Regulation of total mitochondrial Ca\(^{2+}\) in perfused liver is independent of the permeability transition pore

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Regulation of total mitochondrial Ca\(^{2+}\) in perfused liver is independent of the permeability transition pore. Am. J. Physiol. 276 (Cell Physiol. 45): C1297–C1302, 1999.— Triggering of the permeability transition pore (PTP) in isolated mitochondria causes release of matrix Ca\(^{2+}\), ions, and metabolites, and it has been proposed that the PTP mediates mitochondrial Ca\(^{2+}\) release in intact cells. To study the role of the PTP in mitochondrial energy metabolism, the mitochondrial content of Ca\(^{2+}\), Mg\(^{2+}\), ATP, and ADP was determined in hormonally stimulated rat livers perfused with cyclosporin A (CsA). Stimulation of livers perfused in the absence of CsA with glucagon and phenylephrine induced an extensive uptake of Ca\(^{2+}\), Mg\(^{2+}\), and ATP plus ADP by the mitochondria, followed by a release on omission of hormones. In the presence of CsA, the PTP was fully inhibited, but neither the hormone-induced uptake of Ca\(^{2+}\), ATP, or ADP by mitochondria nor their release after washout of hormones was significantly changed. We conclude that the regulation of sustained changes in mitochondrial Ca\(^{2+}\) content induced by hormonal stimulation is independent of the PTP.

Cyclosporin A; permeability transition; mitochondria; calcium; intact rat liver

The inner membrane of mitochondria contains a channel protein, the permeability transition pore (PTP), which, when open, depolarizes the membrane potential and induces a release of matrix ions and solutes with molecular masses up to 1,500 Da (reviewed in Refs. 7, 16, 34). The PTP is activated by Ca\(^{2+}\) from the mitochondrial matrix side and inhibited by the immunosuppressive peptide cyclosporin A (CsA). It has been proposed that the physiological role of the PTP is to mediate mitochondrial Ca\(^{2+}\) release and hence to participate in the regulation of intracellular Ca\(^{2+}\) signaling processes (8, 17, 20, 27). This concept has gained support by several experimental findings, suggesting that, under certain conditions, the PTP may function as a more selective channel, allowing transport of Ca\(^{2+}\) without irreversible deleterious consequences on mitochondrial energy conservation. First, the CsA-sensitive megachannel of the inner mitochondrial membrane has two open conformations distinguished by a twofold difference in conductance, the more narrow of which may represent an ion-selective state of the PTP (32). Second, mitochondria may release Ca\(^{2+}\) by a CsA-sensitive mechanism in the absence of an increased unspecific permeability following a limited Ca\(^{2+}\) uptake that is insufficient for triggering the conventional PTP (13, 21, 22). Third, treatment of cells with CsA may lead to excessive mitochondrial Ca\(^{2+}\) accumulation, suggesting that PTP inhibition by CsA blocks the physiological pathway for Ca\(^{2+}\) efflux from mitochondria (2, 26). Although these data indicate that the PTP may be induced to operate in a selective mode, the physiological setting in which PTP-mediated Ca\(^{2+}\)-selective release takes place remains to be defined. One possibility is that the PTP opens transiently when a cytosolic Ca\(^{2+}\) wave reaches mitochondria (13, 21, 22) and that the PTP participates in mitochondrial modulation of intracellular Ca\(^{2+}\) waves (5, 18, 23). Alternatively, PTP-mediated mitochondrial Ca\(^{2+}\) release may be restricted to more particular conditions related to the induction of cell death (10).

To address the role of the PTP in mitochondrial ion and metabolite homeostasis, we performed measurements of the mitochondrial total Ca\(^{2+}\), Mg\(^{2+}\), and ATP plus ADP content of perfused rat livers following hormonal stimulation with phenylephrine and glucagon. This treatment induces an extensive increase in mitochondrial content of Ca\(^{2+}\), other ions, and adenosine nucleotides (1, 3, 4, 12, 19, 31). To assess the role of the PTP in the regulation of these ions and metabolite fluxes, livers were perfused with CsA to produce PTP inhibition. To ascertain that the PTP was fully inhibited under these conditions, we measured the mitochondrial concentration of CsA and the activity of the PTP in mitochondria isolated from these livers. We found that, in the presence of CsA, neither the hormone-induced uptake of Ca\(^{2+}\), ATP, or ADP nor their net release after omission of hormones was significantly changed. In contrast, CsA caused an inhibition of the uptake of Mg\(^{2+}\) by mitochondria, whereas its release following washout of hormones still occurred in the presence of CsA. These data demonstrated that CsA-induced inhibition of the PTP in mitochondria in situ did not produce any major perturbation of the hormone-induced regulation of mitochondrial ion and metabolite homeostasis. We conclude that regulation of sustained changes in mitochondrial Ca\(^{2+}\) content, induced by hormonal stimulation, is independent of the PTP.

MATERIALS AND METHODS

Perfusions. Male Wistar rats (200–300 g body wt) were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). The rats were treated with heparin (100 IU/kg), the portal vein was cannulated, and the livers were perfused with a buffer containing 119 mM NaCl, 25 mM NaHCO\(_3\), 4.7 mM KCl, 1.3 mM CaCl\(_2\), 0.65 mM MgSO\(_4\), 1.5 mM NaH\(_2\)PO\(_4\), 5.6 mM glucose, 1.7 g BSA/l and 10 mM HEPESS/NaOH, pH 7.4 ± 0.05, equilibrated with O\(_2/\)CO\(_2\) 95/5.
(19:1) at a temperature of 36°C and at a flow rate of 5 ml·min⁻¹·g wet wt⁻¹. The portal vein branches going to the median, left, and caudate lobes of the liver were occluded by ligatures, and the corresponding lobes were removed. The effluent buffer from the remaining lateral liver lobe was collected from a cannula inserted in the caval vein. The experiments were preceded by a 30-min stabilization period. When total tissue extracts were to be analyzed, the lobe (wet wt = 2 g) was excised and freeze-clamped at −196°C. CsA (3 µM) was added to the perfusion buffer as a 25 mM solution in ethanol, and isotope-labeled [³H]CsA was used at an activity of 1 µCi/l. Isotope measurements were carried out in a Wallac Mini B liquid scintillation counter (Wallac, Turku, Finland).

Isolation of mitochondria. Mitochondria were isolated as previously described (12), with the following modifications, to prevent loss of Ca²⁺, Mg²⁺, and adenine nucleotides from mitochondria during the isolation. Before homogenization, the current perfusion buffer was washed out of the vascular system of the liver by perfusing for 1 min with homogenization buffer containing 225 mM mannitol, 75 mM sucrose, 2 mM EGTA, 1 µM ruthenium red, 1 µM CsA, 2 µM BSA, and 10 mM HEPES/KOH, pH 7.2, at a temperature of 10°C. Homogenization was then carried out in the same buffer supplemented with 0.5 mM N-ethylmaleimide to inhibit the mitochondrial phosphate carrier. The washing steps were performed in a buffer containing (in mM) 225 mannitol, 75 sucrose, 2 EGTA, and 10 HEPES/KOH, pH 7.2. When the PTP was to be studied in isolated mitochondria (see Fig. 3) or their CsA content measured, the preparation was carried out in the same way, with the exception that ruthenium red, CsA, and N-ethylmaleimide were omitted from the homogenization buffer. In this way, the extracellular CsA, present in the vascular system during the perfusion, was washed out completely before homogenization; all CsA present in the isolated mitochondria thus represented CsA that had been taken up by cells during perfusion.

Analysis of ions and metabolites. Freeze-clamped livers were ground at −196°C, whereupon the powder was mixed with 20 ml extraction medium containing 8% perchloric acid, 20% ethanol, 10 mM EDTA, and 10 mM HEPES. Freshly prepared mitochondria were mixed with 10 ml of extraction medium at +4°C. The suspension was then sonicated for 1 min at +4°C, the precipitate removed by centrifugation, and the pH adjusted to 6.5–7.0 by addition of 10 M KOH. The sedimented KClO₄ was removed by centrifugation. Ca²⁺ and Mg²⁺ were analyzed by atomic absorption spectroscopy, and adenine nucleotides were measured by chemoluminescence (24) or by ³H- and ³¹P-NMR analysis as described previously (28).

Measurement of the permeability transition. Mitochondria were suspended at a concentration of 1 mg/ml in a buffer containing 300 mM sucrose, 10 mM KPO₄, 5 mM succinate, 10 µM arsenazo III, 2 µM rotenone, and 10 mM HEPES, pH 7.40. CsA (1 µM) was added as a 1 mM solution in ethanol. Light scattering was measured at 570 nm, and Ca²⁺ was measured at the wavelength couple 653–684 nm with a Shimadzu UV-3000 spectrophotometer. Permeability transition was initiated by adding 100 µM Ca²⁺.

Experimental protocol. Three kinds of perfusions were carried out as shown in Fig. 1. First, unstimulated livers were perfused for 30 min in the presence of CsA, whereupon they were homogenized for isolation of mitochondria. Second, hormone-stimulated livers were perfused for 60 min, whereupon 20 mM glucagon and 2 µM phenylephrine were added to the buffer, and the perfusion was allowed to proceed for 30 more min. CsA was added at 30 min and was present until the end of the experiment. Third, washout livers were perfused for 60 min, whereupon 20 mM glucagon and 2 µM phenylephrine were added to the buffer for 30 min. The hormones were then omitted and the experiment was continued for 30 min. CsA was added at 60 min and was present until the end of the experiment. Control perfusions were performed according to these protocols but omitting CsA. Treatment of the perfused livers with CsA for 60 min had no effect on the whole liver content of ATP and ADP (n = 5, results not shown) compared with control livers perfused in the absence of CsA. The hormone-induced uptake and release of Ca²⁺, Mg²⁺, and ATP plus ADP remained unchanged in the presence of the residual 0.012% ethanol used as solvent for CsA (n = 2).

Chemicals. CsA was a kind gift of Dr. Mari Renlund of Sandoz Oy, Finland, and [³H]CsA was purchased from Amersham. All other chemicals were of highest grade and were purchased from Sigma Chemical.

RESULTS

We first addressed the question of whether a sufficient amount of CsA was taken up by the perfused liver to induce complete inhibition of the mitochondrial PTP. For this purpose the CsA concentration in the effluent buffer from the liver was determined from its isotope content. The difference between the CsA concentration of the ingoing and outgoing buffers thus represented the amount of CsA taken up by the liver. Figure 2 shows a plot of the time course of CsA uptake from the buffer, which contained 3 µM CsA. After CsA treatment for 60 min, the liver contained 1.2 µmol CsA, corresponding to ~2 nmol/mg liver protein. In mitochondria isolated from these livers, the concentration of CsA was 460 ± 70 pmol/mg protein (n = 23), which is more than twice the amount required for complete inhibition of permeability transition, i.e., ~200 pmol/mg protein (14). On the basis of these results, we estimated that 30 min of CsA perfusion was necessary to obtain PTP inhibition; therefore CsA was added 30 min before PTP inhibition was desired in the hormone stimulation experiments.

Having shown that mitochondria isolated from CsA-treated perfused rat livers contained a sufficient amount of CsA to fully inhibit the PTP, we studied whether permeability transition was indeed inhibited in these isolated mitochondria. This is of some importance, since CsA is metabolized to a large number of metabolites, many of which are inactive on the PTP (2). Figure...
shows the results of Ca\(^{2+}\) transport and swelling experiments on mitochondria isolated from CsA-treated livers and on control mitochondria isolated from untreated livers. Figure 3A shows that control mitochondria respiring on succinate were able to accumulate Ca\(^{2+}\) from the suspension medium (control). However, after a brief lag, there was a rapid and complete release of the accumulated Ca\(^{2+}\), indicating that mitochondria underwent permeability transition. The results of light-scattering measurements shown in Fig. 3B demonstrated that mitochondria underwent large-amplitude swelling due to passive diffusion of sucrose from the medium to the matrix through the PTP (control). As expected, the addition of 1 µM CsA to these mitochondria had no effect on Ca\(^{2+}\) uptake but completely abolished its release and swelling, due to inhibition of the permeability transition (control + CsA). Similarly, mitochondria isolated from unstimulated livers (n = 2) or hormone-stimulated livers (n = 2) accumulated Ca\(^{2+}\) and underwent a CsA-sensitive permeability transition (data not shown). The same experiments were then repeated on mitochondria isolated from CsA-treated livers (CsA perfused). These mitochondria were able to accumulate Ca\(^{2+}\) from the suspension medium, but neither Ca\(^{2+}\) release nor swelling occurred, showing that they failed to undergo permeability transition (n = 3). These experiments clearly show that CsA treatment of perfused livers resulted in inhibition of the PTP in subsequently isolated mitochondria. We conclude that a sufficient amount of CsA was present in mitochondria in situ in the perfused liver to effectively inhibit opening of the PTP, should that otherwise occur.

Having demonstrated that CsA treatment of the perfused rat liver resulted in inhibition of the PTP, we proceeded to investigate the effect of CsA treatment on hormone-induced transport of ions and adenine nucleotides across the mitochondrial membrane. The results presented in Fig. 4, A–C, show that perfusing the liver with phenylephrine and glucagon for 30 min resulted in an uptake of Ca\(^{2+}\), Mg\(^{2+}\), and adenine nucleotides by mitochondria. Similarly, washout of the hormones for 30 min by perfusion with hormone-free buffer resulted in a release of Ca\(^{2+}\), Mg\(^{2+}\), and adenine nucleotides by the mitochondria. Adding CsA to the perfusion buffer 30 min before the hormones (to obtain inhibition of the PTP at the time of hormone addition) had no effect on Ca\(^{2+}\) transport and swelling experiments on mitochondria isolated from CsA-treated livers and on control mitochondria isolated from untreated livers.
the mitochondrial net uptake of Ca\(^{2+}\) and adenine nucleotides (Fig. 4, A and C, stimulation). Likewise, adding CsA at the same time as hormones (to obtain inhibition of the PTP at the point of hormone withdrawal) had no effect on the release of Ca\(^{2+}\) and adenine nucleotides (Fig. 4, A and C, washout). In contrast, CsA treatment induced an inhibition of mitochondrial Mg\(^{2+}\) uptake, whereas the release of Mg\(^{2+}\) after hormone withdrawal remained unaffected by CsA (Fig. 4B, stimulation and washout).

**DISCUSSION**

In this study we have designed conditions under which perfused rat livers are able to accumulate sufficient amounts of CsA from the perfusion medium to produce full inhibition of the PTP in subsequently isolated mitochondria. Under the reasonable assumption that the PTP was inhibited in situ, we have employed these conditions to study the mitochondrial content of Ca\(^{2+}\), Mg\(^{2+}\), and adenine nucleotides following hormonal stimulation, to address the physiological role of the PTP.

The isotope measurements demonstrated that CsA was readily accumulated in the liver during the entire loading period. The variation in CsA content observed at the end of the loading period might be related to the variations in the size of the liver lobe. The concentration of CsA in isolated mitochondria was lower than in whole liver, but the value may be an underestimate of the actual mitochondrial concentration in the intact liver, since the mitochondria were washed several times during the isolation. The lower concentration of isotope per milligram of protein in mitochondria, compared with whole liver, probably also reflected the fact...
that only a fraction of the cellular total content of cyclolphilin is associated with mitochondria (33). Because full inhibition of the PTP is observed at ~200 pmol/mg mitochondrial protein (9, 11, 14), it can reasonably be assumed that sufficient amounts of the drug were delivered to mitochondria to produce complete inhibition. The measurements of CsA uptake and of the PTP activity in isolated mitochondria thus strongly suggest that the PTP was inhibited in mitochondria in situ. To our knowledge this is the first demonstration that CsA treatment of an intact organ leads to a direct inhibition of the PTP.

Because it has been suggested that the PTP may be involved in mitochondrial Ca\(^{2+}\) transport (8, 13, 17, 21, 22, 27) and thus in the regulation of energy metabolism, we asked the question of whether CsA treatment caused any changes in ion and metabolite transport between mitochondria and the cytosol. Stimulation of the perfused liver with phenylephrine and glucagon has been shown to induce a large mitochondrial uptake of cations and adenine nucleotides that is readily measurable in isolated mitochondria (1, 3, 4, 12, 19, 31). Therefore, we studied the effect of CsA on the mitochondrial content of Ca\(^{2+}\), Mg\(^{2+}\), and adenine nucleotides 1) following a period of stimulation with phenylephrine and glucagon and 2) subsequent to washout of hormones after stimulation. By adding CsA at different times with respect to the hormones, this approach allowed us to distinguish between the effects of CsA on the net uptake of cations and adenine nucleotides, occurring during hormonal stimulation, and the effects of CsA on the net release of ions and metabolites, taking place after washout of hormones.

Stimulation of cells with agonists triggers cytosolic Ca\(^{2+}\) oscillations that are sensed by mitochondria, leading to similar oscillations in the free Ca\(^{2+}\) concentration of the mitochondrial matrix (5, 18, 23, 25, 29). The increase in free mitochondrial Ca\(^{2+}\) concentration is thought to be taking place at the beginning of an oscillation dependent on energization of the inner membrane and is mediated by the Ca\(^{2+}\) uniporter. The subsequent decrease in free matrix Ca\(^{2+}\) concentration may be due to either efflux of Ca\(^{2+}\), or binding of Ca\(^{2+}\) to sites in the matrix, or both. Efflux of Ca\(^{2+}\), mediated by the Na\(^{+}\)/Ca\(^{2+}\) exchanger (5, 29) or possibly by transient PTP opening (13, 21, 22), may be the most important mechanism for free Ca\(^{2+}\) decrease in some types of mitochondria. However, the results of this and earlier studies (1, 12) indicate that, in liver mitochondria, a part of the Ca\(^{2+}\) taken up during an oscillation is bound inside mitochondria, since their total Ca\(^{2+}\) content is increased severalfold. After the washout of hormones, the intramitochondrial total Ca\(^{2+}\) content decreased slowly to resting level by a mechanism that could have been transient PTP opening. However, since our data show that 1) the accumulation of Ca\(^{2+}\) by mitochondria during hormone treatment was not increased in the presence of CsA and 2) the release of Ca\(^{2+}\) following washout was not inhibited by CsA, we conclude that mitochondrial Ca\(^{2+}\) efflux was independent of the PTP. We thus found no evidence for a direct role of the PTP in regulation of mitochondrial total Ca\(^{2+}\) during the agonist-induced Ca\(^{2+}\) signaling process. In fact, the observed efflux rate may be fully accounted for by Ca\(^{2+}\)/Na\(^{+}\) or Ca\(^{2+}\)/H\(^{+}\) exchange, provided that there was no cycling of Ca\(^{2+}\) across the mitochondrial membrane following hormone washout. These findings raise questions concerning the role of the low-conductance state of the PTP. If the low-conductance state is of importance in some Ca\(^{2+}\) signaling process during normal cell life, then it would be logical to expect this state to be regulated differently from the “classic” high-conductance state thought to be implicated in cell death. However, little or no experimental documentation has been obtained supporting the existence of a differential regulation of the conductance states, and further work is thus needed to clarify this important issue.

CsA did not induce any changes in the mitochondrial uptake and release cycle of adenine nucleotides, suggesting that short-time metabolic signaling to mitochondria occurs in a normal way in the presence of CsA. Similarly, we found that, in the presence of CsA, the efflux of Mg\(^{2+}\) following hormone washout remained unchanged and apparently was not mediated by the PTP. The lack of mitochondrial Mg\(^{2+}\) uptake in the presence of CsA may be related to known effects of CsA on the whole cell content of Mg\(^{2+}\) (6).

The PTP may play a role in cell death, particularly in neuronal damage (15, 30), and therefore the demonstration that CsA administration to an intact organ leads to rapid and efficient inhibition of the PTP may be of use in developing strategies to prevent and treat cell and tissue damage.

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