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Inhibition by cyclosporin A of a Ca^{2+}-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress

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The capacity of cyclosporin A to inhibit opening of a Ca^{2+}-dependent pore in the inner membrane of heart mitochondria was investigated. Whereas in the presence of 25 nmol of Ca^{2+}/mg of mitochondrial protein and 5 mM-P_i mitochondria were unable to maintain accumulated Ca^{2+}, inner-membrane potential and sucrose impermeability, all three parameters were preserved when cyclosporin was included. Pore opening was assayed directly by [^{14}C]sucrose entry and entrapment in the matrix space. [^{14}C]Sucrose entry induced by both Ca^{2+} plus P_i and Ca^{2+} plus t-butyl hydroperoxide was almost completely inhibited by 60 pmol of cyclosporin/mg of mitochondrial protein. It is concluded that cyclosporin A is a potent inhibitor of the pore.

INTRODUCTION

Heart mitochondrial Ca^{2+} is controlled by (quasi) steady-state cycling mediated by the Ca^{2+} uniporter and the Na^+–Ca^{2+} carrier (for review, see Crompton, 1985). Several intramitochondrial dehydrogenases are activated by Ca^{2+} over the range 0.1–5 μM, and, most probably, the basic function of the transport cycle is the control of matrix free [Ca^{2+}] according to the regulatory requirements of these enzymes (for review, see Denton & McCormack, 1980). In addition, there are now strong indications of a further action of mitochondrial Ca^{2+} when the limit of dehydrogenase control is exceeded that involves reversible opening of a Ca^{2+}-activated pore in the inner membrane (Al-Nasser & Crompton, 1986*a*,*b*; Crompton *et al.*, 1987*a*,*b*). Although the physiological function of the (hypothetical) pore is obscure, a potential pathological relevance is evident. Since pore opening requires not only increased [Ca^{2+}], but also either increased [P_i] or oxidative stress, all features of reoxygenation-induced injury in heart and other tissues, we have suggested that pore opening may be involved in this type of injury (Al-Nasser & Crompton, 1986*a*; Crompton *et al.*, 1987*b*). Pore opening uncouples energy transduction and leads to equilibration of low-M_r solutes across the inner membrane, so that the consequences for cell recovery would be severe.

Investigations of the possible influence of pore state on the capacity of cells to withstand reoxygenation would be greatly facilitated by pore inhibitors. Fournier *et al.* (1987) have drawn attention to the capacity of cyclosporin to promote retention of accumulated Ca^{2+} by isolated mitochondria. Since, in our view, the losses of mitochondrial Ca^{2+} observed by those authors may reflect pore opening, we have examined the capacity of cyclosporin to inhibit the pore. The study reveals that cyclosporin is a potent pore inhibitor.

METHODS

Mitochondria were prepared from hearts of female Sprague–Dawley rats (250–300 g body wt.) and their protein content was determined as described previously (Crompton *et al.*, 1983). The mitochondria were suspended finally in 70 mM-sucrose/210 mM-mannitol/10 mM-Tris/HCl, pH 7.2.

In all experiments, mitochondria were preincubated at 1 mg of protein/ml and 25 °C in 120 mM-KCl/10 mM-Hepes (K^+ salt, pH 7.2)/1 μM-rotenone/sucrose (to give 10 mM final concn.) until endogenous Ca^{2+} (3–5 nmol of Ca^{2+}/mg of protein) had effluxed (5–8 min). When present, cyclosporin A in ethanolic solution was added at the beginning of the preincubation before transfer to the TPP^+ electrode (below). The [ethanol] in the incubation was maintained below 0.5% (v/v); control experiments confirmed that this amount of ethanol had no effect on pore opening or closure. Pore opening was induced by addition of $CaCl_2$ (to give 25 nmol of total Ca^{2+}/mg of protein), 5 mM-succinate and either 5 mM-KH_2PO_4 or 200 μM-butyl hydroperoxide as indicated.

Ca^{2+} uptake was measured with the indicator arsenazo III, by using a Perkin-Elmer dual-wavelength spectrophotometer, model 356, operating at 675–685 nm.

$\Delta\psi$ was estimated from the accumulation of TPP^+ measured with a TPP^+-sensitive electrode, as described by Al-Nasser & Crompton (1986*a*); 4 μM-TPP^+ was added to the incubation. $\Delta\psi$ was calculated by assuming that only one-eighth of the accumulated TPP^+ was in aqueous solution in the matrix, the remainder being partitioned into the inner membrane (Rottenberg, 1984).

[^{14}C]Sucrose entry and entrapment in the matrix was measured as follows: 1 μCi of ^3H_2O/ml and 0.2 μCi of [U-^{14}C]sucrose/ml were added 1–2 min before pore opening (unless stated otherwise). At intervals after pore

Abbreviations used: $\Delta\psi$, mitochondrial inner-membrane potential; TPP^+, tetraphenylphosphonium ion.

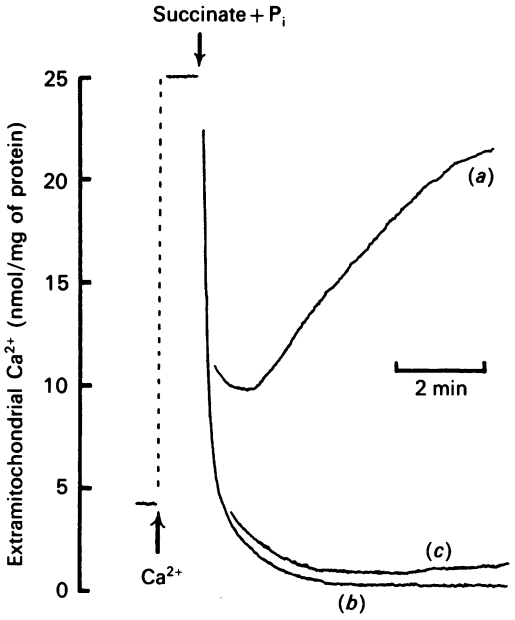

Fig. 1. Effect of cyclosporin on the capacity of heart mitochondria to accumulate and retain Ca^{2+}

Mitochondria were preincubated as described in the Methods section. $CaCl_2$ and succinate plus P_i were added as indicated. Curves: (*a*), 5 mM-P_i; (*b*), 5 mM-P_i and 300 pmol of cyclosporin/mg of protein; (*c*), 0.2 mM-P_i.

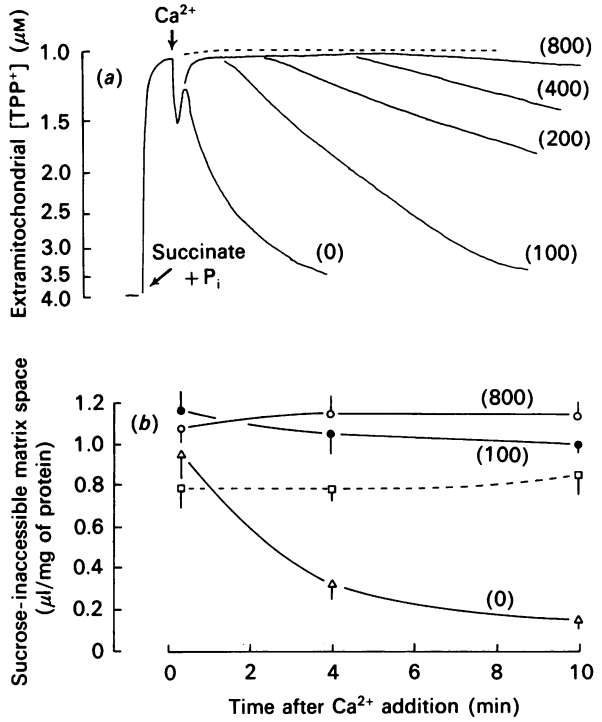

Fig. 2. Effect of cyclosporin on the capacity of heart mitochondria to maintain $\Delta\psi$ and sucrose impermeability

(*a*) TPP^+ uptake: after preincubation (see the Methods section), succinate plus 5 mM-P_i and Ca^{2+} (22 nmol/mg of protein) were added as indicated. (*b*) Sucrose-inaccessible matrix spaces: the spaces were determined on samples of the incubations (values are means ± S.E.M. for four determinations) at the times indicated (abscissa) after addition of Ca^{2+}. Cyclosporin was added during preincubation at the concentrations (pmol of cyclosporin/mg of protein) given in parentheses alongside each curve. Broken lines represent when 2 mM-EGTA was added during preincubation, and Ca^{2+} and cyclosporin were omitted.

opening, 1 mM-EGTA/1 mM-ATP/1 mM-$MgCl_2$ was added to induce pore closure and entrapment of [^{14}C]-sucrose that had entered the matrix. The incubation was diluted 30 s later (when pore closure was complete) in a 10-fold volume of medium (pH 7.2) containing 120 mM-KCl, 10 mM-Hepes, 5 mM-succinate and, when present in the incubation, 5 mM-KH_2PO_4. The diluted incubation was immediately centrifuged in an Eppendorf bench centrifuge for 2 min. The ^{14}C and ^{3}H contents of the mitochondrial pellets and supernatants were determined. The rationale of the procedure, verified previously (Crompton *et al.*, 1987*b*), is that, on dilution, matrix ^3H_2O would be diluted, whereas entrapped [^{14}C]-sucrose would not, leading to an excess of [^{14}C]sucrose over ^3H_2O in the pellet with respect to the supernatant. The pellet ^{14}C values were corrected for ^{14}C in the residual H_2O of the pellet, giving excess [^{14}C]sucrose entrapped. Since the pellet H_2O volume included the matrix space, the excess [^{14}C]sucrose measured in this way undervalued slightly [^{14}C]sucrose entry by an amount equal to the [^{14}C]sucrose contained (after dilution) in a volume of extramitochondrial H_2O equal to the matrix volume. Appropriate corrections were applied by using matrix volumes measured after pore closure. Sucrose-inaccessible matrix volumes were determined in the conventional way from the (^3H_2O minus [^{14}C]sucrose) volumes of the mitochondrial pellets.

RESULTS AND DISCUSSION

Fig. 1 confirms and extends the observations of Fournier *et al.* (1987). In the presence of 5 mM-P_i, the respiration-supported uptake of Ca^{2+} was quickly followed by Ca^{2+} release. Cyclosporin prevented the Ca^{2+} release, so that almost all the Ca^{2+} was accumulated and retained.

The inability to retain accumulated Ca^{2+} depended on [P_i]. Thus, with 0.2 mM-P_i, Ca^{2+} was taken up and retained in the absence of cyclosporin (Fig. 1) and, under these conditions, cyclosporin had no detectable effect on Ca^{2+} uptake (results not shown). Evidently, cyclosporin suppresses the instability of heart mitochondria to Ca^{2+} load in the presence of high [P_i]. Since Ca^{2+} and high [P_i] together, but not separately, induce mitochondrial pore opening (Crompton *et al.*, 1987*b*), the data of Fig. 1 suggest that the action of cyclosporin may be explained by inhibition of pore opening.

This interpretation is supported by Fig. 2. Ca^{2+} plus 5 mM-P_i induced a progressive decrease in the sucrose-inaccessible matrix space, consistent with sucrose entry into the matrix, and this was inhibited by cyclosporin. Parallel measurements were made of TPP^+ uptake, an index of $\Delta\psi$. In the absence of cyclosporin, Ca^{2+} induced a biphasic release of TPP^+ accumulated on commencement of respiration. The initial rapid release of TPP^+ may be attributed to partial depolarization owing to electrophoretic Ca^{2+} influx (which was 80% complete in 10 s; Fig. 1). The subsequent progressive loss of accumu-

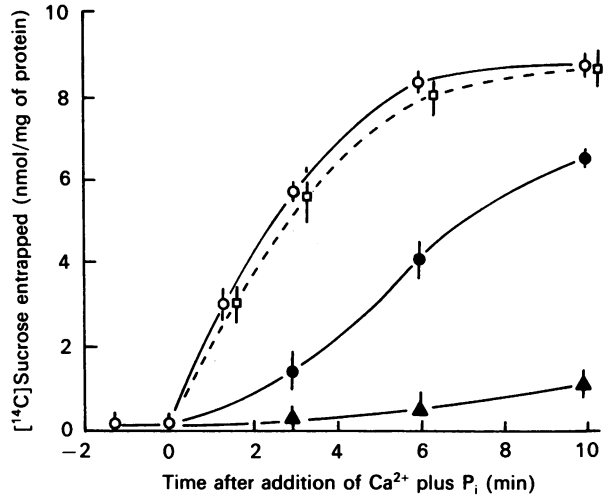

Fig. 3. Time course of pore opening in the presence and absence of cyclosporin

After preincubation (see the Methods section), succinate was added (at −1.5 min in the Figure), followed by Ca^{2+} plus 5 mM-P_i (at zero time in the Figure). Pore closure was induced by EGTA/ATP/Mg^{2+} at the times indicated on the abscissa, and [^{14}C]sucrose entrapment was measured. [Cyclosporin] was as follows: ○, □, none; ●, 40 pmol/mg of protein; ▲, 100 pmol/mg of protein. [^{14}C]Sucrose was added either at −2 min (continuous lines) or 5 s before EGTA/ATP/Mg (broken line). Means ± S.E.M. are given for four replicates with the same mitochondrial preparation.

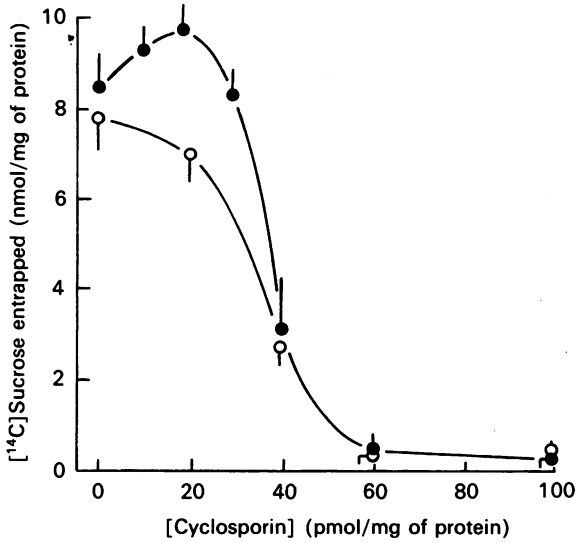

Fig. 4. [Cyclosporin]-sensitivity of pore opening induced by Ca^{2+} and either P_i or t-butyl hydroperoxide

[^{14}C]Sucrose entrapments were measured as in Fig. 3, at 4 min after induction of pore opening by either Ca^{2+} + 5 mM-P_i (●) or Ca^{2+} + 200 μM-t-butyl hydroperoxide (○). Means ± S.E.M. are given for four mitochondrial preparations.

lated TPP^+, coexisting with the loss of sucrose inaccessibility, was inhibited by cyclosporin. Since pore opening uncouples mitochondria and allows entry of sucrose into the matrix, the data of Fig. 2 indicate that cyclosporin inhibits pore opening and thereby permits maintenance of both $\Delta\psi$ and sucrose inaccessibility.

A higher [cyclosporin] was required to maintain accumulated TPP^+ than to prevent sucrose access to the matrix. Nevertheless, even small amounts of cyclosporin markedly delayed the onset of the progressive phase of depolarization, e.g. for about 1 min with 100 pmol of cyclosporin/mg of protein. The values of TPP^+ uptake and matrix space measured in the presence of 800 pmol of cyclosporin/mg of protein yield a $\Delta\psi$ value of 150 mV. This is close to the $\Delta\psi$ value of 160 mV obtained in the absence of Ca^{2+} by using the measured values of TPP^+ uptake and matrix space (broken lines, Fig. 2).

A decrease in sucrose-inaccessible space is ambiguous to the extent that the measured values would be influenced by changes in matrix volume of mitochondria in the closed-pore state. Therefore a more direct assay of pore opening was also used, based on entry and entrapment of [^{14}C]sucrose in the matrix, rather than measurement of the space remaining inaccessible to sucrose. The entrapment assay exploits the fact that Ca^{2+} + P_i-induced pore opening requires the simultaneous presence of both promoters, so that Ca^{2+} chelation with EGTA induces pore closure, and any [^{14}C]sucrose that has entered becomes entrapped (Al-Nasser & Crompton, 1986*a*,*b*; Crompton *et al.*, 1987*a*,*b*). Although the time required for pore closure with EGTA alone is rather variable (Crompton *et al.*, 1987*b*), we have found that ATP plus Mg^{2+} increases the rate of EGTA-induced pore closure (Al-Nasser & Crompton, 1986*b*) such that closure in heart mitochondria is complete within 10 s (M. Crompton & A. Costi, unpublished work; pore closure was measured in exactly the same way as described previously by Crompton *et al.*, 1987*b*). The combination EGTA/ATP/Mg^{2+} was used therefore in this study.

Fig. 3 (upper curve) shows the time course of [^{14}C]-sucrose entrapment with Ca^{2+} and 5 mM-P_i. Since the external [sucrose] before pore closure and dilution (see the Methods section) was 10 mM, the maximal sucrose entrapment of 8.8 nmol of sucrose/mg of protein corresponds to [^{14}C]sucrose entry and equilibration into a matrix space of 0.88 μl/mg of protein. The same [^{14}C]sucrose entrapment was obtained irrespective of whether [^{14}C]sucrose was present throughout the period of pore opening or was added 5 s before pore closure. This indicates that, once each mitochondrion is converted into the open-pore state, sucrose equilibrates rapidly with the matrix space, so that the measured values reflect the true time course of the mitochondria conversion into the open-pore state.

The rate of pore opening was > 50% decreased by 40 pmol of cyclosporin/mg of protein, and was extremely low with 100 pmol/mg. Fig. 4 reports more fully the effect of [cyclosporin] on the 'initial' (4 min) rate at which mitochondria are converted into the open-pore state. In the presence of Ca^{2+} and 5 mM-P_i, the effective range was 30–60 pmol of cyclosporin/mg of protein. t-Butyl hydroperoxide substitutes for P_i in inducing pore opening in the presence of Ca^{2+} (Crompton *et al.*, 1987*b*), and yields the same degree of [^{14}C]sucrose entrapment (Fig. 4, minus cyclosporin). Fig. 4 shows that the range of [cyclosporin] that inhibits Ca^{2+} + P_i-induced pore opening is similarly effective in preventing Ca^{2+} + hydroperoxide-induced pore opening.

Cyclosporin is a highly lipophilic immunosuppressant,

that inhibits T-lymphocyte activation, but whose mechanism of action is not yet understood (for review, see Hess & Colombani, 1986*a*). T-cell activation requires increased cytosolic Ca^{2+}, and there is evidence that cyclosporin may inhibit Ca^{2+}-dependent events that follow this increase (Metcalfe, 1984). Cyclosporin binds to several proteins, including calmodulin, and inhibits Ca^{2+}/calmodulin activation of target enzymes (Hess & Colombani, 1986*a*,*b*). On this basis, it is conceivable that cyclosporin may inhibit mitochondrial pore opening by interacting with a protein that confers Ca^{2+}-sensitivity to the process. Since Ca^{2+} is obligatory for both P_i-induced and hydroperoxide-induced pore opening, cyclosporin would be expected to inhibit both, which it does with similar affinity. There is no evidence, however, that calmodulin itself confers Ca^{2+}-sensitivity to the pore. Indeed, the sensitivity of pore opening to matrix free $[Ca^{2+}]$ ($K_d > 20$ μM; Al-Nasser & Crompton, 1986*a*) is at least an order of magnitude lower than the Ca^{2+} affinity of calmodulin. Also, the affinity of cyclosporin for calmodulin ($K_d = 100$–200 nM; Hess & Colombani, 1986*b*) is rather lower than the apparent affinity for pore inhibition, at least as measured by sucrose entry (Fig. 4). Rather more cyclosporin was required to prevent dissipation of $\Delta\psi$. This may relate to the previous observation that depolarization attributable to the pore precedes significant sucrose entry at mildly acid pH values (e.g. pH 6.5; Al-Nasser & Crompton, 1986*a*). It is possible that minimal pore opening may suffice for uncoupling owing to rapid H^+ back-flow through the inner membrane, in particular when $[H^+]$ is increased.

The use of mitochondria with entrapped Ca^{2+} buffers indicated that > 25 μM matrix free Ca^{2+} is needed for maximal rate of pore opening (Al-Nasser & Crompton, 1986*a*). Such values greatly exceed the Ca^{2+} concentrations that regulate the matrix dehydrogenases and that are thought to exist normally (around 1 μM; Denton & McCormack, 1980; Crompton, 1985). Nevertheless, reoxygenation-induced injury is associated with cellular Ca^{2+} uptake (Poole-Wilson *et al.*, 1984), which raises the question whether the increase in cytoplasmic free $[Ca^{2+}]$ under such conditions might cause mitochondrial Ca^{2+} overload sufficient for pore opening. Analyses of the steady-state behaviour of Ca^{2+} cycling across the inner membrane of isolated heart mitochondria reveal an extremely steep dependence of matrix free $[Ca^{2+}]$ on extramitochondrial free $[Ca^{2+}]$ when the latter is maintained (rather than being transient, as in normal beat-to-beat function) above a critical value close to 1 μM (Crompton, 1985). For example, with extramitochondrial free $[Ca^{2+}]$ constant at 0.8 μM, the analyses predict < 2 μM matrix free Ca^{2+}, whereas with extramitochondrial $[Ca^{2+}]$ maintained constant at 1.6 μM, > 20 μM matrix free $Ca^{2+}$is indicated. Thus there is a critical limit of extramitochondrial free $[Ca^{2+}]$ below which matrix free $[Ca^{2+}]$ differs little from that outside and above which mitochondrial Ca^{2+} overload occurs. Clearly, the limit may differ *in vivo*, but a value around 1 μM is indicated.

These predictions of Ca^{2+}-cycle behaviour may be related to measurements of cytoplasmic free $[Ca^{2+}]$ in single heart cells subject to anoxia/reoxygenation (Allshire *et al.*, 1987). These revealed an eventual rise in cytoplasmic free $[Ca^{2+}]$ after prolonged anoxia; this rise was preceded by cell shortening, taken to indicate rigor caused by lack of ATP. The eventual progressive increase in cytoplasmic $[Ca^{2+}]$ was reversed by reoxygenation, provided that a limiting free $[Ca^{2+}]$ (1.5–3 μM) was not exceeded. This limit is sufficiently close to that which determines the behaviour of isolated mitochondria to suggest to us the following hypothesis. (*a*) When cytoplasmic $[Ca^{2+}]$ is below this limit, reoxygenation leads to little or no net Ca^{2+} uptake by mitochondria, so that the pore remains in the closed state, and oxidative phosphorylation regenerates the phosphorylation potential necessary to drive the active Ca^{2+}-transport systems of the plasma membrane and sarcoplasmic reticulum that restore Ca^{2+} homoeostasis. (*b*) When cytosolic $[Ca^{2+}]$ is maintained beyond this limit, reoxygenation induces mitochondrial Ca^{2+} uptake and overload sufficient for pore opening; this would be favoured by the increased $[P_i]$ that arises during anoxia (Kammermeier *et al.*, 1982) and by the oxidative stress that occurs on reoxygenation (Poole-Wilson *et al.*, 1984). Pore opening would bring about not only uncoupling of oxidative phosphorylation but also exposure of uncoupled mitochondrial F_1-ATPase to the cytoplasm, and the generation of the high cytoplasmic phosphorylation potential required (directly or indirectly) for active Ca^{2+} extrusion from the cell and accumulation by sarcoplasmic reticulum would be effectively compromised. In this sense, pore opening might be a critical event beyond which irreversible injury ensues. Our finding of a potent inhibitor of pore opening offers a means by which this proposal might be tested *in vivo*.

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