitochondrial *state 3* respiration as a function of the matrix adenine nucleotide content. The mitochondrial adenine nucleotide content was manipulated in preincubation to values between 2.67 and 21.98 nmol/mg protein and *state 3* respiration (in the presence of 0.2 mM ADP) measured for each preparation as described under MATERIALS AND METHODS.

tide concentration and composition dynamically regulate permeability transition. A moderate decrease in the matrix adenine nucleotide content, as observed under conditions of transient hypoxia or ischemia, without complete dephosphorylation of cytosolic adenine nucleotides, should be readily reversible for two reasons: first, because mitochondrial permeability transition is inhibited by cytosolic ATP and ADP, and second, because reuptake of adenine nucleotides by the mitochondria can occur on reoxygenation if cytosolic ATP is available as a substrate for the ATP-Mg/P<sub>i</sub> carrier. Severe or prolonged hypoxic/ischemic stress ultimately leads to a dramatic decrease in cytosolic ATP and ADP and a concomitant increase in AMP. As a result, uncontrolled mitochondrial permeability transition will be more likely, leading to mitochondrial depolarization and release of proapoptotic proteins, e.g., cytochrome *c* and apoptosis-inducing factor as well as to complete depletion of matrix adenine nucleotides. Dephosphorylation of cytosolic adenine nucleotides also precludes reuptake of adenine nucleotides by the mitochondria via the ATP-Mg/P<sub>i</sub> carrier on reoxygenation/reperfusion, making these changes irreversible. It is thus postulated that via modulation of mitochondrial permeability transition, changes in matrix and cytosolic adenine nucleotide concentrations play a role in hepatocyte adaptation to and recovery from transient hypoxia, as well as in cellular dysfunction under conditions of extreme hypoxia and ischemia that can ultimately lead to apoptotic or necrotic cell death. The ATP-Mg/P<sub>i</sub> carrier, when activated by elevated cytosolic Ca<sup>2+</sup>, mediates the shift of adenine nucleotides from mitochondria to the cytosol and may therefore play an important role in the regulation of mitochondrial permeability transition under conditions of hypoxia and ischemia in hepatocytes.

#### DISCLOSURES

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