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SENSITIVITY OF MITOCHONDRIAL PEPTIDYL-PROLYL *CIS–TRANS* ISOMERASE, PYRIDINE NUCLEOTIDE HYDROLYSIS AND Ca^{2+} RELEASE TO CYCLOSPORINE A AND RELATED COMPOUNDS

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Abstract—Prooxidants activate a specific Ca^{2+} release pathway from mitochondria. Here we investigate the inhibitory potency of cyclosporine A and six related compounds with respect to peptidyl-prolyl *cis–trans* isomerase (PPIase), pyridine nucleotide hydrolysis and Ca^{2+} release. Whereas the absolute inhibitory potency of the compounds varies by about three orders of magnitude, a given compound is always most effective on PPIase, followed by pyridine nucleotide hydrolysis, and least effective in Ca^{2+} release inhibition. The data show that pyridine nucleotide hydrolysis is a prerequisite but not a consequence of Ca^{2+} release. They also strongly suggest that PPIase participates in the Ca^{2+} release mechanism from intact mitochondria by regulating the intramitochondrial NAD^+ glycohydrolase, and thereby ascribe a physiological function to the protein. Furthermore, a complete lack of correlation between the inhibitory potencies described here and the reported immunosuppressive activities of the drugs is evident.

Transport of Ca^{2+} through the inner mitochondrial membrane has been studied for more than 3 decades, but a detailed understanding of it is not yet achieved. This is mainly because no protein component involved in Ca^{2+} uptake or release has been identified with certainty.

We have shown previously that prooxidants stimulate Ca^{2+} release from intact mitochondria (reviewed in Ref. 1). Hydrolysis of NAD^+ to ADPribose and nicotinamide is necessary but not sufficient for the prooxidant-induced Ca^{2+} release, and there is evidence that protein mono(ADP-ribosyl)ation regulates the release pathway. We also demonstrated recently that this pathway is specific because it allows Ca^{2+}, but not K^+ or sucrose to cross the inner mitochondrial membrane [2].

Here we show that seven different immunosuppressants, six cyclosporine A (CSA†) derivatives and FK506, have the same relative inhibitory potency towards mitochondrial peptidyl-prolyl *cis–trans* isomerase (PPIase), prooxidant-dependent pyridine nucleotide hydrolysis and Ca^{2+} release. We conclude that PPIase is a protein involved in the Ca^{2+} release mechanism. Our results also show that pyridine nucleotide hydrolysis is likely to be a prerequisite and not a consequence of mitochondrial Ca^{2+} release.

MATERIALS AND METHODS

Materials. CSA and related substances, namely MeVal-4-CS, CSG, dihydro-CSD, D-Lys-8-CS and CSH were a gift of Sandoz Pharma Preclinical Research, Basel, Switzerland, and FK506 was a gift of Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan. They were stored in solid form at −20° and dissolved in ethanol immediately prior to use. All other chemicals were purchased from standard suppliers, and were of the highest purity commercially available.

Isolation of mitochondria. The isolation of rat liver mitochondria was performed by differential centrifugation as described [2].

Preparation of mitochondrial matrix fraction. The matrix fraction of liver mitochondria was prepared by digitonin treatment and sonication as described [3].

Standard incubation procedure. Mitochondria (2 mg protein/mL) were incubated at 25° in 3 mL of 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4 (MSH buffer) containing 5 μM rotenone and 2.5 mM potassium succinate with continuous stirring and oxygenation. CSA or related compounds dissolved in ethanol were added 30 sec before rotenone. The highest ethanol concentration used was 0.35%, low enough not to affect the results. Ca^{2+} was then added to give a total load of 45 nmol/mg mitochondrial protein, and Ca^{2+} uptake was allowed to proceed for 2 min. Finally, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) alone or in combination with *t*-butylhydroperoxide (tbh) was added at time zero as indicated in the figures.

Determination of Ca^{2+} *uptake and release by mitochondria.* The standard incubation procedure was followed. Ca^{2+} movements across the inner

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† Abbreviations: CSA, cyclosporine A; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; tbh, *t*-butylhydroperoxide; PPIase, peptidyl-prolyl *cis–trans* isomerase.

mitochondrial membrane were monitored by the isotope technique as described [2].

Determination of the reversibility of pyridine nucleotide oxidation as an indicator of pyridine nucleotide hydrolysis. The standard incubation procedure was followed. The redox state of mitochondrial pyridine nucleotides was determined spectrophotometrically in an Aminco DW2A spectrophotometer at 340–370 nm. The extent of pyridine nucleotide hydrolysis was judged from the extent of their re-reduction [4].

Determination of PPIase activity. The assay was performed as described in Ref. 5 with the following modifications: the total volume was 3 mL, the concentration of mitochondrial matrix proteins was 33 μg/mL, the temperature was 6°, and the spectrophotometer was operated in the dual mode at 384–460 nm. The changes in absorption between 30 and 60 sec after addition of the substrate in the absence or presence of matrix fraction (corresponding to 0 and 100% PPIase activity, respectively) and in the presence of matrix fraction together with the inhibitor were used for the calculation of PPIase activity.

Determination of IC_{50}. (i) Ca^{2+} release: the quantity of Ca^{2+} remaining in mitochondria 10 min after initiation of its release was used to calculate the inhibition. The value obtained in the presence of 1 μM CSA (used in each experiment as a standard) was taken as 100% inhibition (cf. Refs 6 and 7), that obtained in the absence of inhibitor as 0% inhibition. The value of 50% inhibition was determined by using six to eight different inhibitor concentrations followed by graphical interpolation of the results thus obtained. (ii) Reversibility of pyridine nucleotide oxidation: pyridine nucleotides remaining after complete consumption of tbh (judged from pyridine nucleotide re-reduction; cf. Fig. 2A) were determined by measuring the absorbance difference of the mitochondrial suspension at 340–370 nm. Complete re-reduction was achieved 10–15 min after EGTA and tbh addition, the variation of time depending on the intramitochondrial glutathione content [8]. As for Ca^{2+} release (see above), 1 μM CSA was used to achieve 100% inhibition, and the value obtained in the absence of inhibitor was taken as 0% inhibition. The potency of the inhibitors was calculated as described for Ca^{2+} release. (iii) PPIase inhibition was determined analogously.

Ca^{2+} release and pyridine nucleotide hydrolysis studies were done in parallel on identical mitochondrial preparations.

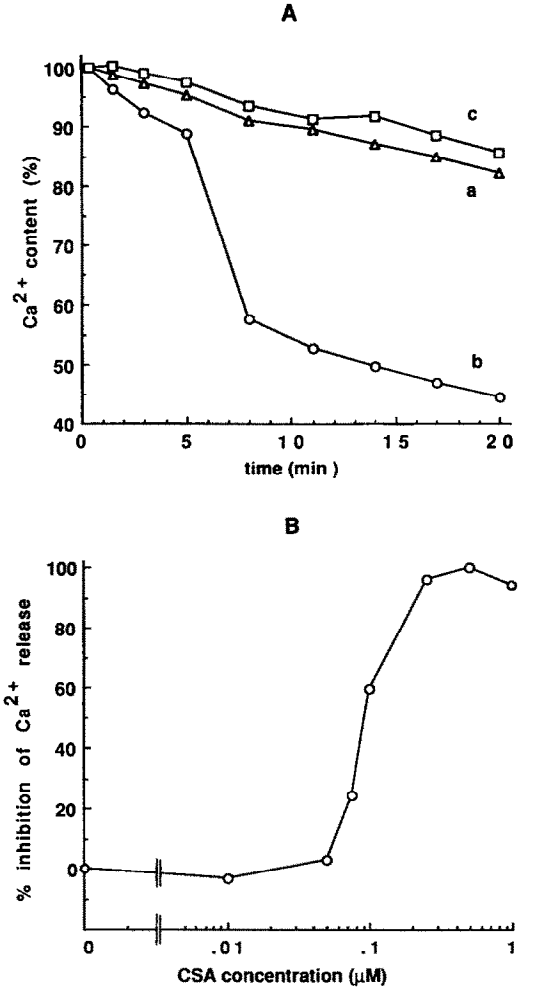

Fig. 1. tbh-induced Ca^{2+} release from rat liver mitochondria in the presence or absence of cyclosporine A. Mitochondria were incubated under standard conditions. (A) Ca^{2+} release was started (time 0 min) by the addition of 10 mM EGTA (trace a), or 100 μM tbh plus 10 mM EGTA (trace b). Trace c: conditions as for trace b except for the presence of 1 μM cyclosporine A. 100% Ca^{2+} corresponds to 45 nmol Ca^{2+}/mg protein. (B) Per cent inhibition of Ca^{2+} release determined as described in Materials and Methods. The results shown are from one experiment typical of four.

RESULTS

When the Ca^{2+} chelator EGTA is added to mitochondria energized with succinate and loaded with Ca^{2+}, release of the ion from mitochondria occurs (Fig. 1A, trace a). This release is stimulated by the prooxidant tbh (Fig. 1A, trace b). In the presence of CSA (Fig. 1A, trace c) the tbh-dependent Ca^{2+} release is totally prevented, and mitochondria retain the ion at levels even higher than in the presence of EGTA alone. These results are in accordance with our previous reports that CSA prevents Ca^{2+} release from rat liver and kidney mitochondria [6, 7]. As shown in Fig. 1B, inhibition of Ca^{2+} release is dependent on the CSA concentration up to about 0.5–1 μM, with 50% inhibition (IC_{50}) reached at about 104 nM.

CSA inhibits mitochondrial Ca^{2+} release by preventing NAD^+ hydrolysis [6, 7]. Accordingly, we analysed spectrophotometrically in Ca^{2+}-loaded mitochondria to what extent the oxidation of pyridine nucleotides is reversible in order to gauge their hydrolysis (Fig. 2A). CSA inhibits the hydrolysis in a concentration-dependent manner, with IC_{50} reached at about 81 nM (Fig. 2B).

The mitochondrial PPIase activity cannot presently be measured in intact mitochondria, but with an artificial substrate in mitochondrial matrix extracts.

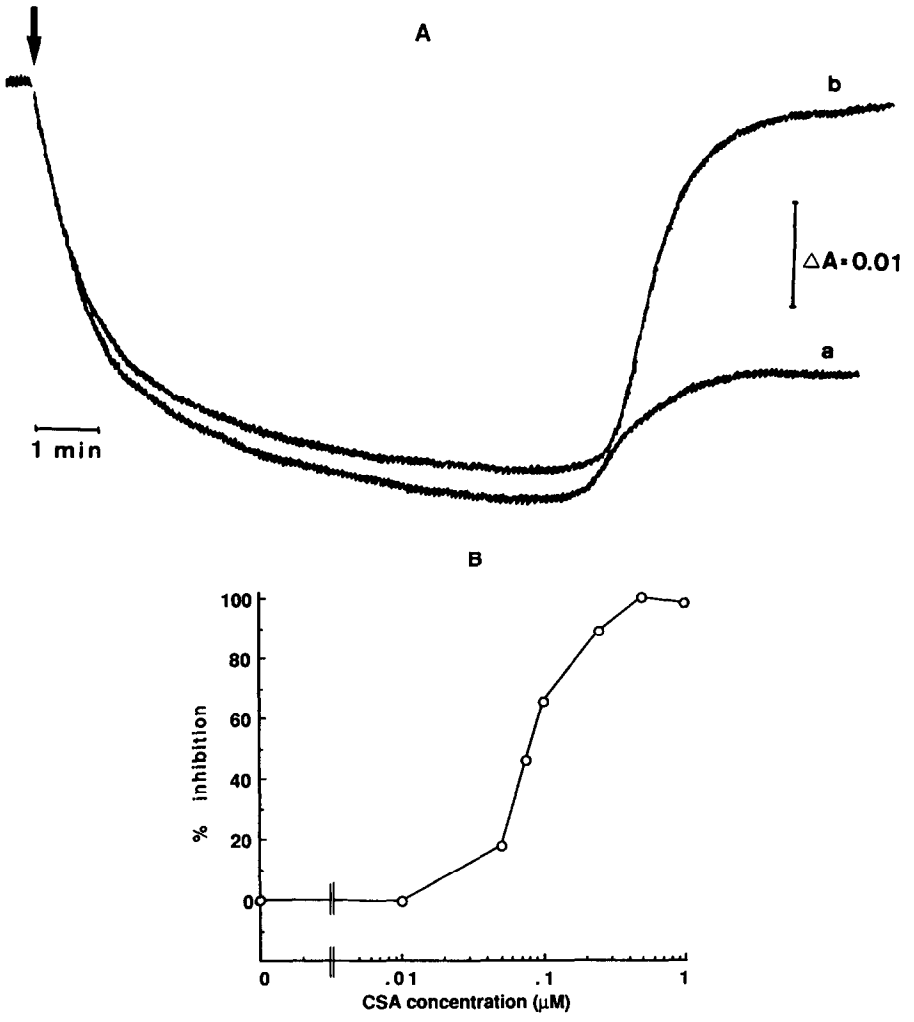

Fig. 2. tbh-induced oxidation and re-reduction of mitochondrial pyridine nucleotides as an indicator of their hydrolysis. Mitochondria were incubated under standard conditions. (A) The change in the redox level of mitochondrial pyridine nucleotides was monitored at 340–370 nm. At the arrow, 100 μM tbh plus 10 mM EGTA (trace a) was added. Trace b: conditions as for trace a except for the presence of 1 μM cyclosporine A. (B) Per cent inhibition of pyridine nucleotide hydrolysis determined as described in Materials and Methods. The results shown are from one experiment typical of four.

Figure 3A shows complete inhibition of its activity by 1 μM CSA. Under the assay conditions used here, IC_{50} is reached at about 12.4 nM (Fig. 3B).

The sensitivity of PPIase, pyridine nucleotide hydrolysis and Ca^{2+} release to small amounts of CSA suggested a causal relationship between these parameters. Since Ca^{2+} release is slightly less sensitive to CSA than pyridine nucleotide hydrolysis it also appeared that Ca^{2+} release is secondary to the latter. To further substantiate these interpretations we analysed, as described for CSA, the inhibitory potency of several of its derivatives and other immunosuppressants. The results are compiled in Table 1. The IC_{50} values of the compounds for a given parameter differ by about three orders of magnitude. Unless solubility problems precluded their determinations, we found that with a given compound PPIase is most sensitive to the drugs. Pyridine nucleotide hydrolysis and Ca^{2+} release are, under the present experimental conditions, more resistant to inhibition, the latter about 1.3–1.8-fold more than the former.

DISCUSSION

Ca^{2+} release from mitochondria was first associated with the oxidation of mitochondrial pyridine nucleotides by the work of Lehninger *et al.* [9]. This association has then been confirmed by others (reviewed in Ref. 1). Three types of inhibitors gave the conclusion that pyridine nucleotide oxidation and hydrolysis are necessary but not sufficient for Ca^{2+} release: (i) in early studies it was shown that in the presence of ATP the tbh-stimulated pyridine nucleotide oxidation is accelerated, yet their hydrolysis and Ca^{2+} release are inhibited [10]. (ii)

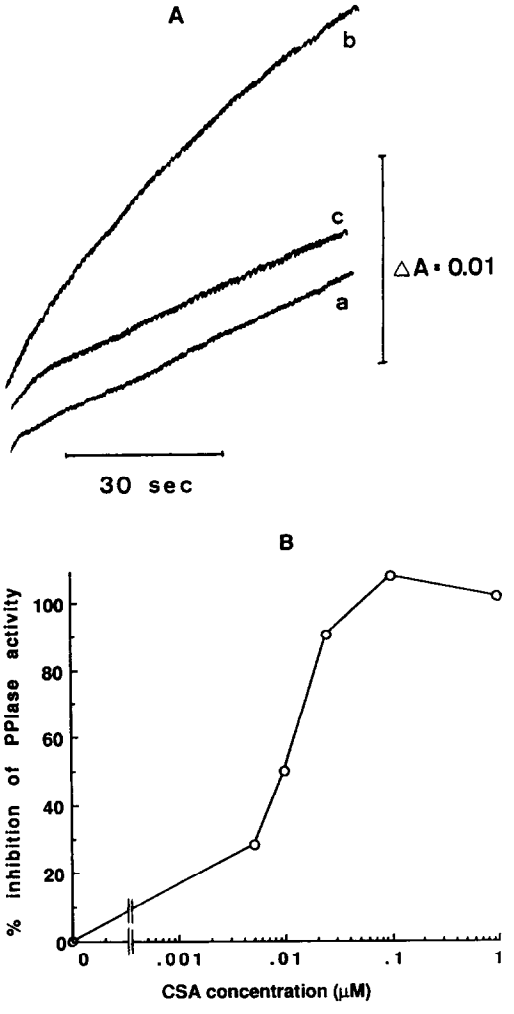

Fig. 3. Inhibition of PPIase activity by cyclosporine A. The assay was performed as described in Materials and Methods. (A) The figure shows the recordings of optical changes during incubation without matrix proteins (trace a), with matrix proteins (trace b) and with matrix proteins in the presence of 1 μM CSA (trace c). (B) Per cent inhibition of PPIase activity determined as described in Materials and Methods. The results shown are from one experiment typical of four.

A second class of inhibitors, e.g. 4-hydroxynonenal [11] and CSA [6, 7] do not influence the tbh-stimulated pyridine nucleotide oxidation but prevent their hydrolysis and Ca^{2+} release. (iii) *m*-Iodobenzylguanidine affects neither pyridine nucleotide oxidation nor their hydrolysis but prevents Ca^{2+} release [12]. Thus, hydrolysis of oxidized mitochondrial pyridine nucleotides is an obligatory but not sufficient prerequisite for the prooxidant-dependent Ca^{2+} release from mitochondria.

The IC_{50} values of Ca^{2+} release obtained in this study with a given compound are always somewhat higher than those of pyridine nucleotide hydrolysis. Although mechanistic arguments based on correlations do not prove a case, the results are consistent with the previously proposed causal relationship between the two events (see above). The results further document that pyridine nucleotide hydrolysis is likely to be a prerequisite and not the consequence of mitochondrial Ca^{2+} release.

PPIase has previously been found in mitochondria of *Neurospora crassa* [13], *Saccharomyces cerevisiae* [13, 14] and rat heart and liver [5, 15], but the physiological function(s) of the protein remained unclear. It was postulated that it regulates a Ca^{2+}-dependent unspecific "pore" in the inner mitochondrial membrane [5, 15–18], possibly by interacting with the adenine nucleotide translocator [18]. However, we recently reported [2] that "pore" formation is a consequence of excessive Ca^{2+} cycling, secondary to the prooxidant-stimulated, specific Ca^{2+} release investigated in the present study.

PPIase activity, measured in the mitochondrial matrix fraction with an artificial substrate, is particularly sensitive to the inhibitors, and the IC_{50} values determined for PPIase lie relatively close together. This may not reflect the sensitivity of the enzyme in intact mitochondria, where more sites compete for the mostly highly lipophilic inhibitors, and where the (so far) unidentified natural substrate(s) of the enzyme may render it more selective due to e.g. topological constraints.

The relative inhibitory capacity of all drugs tested ($1 > 2 > 3 > 4 > 5 > 6 \geq 7$) is the same for PPIase inhibition, for pyridine nucleotide hydrolysis and for Ca^{2+} release. This strongly suggests that PPIase has an important role in the regulation of Ca^{2+} release from intact mitochondria, and may thereby ascribe a physiological function, namely control of mitochondrial pyridine nucleotide hydrolysis and Ca^{2+}

Table 1. Inhibition of mitochondrial Ca^{2+} release, pyridine nucleotide hydrolysis and matrix PPIase

Drug	1	2	3	4	5	6	7
IC_{50}, Ca (nM)	82.1 ± 3.5	104.2 ± 17.4	300 ± 13	374 ± 17	827 ± 103	>30,000*	>30,000*
IC_{50}, PN (nM)	62.9 ± 6.8	80.8 ± 8.7	194 ± 21	237 ± 29	444 ± 53	>30,000*	>30,000*
IC_{50}, PPIase (nM)	11.4 ± 0.7	12.4 ± 0.6	20.3 ± 2.4	22.3 ± 3.9	49.6 ± 2.2	>30,000†	>30,000*

The IC_{50} values for Ca^{2+} release from rat liver mitochondria (IC_{50}, Ca), for pyridine nucleotide hydrolysis (IC_{50}, PN) and for PPIase activity (IC_{50}, PPIase) are shown as mean ± SD (N = 4) for CSA and related compounds.

Compounds used: **1**, MeVal-4-CS; **2**, CSA; **3**, CSG; **4**, dihydro-CSD; **5**, D-Lys-8-CS; **6**, CSH; **7**, FK506.

* No inhibition up to 30 μM, the solubility limit of the drugs.

† Weak inhibition at 30 μM, the solubility limit of the drug.

release, to the mitochondrial enzyme. Further work is necessary to determine whether the inhibitory action of PPIase on NAD^+ glycohydrolase is a direct one, e.g. by changing the conformation of the enzyme at the active site, or if the inhibition occurs via a yet unknown regulatory protein.

For some drugs, the IC_{50} values for mitochondrial PPIase (Table 1) do not parallel the cyclophilin A binding affinity (Sandoz Pharma Preclinical Research, unpublished results). Therefore, it seems very likely that mitochondrial PPIase is different from cyclophilin A. This is consistent with the finding of Connern and Halestrap [15] that the mitochondrial and cytosolic PPIases have different amino acid sequences and with the authors' suggestion that the two enzymes are encoded for by distinct nuclear genes.

Whereas an excellent relationship between the inhibitory potency of the drugs is found with PPIase activity, pyridine nucleotide hydrolysis and Ca^{2+} release, no such relationship between these three parameters and the immunosuppressive activities of the drugs [19] is evident. This is particularly evident with two compounds: (i) FK506 is about 100-fold more potent than CSA as an immunosuppressant but at least 3000-fold less effective as an inhibitor of mitochondrial Ca^{2+} release. (ii) MeVal-4-CS, on the other hand, is more than 1000-fold less potent in immunosuppression but about 20% more effective on Ca^{2+} release. Therefore, the mechanisms by which CSA and related compounds cause immunosuppression and inhibition of Ca^{2+} release from mitochondria are not directly related.

Whether inhibition of Ca^{2+} release from mitochondria is important for the cytotoxicity of CSA and related compounds (for review, see Ref. 20), or for the beneficial effects of CSA in hypoxia/reperfusion injury [21] requires further testing.

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