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THE NONSPECIFIC INNER MEMBRANE PORE OF LIVER MITOCHONDRIA: MODULATION OF CYCLOSPORIN SENSITIVITY BY ADP AT CARBOXYATRACTYLOSIDE-SENSITIVE AND INSENSITIVE SITES

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Cyclosporin A prevents the opening of a nonspecific pore in the inner membrane of liver mitochondria when added prior to Ca^{2+}. In the presence of 10 μM Ca^{2+} cyclosporin is unable to close the pore and restore the original permeability unless ADP is also added. ADP acts at a high-affinity site (K_m 5 μM), corresponding to the adenine nucleotide transporter. This effect of ADP is prevented and reversed by carboxyatractyloside. In the presence of carboxyatractyloside, cyclosporin added with higher concentrations of ADP (K_m 70 μM) also can close the pore. This suggests that a lower-affinity ADP-binding component as well as cyclophilin and the adenine nucleotide transporter can modulate the sensitivity of the pore to cyclosporin.

In addition to a number of highly selective ion carriers, the inner membrane of the mitochondrion contains a system which permits nonspecific distribution of compounds with M_r < 1500 [1,2]. This so-called nonspecific pore has an internal diameter of about 2-3 nm [3-5] and has recently been identified with the 1.3 nS megachannel seen in patch-clamp studies with mitoplasts [6]. Pore opening is reversible and associated with Ca^{2+} binding at an intramitochondrial trigger site [7,8]. Cyclosporin A is a potent inhibitor that can prevent pore opening at very low concentrations [9-11] and can also close the open pore under some conditions [12,13]. This has led to the postulate that cyclosporin reacts with the pore itself or with a component directly involved in pore regulation [9-11]. Recently, there have been indications that the action of cyclosporin on the pore may be modulated by [Ca^{2+}], by adenine nucleotides, and by the redox state of mitochondrial thiols [13,14]. In the present work the ability of ADP to modify the sensitivity of the pore to cyclosporin was examined and two classes of ADP-binding sites have been identified.

The abbreviations used are as follows: cATR, carboxyatractyloside; ANT, adenine nucleotide translocator; $\Delta\psi$, mitochondrial membrane potential; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; TPP^+, tetraphenylphosphonium ion; CSA, cyclosporin A.

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METHODS

Rat liver mitochondria were prepared as previously described [15] in a medium of sucrose (70 mM), mannitol (210 mM), HEPES (5 mM, pH 7.4 with KOH), bovine serum albumin (0.5%) and EGTA (0.5 mM). The final washing was performed in the same medium but without EGTA or albumin. Protein concentration was determined by the biuret method using bovine serum albumin as a standard. Membrane potential ($\Delta\psi$) and mitochondrial swelling were monitored simultaneously at 25^{0} in a stirred cell. Membrane potential was followed by the accumulation of TPP^{+} using a TPP^{+}-selective electrode [16] and swelling by absorbance at 520 nm using a Brinkman probe colorimeter. Mitochondria (0.2 mg/ml) were incubated in a medium of sucrose (250 mM), HEPES (10 mM, pH 7.4 with Tris), succinate (5 mM), P_i (5 mM), TPP^{+} chloride (2 μM) and rotenone (2 μM). Cyclosporin A was a gift from Dr. David L. Winter of Sandoz.

RESULTS AND DISCUSSION

Addition of Ca^{2+} (10 μM) to liver mitochondria in the presence of P_i leads to a rapid drop in $\Delta\psi$ due to Ca^{2+} accumulation, followed by a slower spontaneous decrease in $\Delta\psi$ (Fig.1A, trace 3). This second phase is accompanied by high-amplitude swelling which is indicative of nonspecific permeability (Fig. 1B, trace 3). Cyclosporin completely prevents this loss of $\Delta\psi$ and swelling when added prior to Ca^{2+}, but does not prevent Ca^{2+} accumulation (Fig. 1A and B, trace 1).

The mitochondrial population does not respond homogeneously during the permeability transition [see 1,2]. A more susceptable sub-population of mitochondria appears to undergo the transition initially and the Ca^{2+} released from this fraction becomes available for accumulation by the more stable sub-population. As these in turn become destabilized the entire population moves toward the open-pore state. Addition of cyclosporin at a stage when over 50% of the mitochondria population is in the open-pore state not only arrests furthur transition (Fig. 1B, trace 2) but restores the original permeability of the entire population and permits restoration of $\Delta\psi$ (Fig. 1A, trace 2). Addition of cATR to mitochondria resealed with cyclosporin produces a rapid return to the open-pore state (Fig. 1, trace 2). In contrast, cATR has no effect on mitochondria in which the permeability transition has been prevented by cyclosporin (Fig. 1, trace 1). In addition, cyclosporin is unable to reverse the transition in mitochondria that have been in the open-pore state for a short time (Fig. 1, trace 3). This loss of sensitivity to cyclosporin has also been reported in mitochondria loaded with Ca^{2+} and challenged with t-butylhydroperoxide [17]. Insensitivity to cyclosporin could result from the opening of a second nonspecific pathway as a result of phospholipid hydrolysis [17], but it is also possible that some factor that modulates the sensitivity of the pore to cyclosporin is lost from the matrix in the open-pore state.

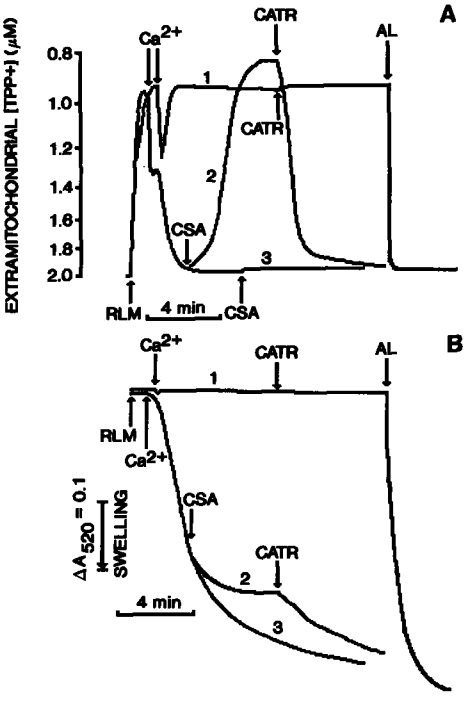

Fig. 1. The influence of cyclosporin A (CSA) and of carboxyatractyloside (CATR) on the Ca^{2+}-induced loss of $\Delta\psi$ (A) and high-amplitude swelling (B). Mitochondria were incubated as described under Methods. Trace 1: CSA (1 μM) was present initially. Where indicated, $CaCl_2$ (10 μM or 50 nmol/mg) and CATR (2.5 μM) were added. Alamethicin, a pore-forming peptide (AL, 7 μg/ml) was added to induce permeabilization. Traces 2 and 3: CSA was omitted initially and added where indicated. Other additions as for Trace 1.

The latter possibility gains support from the sensitivity of the cyclosporin effect to changes in the conformational state of the ANT as the transition progresses. cATR stabilizes the ANT in the c-configuration [18] and can fully reverses the effect of cyclosporin on the restoration of $\Delta\psi$ (Fig. 1A, trace 2). There have been indications that ADP, which is rapidly released from mitochondria in the open-pore state, may modulate the interaction of cyclosporin with the pore or a regulatory component [14]. The relationship between the effects of ADP and cyclosporin on the state of the pore is explored further in the study shown in Fig. 2. Addition of ADP at concentrations up to 100 μM fails to restore $\Delta\psi$ in mitochondria that have undergone the permeability transition (trace 5). Cyclosporin alone has little effect in these mitochondria (trace 4), but the combination of cyclosporin and ADP restores $\Delta\psi$ effectively (traces 1-3). Both the extent of restoration of $\Delta\psi$ and the response to cATR depend on the concentration of ADP. ADP at 100 μM (trace 1) restores $\Delta\psi$ more effectively than 10 μM ADP (trace 2). Addition of cATR causes a rapid return to the open-pore state in the presence of the lower concentration of ADP (trace 2), but has only a slight effect at 100 μM ADP

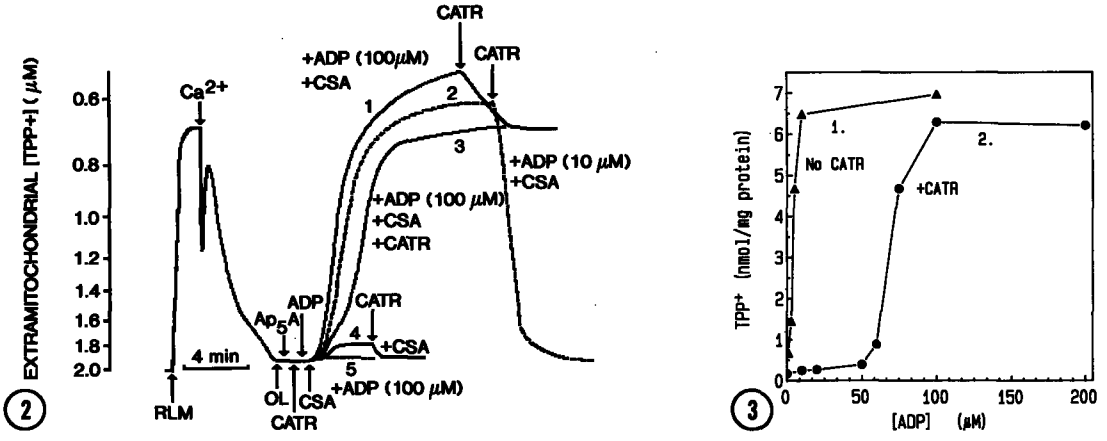

Fig. 2. The influence of ADP on the cyclosporin (CSA)-induced restoration of $\Delta\psi$ in Ca^{2+}-deenergized liver mitochondria. Mitochondria were incubated as described in Fig. 1. Where indicated the following additions were made: oligomycin (OL, 5 μg/mg), P^1, P^5-di (adenosine-5′)pentaphosphate (Ap_5A, 50 μM; added to inhibit adenylate kinase), CATR (5 μM), ADP (10 μM in Trace 2; 100 μM Traces 1,3 and 5; omitted in Trace 4), and CSA (1 μM in Traces 1-4; omitted in Trace 5).

Fig. 3. Restoration of $\Delta\psi$ by cyclosporin as a function of ADP concentration. Experiments followed the protocol of Fig. 2, Traces 1 and 2 in which deenergized mitochondria are treated with varying amounts of ADP, followed by CSA (1 μM). The accumulation of TPP^+ as an indication of $\Delta\psi$ is plotted vs [ADP] in the absence of CATR (Trace 1). The effect of ADP on $\Delta\psi$ in the presence of CATR (5 μM) is shown in trace 2.

(trace 1). cATR added before ADP and cyclosporin (trace 3) produces virtually the same final $\Delta\psi$ as is seen with the opposite order of addition (trace 1).

The ability of ADP to restore $\Delta\psi$ in the presence of cyclosporin can be expressed as an accumulation of TPP^+ [12]. Plots of TPP^+ uptake vs ADP concentration in the presence and absence of cATR (Fig. 3) indicate that two different binding sites for ADP are involved. In the absence of cATR the $\Delta\psi$ is restored by low concentrations of ADP with a half-maximal effect at 5 μM (Fig. 3, trace 1). This compares well with the K_m of the ANT [18] and the rapid reversal of pore closure by cATR (Fig. 2, trace 2) under these conditions establishes that the adenine nucleotide translocator is involved in modulation of the sensitivity of the pore to cyclosporin. Higher concentrations of ADP promote the restoration of $\Delta\psi$ by cyclosporin in the presence of cATR (Fig. 3, trace 2) with a half-maximal effect at about 70 μM. ADP at 100 μM confers full sensitivity to cyclosporin to the nonspecific pore. ATP at 100 μM and AMP at 300 μM are without effect in these protocols, but higher levels of ATP (300 μM) permit the cATR-insensitive restoration of $\Delta\psi$ by cyclosporin (not shown). ATP at 300 μM has no effect in the absence of cyclosporin. There is a gradual increase in the concentration of ADP needed to permit full restoration of $\Delta\psi$ as the time interval between the completion of the permeability transition and the addition of ADP is increased in the

protocol of Fig 2 (not shown). This indicates that the K_m for ADP at the cATR-insensitive site increases with time when mitochondria are in the open-pore state.

It has been proposed that the permeability transition is triggered by the formation of a complex between the ANT and the matrix protein, cyclophilin (peptidyl-prolyl cis/trans isomerase) [11]. Cyclosporin is thought to inhibit pore opening by interacting with cyclophilin and dissociating the complex [11]. In the model of Halestrap and Davison [11] Ca^{2+} interaction with ANT induces a conformational change that permits interaction with cyclophilin and opening of the pore. ADP reverses this conformational change and the ADP effect is prevented by cATR [11]. The present studies do not exclude such a role for ANT. However, it is clear that higher levels of ADP can modulate the ability of cyclosporin to bring about closure of the pore and this closure can occur when the ANT is locked in the c-configuration by cATR (Fig 3). This suggests that a low-affinity ADP-binding component, as well as ANT, may modulate the interaction of cyclosporin with the pore. The presence of an additional ADP-binding component has also been suggested by studies of an adenine nucleotide-induced contraction of the inner membrane [19] and by effects of ADP on mitochondrial permeability in the absence of cyclosporin [8,20]. Further work will be necessary to define the nature and exact role of this low-affinity ADP-binding component.

An addition conclusion of this study is that cyclosporin-sensitivity is not always a reliable criterion for solute flux through the Ca^{2+}-dependent inner membrane pore. It is clear that the sensitivity of the pore to cyclosporin is highly dependent on the adenine nucleotide status of the mitochondria as well as other factors, such as the concentration of Ca^{2+} [13] and the redox state of thiol groups [14].

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