Cyclosporin A-sensitive release of Ca^{2+} from mitochondria in intact thymocytes

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Received 6 October 1997

Abstract Release of Ca^{2+} from mitochondria into cytosol in intact thymocytes was studied using the fluorescent dye Fluo-3. It was shown that the release of Ca^{2+} induced by the dithiol cross-linking agent phenylarsine oxide or by uncoupler was strongly inhibited by cyclosporin A, a specific inhibitor of the permeability transition pore (PTP) in mitochondria. Oxidative stress sensitized the pore so even partial uncoupling caused rapid cyclosporin A-sensitive release of Ca^{2+}. The experiments on digitonin-permeabilized cells confirmed that uncoupling induced opening of the PTP, which forms the major pathway for rapid release of Ca^{2+} from thymocyte mitochondria.

Key words: Thymocyte; Mitochondrion; Ca^{2+}; Cyclosporin A; Permeability transition pore

1. Introduction

It is now well established that mitochondria can play a dominant role in clearance of cytosolic Ca^{2+} overload in different physiological conditions [1,2]. Upon hormonal stimulation probably only a specific fraction of mitochondria accumulates Ca^{2+} [3] but in any case the release of Ca^{2+} is a necessary step which returns the system to the 'resting state'. Recent observations indicate that accumulation and release of Ca^{2+} by mitochondria modulate the amplitude and spatiotemporal organization of the cytosolic Ca^{2+} signals [4–6]. The mechanisms that regulate mitochondrial Ca^{2+} fluxes in the cell remain obscure. In isolated mitochondria accumulation of Ca^{2+} can induce opening of the permeability transition pore (PTP) in the inner membrane (for review, see [7]). This phenomenon was also observed in permeabilized cells [8,9]. The PTP opening is regulated by Ca^{2+} and pH in the matrix, by membrane potential and by the redox state of mitochondrial components. This pore was suggested to form the major pathway for rapid release of Ca^{2+} from mitochondria in living cells [10]. The data presented below strongly support this hypothesis.

2. Materials and methods

Thymocytes were isolated from rat thymuses and washed twice in Hanks' medium (HBS, Sigma) supplemented with 10 mM glucose and 10 mM HEPES pH 7.4. Cells (approx. 10^8/ml) were loaded with 10 mM Fluo-3 AM (Molecular Probes) in the same medium with 1 mM probenecid at 37°C for 15 min, washed twice and stored on ice. Viability of the cells (more than 90% initially, determined with trypan blue staining) did not decrease after loading and during experiments. Confocal images confirmed that thymocytes were labelled homogeneously: no accumulation of the dye in mitochondria or exclusion from the nucleus was observed.

Fluorescence (excitation at 500 nm and emission at 526 nm) was measured in HBS (without probenecid) at 25°C and 5×10^7 cells/ml using a Perkin-Elmer spectrofluorimeter with constant stirring. For calibration F_0 (in the medium with 5 mM EGTA) and F_m (in the medium with 1 mM free Ca^{2+}) were measured in the presence of digitonin (5 mM) and $K_D = 425$ nM was used in calculations of the cytosolic concentration of Ca^{2+}.

Thymocytes (10^8/ml) were permeabilized by digitonin (5 mM) in the medium that contained 100 mM KCl, 5 mM KP_i, 5 mM EGTA, 20 mM HEPES, 5 mM 2-oxybutyrate, 2 mM rotenone, 1 mg/ml oligomycin, 25 mM Arsenazo III, pH 7.4 at 25°C. Changes in absorbance at 690 nm vs. 650 nm, measured with an Aminco DW-2 spectrophotometer, were proportional to concentration of added Ca^{2+} up to 20 mM.

Cyclosporin A and its analogs were gifts from Sandoz.

3. Results and discussion

3.1. Slow accumulation of Ca^{2+} in mitochondria

Treatment of thymocytes with thapsigargin (TG) (the specific inhibitor of Ca^{2+}-ATPase of endoplasmic reticulum) caused a rapid increase of Ca^{2+} concentration in cytosol (Ca_{cyt}) that remained constant for at least 20 min (Fig. 1). Only a small and transient increase of Ca_{cyt} was induced by TG in the absence of Ca^{2+} in the external medium (not shown). Thus continuous increase of Ca_{cyt} was caused by opening of Ca^{2+} channels in the outer membrane and so-called capacitative Ca^{2+} entry [11]. These channels remained open for the full incubation time, so the subsequent addition of EGTA (external Ca^{2+} concentration drops to approx. 10 nM) resulted in a rapid decrease of Ca_{cyt} to a level that was even lower than the initial one. During incubation with TG mitochondria in thymocytes accumulated a significant amount of Ca^{2+}, so their deenergization with uncoupler (FCCP) induced a rapid release of Ca^{2+} into cytosol and an increase of Ca_{cyt} (Fig. 1A). This effect did not depend on the decrease of the cellular ATP, since addition of oligomycin (a specific inhibitor of mitochondrial ATPase) neither caused any increase of Ca_{cyt} per se, nor changed the effect of the uncoupler (not shown). The increase of Ca_{cyt} after release of Ca^{2+} from mitochondria was stable for several minutes. This means that under the conditions described both the Ca^{2+}-ATPase and Ca^{2+} channels did not operate in the plasma membrane. The amplitude of the uncoupler-induced response slowly increased with time of incubation and reached a plateau in approx. 20 min (Fig. 1C). These data indicate that accumulation of Ca^{2+} in mitochondria was very slow (that is in agreement with the low affinity of mitochondrial Ca^{2+} transport)

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Abbreviations: PTP, permeability transition pore; CsA, cyclosporin A; TG, thapsigargin; PhAsO, phenylarsine oxide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; t-BOOH, *tert*-butylhydroperoxide; 6-Kch, 6-ketocholestanol

PII S0014-5793(97)01361-6

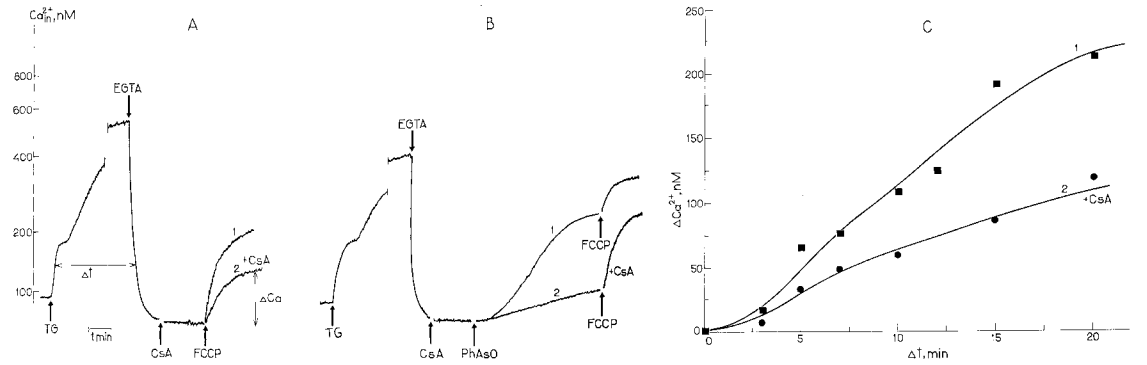

Fig. 1. Cyclosporin A-sensitive increase of Ca_{cyt} induced by FCCP (A,C) or PhAsO (B) in intact thymocytes. Thymocytes were loaded with Fluo-3 AM, fluorescence was measured and Ca_{cyt} was calculated as described in Section 2. After addition of thapsigargin (TG, 1 mM) and incubation for 20 min (A,C) or 15 min (B) EGTA (5 mM) was added. CsA (2 mM) was added where indicated 2 min before FCCP or PhAsO. A: FCCP (2 mM) was added in the absence (trace 1) or in the presence (trace 2) of CsA. B: PhAsO (10 mM) and FCCP (2 mM) were added when indicated in the absence (trace 1) or in the presence (trace 2) of CsA. C: Amplitude of increase of Ca_{cyt} induced by FCCP (2 mM) and time of incubation were determined as shown in A. Curve 1, without CsA; curve 2, with CsA.

and possible 'rapid uptake mode(s)' [12] did not play any significant role.

3.2. Cyclosporin A-sensitive release of Ca^{2+} from mitochondria

The release of Ca^{2+} accumulated in mitochondria was induced by phenylarsine oxide (PhAsO) (Fig. 1B). This agent causes cross-linking of vicinal dithiols and is known to be a powerful inducer of the permeability transition in mitochondria [7]. The increase of Ca_{cyt} induced by PhAsO was strongly inhibited by cyclosporin A (CsA), a specific inhibitor of the permeability transition pore (PTP). The same effect was observed with an analog of CsA-MeVal-cyclosporin but not with cyclosporin H (not shown). The first analog, like CsA, blocks the mitochondrial pore but has no immunosuppressive effect while cyclosporin H is ineffective as an inhibitor of the pore [13]. These data indicate that PhAsO induced the opening of the PTP and release of Ca^{2+} from mitochondria via this pathway. In agreement with this conclusion the uncoupler-sensitive Ca^{2+} stores were almost emptied after incubation with PhAsO (Fig. 1B). PhAsO probably also induced the release of Ca^{2+} from the other intracellular stores [14] but this effect did not shadow the release of Ca^{2+} from mitochondria since (a) the amplitude of increase of Ca_{cyt} induced by PhAsO was only slightly higher than that induced by the uncoupler; and (b) no increase of Ca_{cyt} was observed if PhAsO was added after the uncoupler (not shown).

3.3. Uncoupler-induced release of Ca^{2+} from mitochondria

The increase of Ca_{cyt} caused by the uncoupler was partially inhibited by CsA (Fig. 1A,C). Inhibition did not exceed 50–60% and did not depend on time of incubation with TG. The effect of MeVal-CsA coincided with the effect of CsA while CsH was ineffective (not shown). This partial inhibition indicates that two or more mechanisms are involved in uncoupler-induced release of Ca^{2+} from mitochondria and the PTP is one of them. As the permeability transition leads to rapid and complete release of Ca^{2+} from individual mitochondria, it can be proposed that the uncoupler-induced immediate PTP opening occurs only in a fraction of mitochondria in thymocytes. Ca^{2+} release via the reversal of Ca^{2+} uniport was not predominant probably because the Ca^{2+} uniporter was inactive at the very low (physiological) concentration of Ca^{2+} in cytosol (as was shown in isolated mitochondria [15]). The experiments with permeabilized cells (see below) support this proposal and indicate that the activity of alternative mechanisms of Ca^{2+} release is negligible in mitochondria of thymocytes.

In the following experiments the conditions of oxidative stress were modelled using *tert*-butylhydroperoxide (t-BOOH) (Fig. 2) or diamide (not shown) as prooxidants. In-

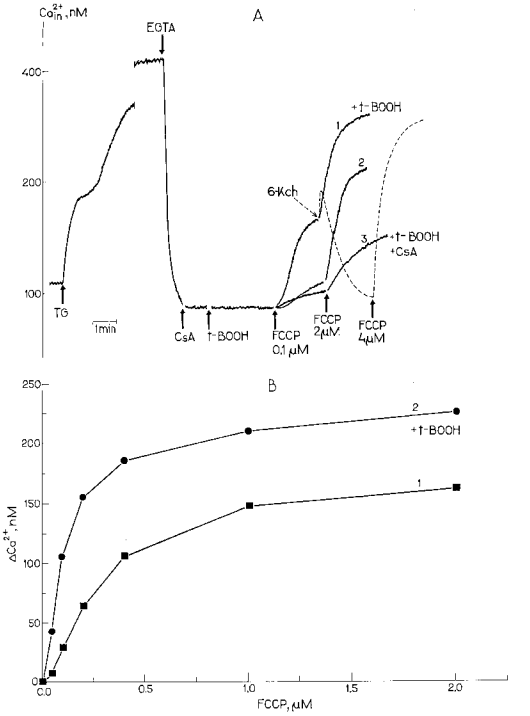

Fig. 2. FCCP-induced increase of Ca_{cyt} under conditions of oxidative stress. The conditions of incubation were the same as in Fig. 1. After 10 min preincubation with TG and addition of EGTA *tert*-butylhydroperoxide (t-BOOH, 0.2 mM) was added followed by FCCP after 3 min. A: Trace 1, two additions of FCCP (0.1 mM and 2 mM) were done when indicated. Trace 2, the same additions of FCCP in the absence of t-BOOH. Trace 3, CsA (2 mM) was added 1 min before t-BOOH followed by the same additions of FCCP. Dashed trace, after additions of t-BOOH and FCCP (0.1 mM) 6-ketocholestanol (6-Kch, 20 mM) was added. The final addition of FCCP (4 mM) was done 2 min after 6-Kch. B: FCCP at indicated concentration was added as in A after preincubation in the presence (curve 1) or in the absence of t-BOOH (curve 2).

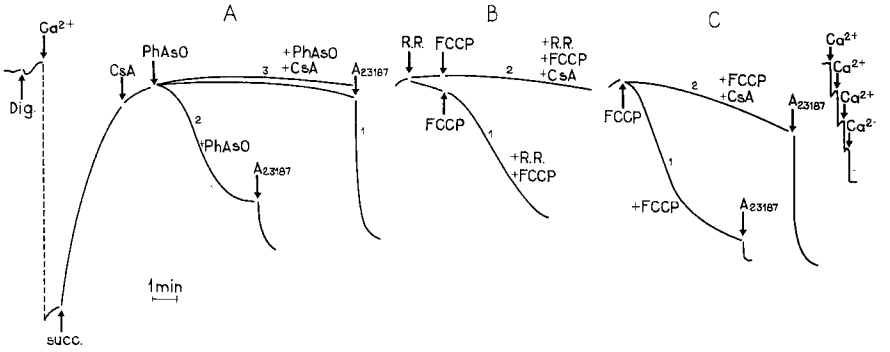

Fig. 3. Ca^{2+} release from mitochondria in permeabilized thymocytes. Thymocytes were incubated and concentration of Ca^{2+} was measured as described in Section 2. Digitonin (Dig., 5 mM), $CaCl_2$ (20 mM) and succinate-Tris (Succ., 5 mM) were added in all assays as indicated in A. CsA (2 mM) was added where indicated 1 min before PhAsO (A), ruthenium red (B) or FCCP (C). The Ca^{2+} ionophore A_{23187} (2 mM) was added to demonstrate the maximal amplitude of Ca^{2+} release. Calibration with additions of $CaCl_2$ (2 mM each addition) is shown. A: Trace 1, no inducers. Traces 2 and 3, PhAsO (40 mM) was added in the absence (trace 2) or in the presence (trace 3) of CsA. B: Ruthenium red (RR, 0.1 mM) was added 1 min before FCCP (0.4 mM) in the absence (trace 1) or in the presence (trace 2) of CsA. C: FCCP (0.4 mM) was added in the absence (trace 1) or in the presence (trace 2) of CsA.

cubation of thymocytes for 2 min with these compounds (after Ca^{2+} accumulation in mitochondria) did not cause any increase of Ca_{cyt} but strongly enhanced the effect of the uncoupler. CsA completely blocked this effect indicating that PTP opening was practically the only mechanism of Ca^{2+} release from partially uncoupled mitochondria under the conditions of oxidative stress (Fig. 2A, traces 1 and 3). These data are in perfect agreement with the experiments on isolated mitochondria which demonstrated the shift in the apparent PTP gating potential to more negative values imposed by various oxidative agents [16]. The effect of FCCP at maximal concentration was also slightly higher after preincubation of thymocytes with t-BOOH (Fig. 2B) or diamide (not shown). Probably even maximal deenergization caused PTP opening only in a fraction of mitochondria in living cells and this fraction was increased by prooxidants.

Our experimental model allowed us to demonstrate the reversible modulation of Ca_{cyt} by mitochondria. It was shown recently that 6-ketocholestanol (6-Kch) restored mitochondrial membrane potential both in isolated mitochondria and in intact thymocytes after partial depolarization by various uncouplers [17]. When 6-Kch was added to thymocytes treated with t-BOOH and FCCP (100 nM) a rapid decrease in Ca_{cyt} was observed (Fig. 2A, dashed trace). The maximal increase of Ca_{cyt} was further induced by higher concentrations of uncoupler in agreement with the results of the measurements of mitochondrial membrane potential [17]. These data indicate that Ca^{2+} released after permeability transition was accumulated by mitochondria reenergized by the addition of 6-Kch. We have shown that 6-Kch did not restore the membrane potential in mitochondria with the open pore (unpublished results), so Ca^{2+} was, probably, taken up by mitochondria that were not permeabilized at the previous step.

3.4. *Release of Ca^{2+} from mitochondria in permeabilized thymocytes*

The role of PTP in release of mitochondrial Ca^{2+} was confirmed in experiments with thymocytes treated with digitonin to permeabilize the plasma membrane. Cells were incubated in cytosol-like medium supplemented with 2-oxybutyrate and rotenone to support the reduced state of mitochondrial components. Energization of mitochondria by succinate oxidation caused a rapid uptake of added Ca^{2+} (Fig. 3) that was completely inhibited by the specific inhibitor of Ca^{2+} uniporter, ruthenium red (not shown). The opening of the PTP induced by PhAsO caused a complete release of Ca^{2+} which was sensitive to CsA and MeVal-CsA (Fig. 3A). t-BOOH (0.2 mM) was ineffective under these conditions and caused a slow CsA-sensitive release of Ca^{2+} only in the absence of 2-oxybutyrate when oxidation of pyridine nucleotides and glutathione in mitochondria was induced (not shown). The uncoupler caused a rapid release of Ca^{2+} which was partially inhibited by CsA and completely blocked by a combination of CsA and ruthenium red (Fig. 3B,C). Ruthenium red per se induced a slow release that was strongly accelerated by the subsequent addition of uncoupler (Fig. 3B). This set of data allows us to conclude that: (a) PTP contributes significantly to the bulk process of uncoupler-induced Ca^{2+} release from mitochondria in thymocytes; (b) PTP opening is induced by uncoupler; (c) Ca^{2+} release via other possible pathways (putative Ca^{2+}/H^+ antiport, for example) is much slower than the release caused by the reversal of Ca^{2+} uniport and by PTP opening. These conclusions are in perfect agreement with the results of experiments with intact thymocytes.

4. Conclusion

Rapid release of Ca^{2+} from mitochondria is an important physiological event and the non-selective PTP is, probably, the major specific pathway for Ca^{2+} release. The data presented here indicate that the PTP in intact thymocytes can be induced by oxidation (or cross-linking) of critical dithiols in the mitochondrial membrane or by uncoupling (Fig. 1). These same events can modulate Ca^{2+}-induced opening of the PTP that is involved in Ca^{2+} signalling.

Physiological mechanisms of partial ('mild') uncoupling were suggested to serve as protection against overproduction of superoxide radicals and oxidative damage of mitochondria [18]. Oxidative stress sensitized the PTP triggering so even mild uncoupling caused the pore to open and release Ca^{2+}. PTP was induced in only a fraction of mitochondria and after reenergization the intact mitochondria were able to restore low levels of Ca_{cyt} (Fig. 2). It can be proposed that a decrease of Ca_{cyt} leads to closure of PTP and returns the cell to the

'resting state'. Under more drastic conditions the opening of PTP could become irreversible. This causes the release of some mitochondrial proteins into cytosol and triggers programmed cell death [19].

Acknowledgements: The author is grateful to Professor P. Bernardi and all members of his laboratory at the University of Padua for their help and support during his visit to Italy. This work was supported by a Grant from the Russian Fund for Fundamental Investigations and by a Fellowship from the Rector of the University of Padua.

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