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CYCLOSPORINE A INHIBITS MITOCHONDRIAL PYRIDINE NUCLEOTIDE HYDROLYSIS AND CALCIUM RELEASE

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Abstract—Mitochondria participate in the maintenance of cellular Ca^{2+} homeostasis. Here we show that the immunosuppressive drug cyclosporine A at low concentrations inhibits release but not uptake of Ca^{2+} by mitochondria. Prevention of Ca^{2+} release is due to inhibition of intramitochondrial enzymatic hydrolysis of NAD to ADP-ribose and nicotinamide. These findings suggest a mechanism by which cyclosporine A interferes with cellular Ca^{2+} homeostasis, and may be related to the immunosuppressive and cytotoxic properties of the drug.

Intracellular Ca^{2+} is of central importance for many vital cellular processes such as cell growth and division, signal transduction, movement, secretion and short-term enzyme regulation [1]. To this end, fine tuning of its concentration is required. The ionized cytosolic Ca^{2+} in resting cells is kept between 0.1 and 0.2 μM against millimolar concentrations in extracellular fluids. The intracellular Ca^{2+} concentration is modulated by binding the ion to nonmembraneous proteins, and by the operation of membrane-bound Ca^{2+} transport systems. The existence of the latter has been established in the plasma membrane, the endoplasmic (sarcoplasmic) reticulum, and in the mitochondria. The transport systems differ in calcium affinity and transport capacity, and probably also in importance for the maintenance and development of physiological and pathological states of the cell [2]. Due to their high calcium storage capacity combined with a relatively low calcium affinity mitochondria act as safety devices against toxic increases of cytosolic Ca^{2+} [3].

Ca^{2+} enters and leaves energized mitochondria by separate routes. It is taken up electrophoretically in response to the mitochondrial membrane potential, negative inside, via a site that can be inhibited by ruthenium red. Release of Ca^{2+} is electroneutral and, as shown mainly with various prooxidants, most likely regulated to protein mono(ADP-ribosylation) (reviewed in Ref. 3). However, even without added prooxidants this regulatory mechanism appears to operate [4].

Cyclosporine A, a cyclic unadecapeptide of the fungus *Tolypocladium inflatum Gams*, has several pharmacologic properties including antiparasitic and antimalarial activities as well as the potential for reversing multidrug resistance in tumors. Most important and clinically most relevant is its unique immunosuppressive effects on certain immunocompetent cells, and the nephrotoxicity encountered upon cyclosporine A treatment. Whereas a good deal has been learnt about the cell biological aspects of cyclosporine A [5, 6], the biochemical basis of the drug's action remains unexplored. We now report that cyclosporine A prevents Ca^{2+} release from mitochondria by inhibiting the intramitochondrial hydrolysis of NAD to ADP-ribose and nicotinamide, and discuss possible consequences of this inhibition on cellular Ca^{2+} homeostasis as related to cyclosporine A's cytotoxicity and immunosuppression.

MATERIALS AND METHODS

Isolation of mitochondria. Female Wistar rats (180–250 g) were fasted overnight and killed by decapitation. Liver mitochondria were isolated by a conventional differential centrifugation method using 210 mM mannitol, 70 mM sucrose, 5 mM Hepes [4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid†], pH 7.4 (MSH buffer), and 1 mM EDTA as isolation medium. Mitochondria were washed twice in MSH buffer. The protein content of the final mitochondrial suspension was determined by the biuret method with bovine serum albumin as standard.

Labeling of mitochondrial pyridine nucleotides in vivo. Overnight fasted rats were injected intravenously with [carboxyl-^{14}C]nicotinic acid (12.5 μCi, 0.223 μmol). After 3 hr, the animals were killed and liver mitochondria isolated [7].

Standard incubation procedure. Mitochondria, 2 mg of protein/mL, were incubated at 25° in 3 mL of MSH buffer under constant stirring. When appropriate, cyclosporine A was added at the indicated concentrations. A fine jet of oxygen was blown onto the surface of the suspension to prevent anaerobiosis. Reduction of mitochondrial pyridine nucleotides and release of endogenous Ca^{2+} was induced by 5 μM rotenone. Mitochondria were then energized with 2.5 mM K^+-succinate. Ca^{2+} (60 nmol/mg of protein) was added and Ca^{2+} uptake allowed to proceed for 2 min. Finally, *t*-butylhydroxyperoxide or alloxan

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† Abbreviations: Hepes, [4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid]; MSH buffer, 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4; ruthenium red, $Ru_2(OH)_2Cl_4 \cdot 7NH_3 \cdot 3H_2O$; arsenazo III, 2,2'-(1,8-dihydroxy-3,6-disulfonaphthalene-2,7-bis-azo)bis(benzenearsenic acid).

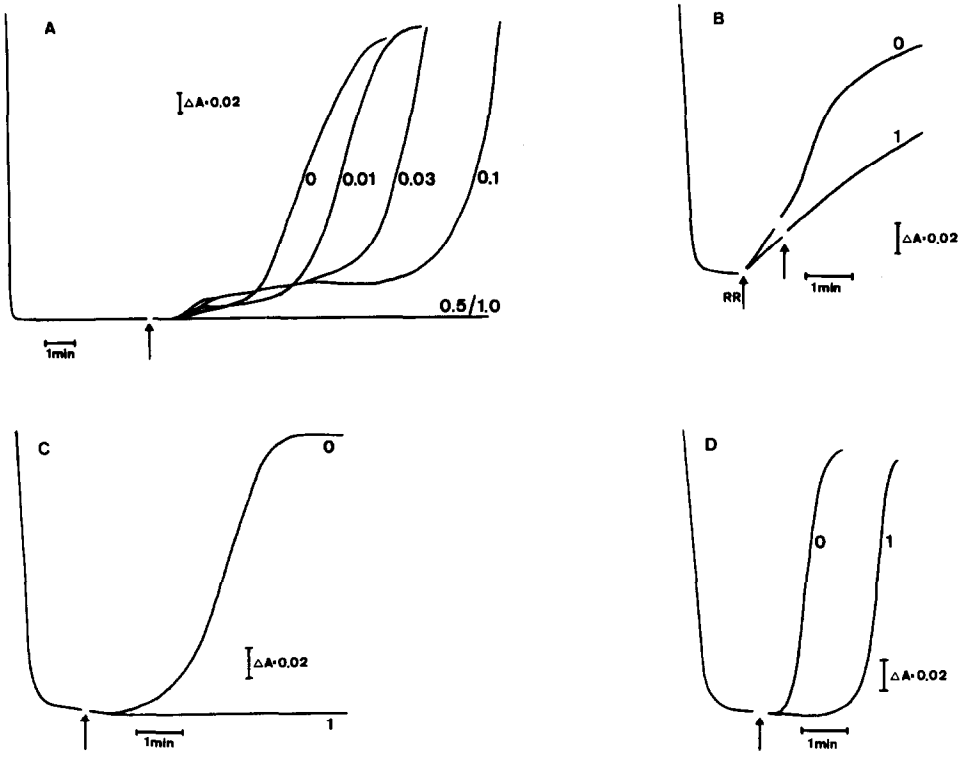

Fig. 1. Prooxidant-induced release of Ca^{2+} from rat liver mitochondria. In the presence of arsenazo III, mitochondria were loaded with 60 nmol Ca^{2+}/mg of protein. (A) At the arrow, Ca^{2+} release was initiated by the addition of 100 μM *t*-butylhydroperoxide. (B) At the left arrow, 2 nmol of ruthenium red (RR)/mg of protein, followed by 100 μM *t*-butylhydroperoxide (right arrow) were added. (C) and (D) At the arrow, Ca^{2+} release was initiated by the addition of 1.7 (C) or 5 (D) mM alloxan. Ca^{2+} movements across the inner mitochondrial membrane were followed spectrophotometrically at 685–675 nm. Numbers next to the traces indicate cyclosporine A (μM).

were added (time zero), preceded, when appropriate, by 2 nmol ruthenium red/mg of protein.

Determination of Ca^{2+} *uptake and release by mitochondria.* Ca^{2+} movements across the inner mitochondrial membrane were followed by dual wavelength spectrophotometry in MSH buffer containing 50 μM arsenazo III [8].

Determination of mitochondrial pyridine nucleotides. Mitochondrial pyridine nucleotides were determined spectrophotometrically in an Aminco DW 2A spectrophotometer at 340–370 nm.

Determination of nicotinamide release. Pyridine nucleotides were labeled *in vivo* at the nicotinamide moiety [7]. Mitochondria from the livers of these animals were incubated according to the standard procedure, and at zero time *t*-butylhydroperoxide was added. Release of nicotinamide was followed by determining the radioactivity remaining intramitochondrially. To this end, 150 μL aliquots were withdrawn at the times indicated, filtered through Millipore filters (0.45 μm pore size), and rinsed with 150 μL of cold MSH buffer. The radioactivity remaining on the filters was determined in a liquid scintillation counter.

RESULTS

Figure 1 reports uptake of Ca^{2+} by energized rat liver mitochondria followed by prooxidant-induced Ca^{2+} release. Cyclosporine A does not affect Ca^{2+} uptake. However, the *t*-butylhydroperoxide-induced release, which requires the concerted operation of glutathione peroxidase, glutathione reductase and the energy-linked transhydrogenase in mitochondria [3], is very sensitive to nanomolar concentrations of cyclosporine A (Fig. 1A). When ruthenium red is added to mitochondria after completion of Ca^{2+} uptake (Fig. 1B), i.e., when re-uptake of any released Ca^{2+} ("Ca^{2+} cycling" [9]) is prevented, net Ca^{2+} release is observed even without addition of *t*-butylhydroperoxide. This "spontaneous" release can to a certain extent be prevented by cyclosporine A. In accordance with previous findings [10] *t*-butylhydroperoxide causes also, in the presence of ruthenium red, a stimulation of the Ca^{2+} release pathway. The stimulation is totally prevented by cyclosporine A (Fig. 1B).

Like *t*-butylhydroperoxide, the prooxidant alloxan induces net Ca^{2+} release from intact mitochondria by activation of the specific pyridine nucleotide-dependent release pathway. However, alloxan does not engage the glutathione peroxidase/transhydrogenase enzyme cascade. Rather, this prooxidant oxidizes intramitochondrial pyridine nucleotides predominantly non-enzymatically [8]. Figures 1C and D show that 1 μM cyclosporine A prevents completely

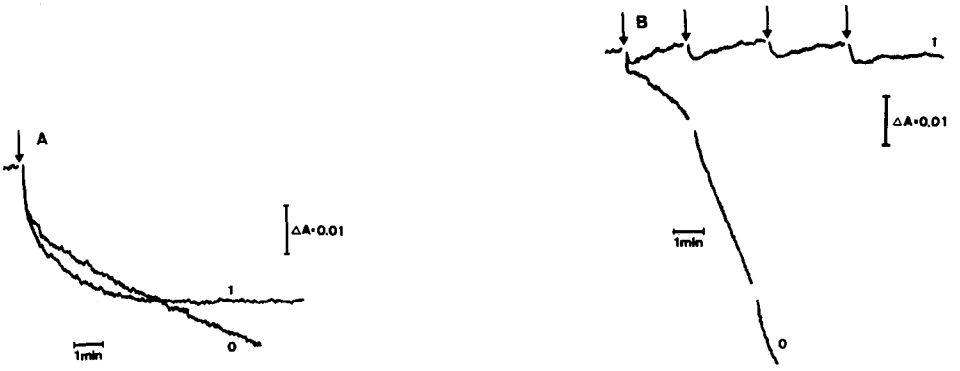

Fig. 2. Changes in the redox level of mitochondrial pyridine nucleotides. The redox level of mitochondrial pyridine nucleotides was followed spectrophotometrically at 340–370 nm. (A) Mitochondria were energized with succinate, and no Ca^{2+} was added. At the arrow, 100 μM *t*-butylhydroperoxide was added. (B) Mitochondria were energized with succinate and loaded with 60 nmol Ca^{2+}/mg of protein. At the arrows, successive pulses of 10 nmol *t*-butylhydroperoxide were added. Numbers next to the traces indicate cyclosporine A (μM).

Ca^{2+} release induced by 1.7 mM, and retards the release induced by 5 mM alloxan.

Pyridine nucleotide oxidation and hydrolysis is a prerequisite for the prooxidant-induced mitochondrial Ca^{2+} release, with hydrolysis requiring intramitochondrial Ca^{2+} [3]. Since *t*-butylhydroperoxide and alloxan cause pyridine nucleotide oxidation by different pathways (see above), the inhibition of Ca^{2+} release induced by either of these compounds suggested that cyclosporine A does not interfere with the prooxidant-induced pyridine nucleotide oxidation. Indeed, the drug clearly does not slow down the initial rate of oxidation induced by *t*-butylhydroperoxide, provided that mitochondria are not loaded with Ca^{2+} (Fig. 2A). The reason for the slightly larger extent of oxidation in the absence of cyclosporine A (Fig. 2A) was not explored. It most probably reflects the very limited hydrolysis of pyridine nucleotides that already takes place in the presence of endogenous Ca^{2+} [3]. Also the oxidation of pyridine nucleotides by alloxan (not shown) was not affected by cyclosporine A.

In Ca^{2+}-loaded mitochondria, intramitochondrial pyridine nucleotides are extensively irreversibly oxidized due to Ca^{2+}-dependent enzymatic splitting of NAD to ADP-ribose and nicotinamide [3, 11]. As reported previously [11], hydrolysis can be provoked in a step-wise manner when small pulses of prooxidant are used repeatedly. This is shown in the control experiment in Fig. 2B (lower trace), where small quantities of *t*-butylhydroperoxide cause a progressive and irreversible loss of reduced pyridine nucleotides from Ca^{2+}-loaded mitochondria. In the presence of 1 μM cyclosporine A the oxidation is reversible, indicating absence of pyridine nucleotide hydrolysis under these conditions (Fig. 2B, upper trace).

As mentioned, the spectrophotometric analysis of intramitochondrial pyridine nucleotides strongly suggested that cyclosporine A inhibits the prooxidant-induced Ca^{2+} release by preventing pyridine nucleotide hydrolysis. To give unequivocal proof of this we measured release of nicotinamide from mitochondria as indicator for intramitochondrial pyridine nucleotide hydrolysis [7] (Fig. 3). In the absence but not in the presence of 1 μM cyclosporine A extensive hydrolysis is observed. Fifty per cent inhibition of hydrolysis was achieved with about 0.05 μM, i.e., 25 pmol of cyclosporine A/mg of mitochondrial protein.

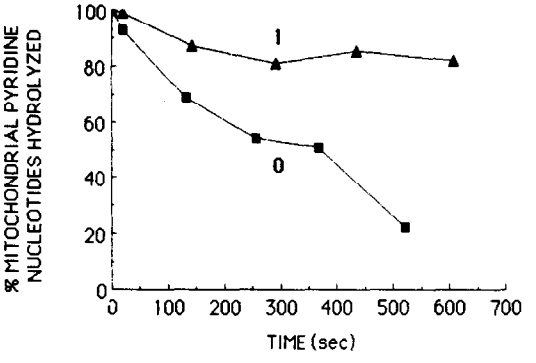

Fig. 3. Hydrolysis of pyridine nucleotides in rat liver mitochondria. Intramitochondrial pyridine nucleotides were labelled *in vivo* at the nicotinamide moiety. Incubation was according to the standard procedure. Mitochondria were loaded with 60 nmol of Ca^{2+}/mg of protein. At zero time, 100 μM *t*-butylhydroperoxide was added. The release of nicotinamide from mitochondria was measured by Millipore filtration. Numbers next to the traces indicate cyclosporine A (μM).

DISCUSSION

There is convincing evidence that protein mono(ADP-ribosylation) regulates Ca^{2+} release from intact respiring mitochondria. Release requires both oxidation of pyridine nucleotides and hydrolysis to ADP-ribose and nicotinamide [2], and is accompanied by a transient modification of intramitochondrial proteins with mono(ADP-ribose) [12]. As shown in the present report, cyclosporine

A inhibits the prooxidant-induced as well as the "spontaneous" Ca^{2+} release from mitochondria by preventing the initial reaction during protein ADP-ribosylation, i.e., pyridine nucleotide hydrolysis. The action of cyclosporine A is, therefore, comparable to that of ATP which also inhibits pyridine nucleotide hydrolysis [13], but different from that of the other known Ca^{2+} release inhibitor, *meta*-iodobenzylguanidine, which inhibits presumably by competing with the natural acceptor proteins for ADP-ribose [4].

Our data confirm and extend previous experiments in which cyclosporine A promoted Ca^{2+} retention in mitochondria [14], inhibited *t*-butylhydroperoxide-induced Ca^{2+} release from heart mitochondria [15], or Ca^{2+}-dependent mitochondrial swelling induced by a variety of agents [16]. They are also accordant with the increased content of the mitochondrial Ca^{2+} pool in isolated hepatocytes treated with cyclosporine A [17].

Our data may also explain the cytotoxicity of cyclosporine A. Since several mitochondrial dehydrogenases are regulated by micromolar concentrations of Ca^{2+} [18], long-term overloading of mitochondria with Ca^{2+} due to a deactivated release pathway may severely compromise these enzymes. In addition, ATP synthesis is inhibited by increased amounts of mitochondrial Ca^{2+} [19]. Cyclosporine A may, therefore, be cytotoxic by shutting off the cellular energy supply.

As to immunosuppression by cyclosporine A it should be recalled that lymphocyte proliferation depends on a sustained increase in the cytosolic Ca^{2+} level. Whether the inhibition by cyclosporine A of mitochondrial pyridine nucleotide hydrolysis and Ca^{2+} release is related to immunosuppression and altered lymphocyte function induced by this compound requires further investigation. Therefore, future studies will be directed towards the effect of cyclosporine A on mitochondrial Ca^{2+} transport in lymphocytes.

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