Inhibition of Ca^{2+}-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis–trans isomerase and preventing it interacting with the adenine nucleotide translocase

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1. Isolated rat liver and heart mitochondria incubated in 150 mM-KSCN or sucrose medium in the presence of respiratory-chain inhibitors showed a large increase in swelling when exposed to 250 μM-Ca^{2+}. Swelling was inhibited by bongkrekic acid and cyclosporin A in both media and by ADP in KSCN medium; the effect of ADP was reversed by carboxyatractyloside. These results demonstrate that this is a suitable technique with which to study the opening of the Ca^{2+}-induced non-specific pore of the mitochondrial inner membrane and implicate the adenine nucleotide carrier in this process. 2. Titration of the rate of swelling with increasing concentrations of cyclosporin showed the number of cyclosporin-binding sites ($\pm$ S.E.M.) in liver and heart mitochondria to be respectively 113.7 ± 5.0 ($n = 9$) and 124.3 ± 11.2 ($n = 10$) pmol/mg of protein, with a K_i of about 5 nM. 3. Liver and heart mitochondrial-matrix fractions were prepared free of membrane and cytosolic contamination and shown to contain cyclosporin-sensitive peptidyl-prolyl cis–trans isomerase (cyclophilin) activity. Titration of isomerase activity with cyclosporin gave values ($\pm$ S.E.M.) of 110.6 ± 10.1 ($n = 5$) and 165.4 ± 15.0 ($n = 3$) pmol of enzyme/mg of liver and heart mitochondrial protein respectively, with a K_i of 2.5 nM. The similarity of these results to those from the swelling experiments suggest that the isomerase may be involved in the Ca^{2+}-induced swelling. 4. The rapid light-scattering change induced in energized heart mitochondria exposed to sub-micromolar Ca^{2+} [Halestrap (1987) Biochem. J. **244**, 159–164] was inhibited by ADP and bongkrekic acid, the former effect being reversed by carboxyatractyloside. These results suggest an interaction of Ca^{2+} with the adenine nucleotide carrier when in the 'c' conformation. 5. A model is proposed in which mitochondrial peptidyl-prolyl cis–trans isomerase interacts with the adenine nucleotide carrier in the presence of Ca^{2+} to cause non-specific pore opening. The model also explains the involvement of the adenine nucleotide translocase in the PP_i-mediated cyclosporin-insensitive increase in K^+ permeability described in the preceding paper [Davidson & Halestrap (1990) Biochem. J. **268**, 147–152]. 6. The physiological and pathological implications of the model are discussed in relation to reperfusion injury and cyclosporin toxicity.

INTRODUCTION

In the preceding paper (Davidson & Halestrap, 1990) we provided evidence that the swelling of mitochondria caused by sub-micromolar $[Ca^{2+}]$ (Halestrap et al., 1986; Davidson & Halestrap, 1987; Halestrap, 1989) might involve both a PP_i-dependent mechanism that is insensitive to inhibition by cyclosporin and an additional effect of Ca^{2+} that is inhibited by cyclosporin. It was suggested that the cyclosporin-sensitive mechanism might be similar to that associated with the opening of a non-specific pore at high (supra-physiological) Ca^{2+} loading of mitochondria, which is also known to be inhibited by cyclosporin (Fournier et al., 1987; Crompton et al., 1987, 1988; Broekemeier et al., 1989). However, at lower $[Ca^{2+}]$ the pore probably remains open only for short periods of time. These are long enough to allow sufficient electrogenic entry of K^+ to produce the observed swelling but insufficient to allow collapse of the membrane potential and loss of accumulated ions.

It has been shown by several workers that the massive swelling of mitochondria at high $[Ca^{2+}]$ that is associated with the opening of the non-specific pore can be blocked by addition of ADP, ATP and bongkrekic acid and activated by phosphate, palmitoyl-CoA and carboxyatractyloside (Chappell & Crofts, 1965; Haworth & Hunter, 1979; Hunter & Haworth, 1979; Harris et al., 1979; Crompton & Costi, 1988; Le Quoc & Le Quoc, 1988). These effectors all bind to the adenine nucleotide carrier, and this has

led to the suggestion that the conformation of this carrier influences the ability of Ca^{2+} to open the pore (Le Quoc & Le Quoc, 1988). The inhibition of the pore opening by cyclosporin is unlikely to be by binding to the carrier, however, since the number of cyclosporin-binding sites is about 50–100 pmol/mg of protein (Crompton et al., 1988; Broekemeier et al., 1989), which is substantially less than the number of adenine nucleotide translocase molecules, especially in heart mitochondria (Klingenberg, 1976). The only well-characterized cyclosporin-binding protein is cyclophilin, which is now known to be identical with the enzyme peptidyl-prolyl cis–trans isomerase (Fischer et al., 1989; Takahashi et al., 1989). In Neurospora crassa it is known that this enzyme is present in the mitochondrial matrix as well as the cytosol (Tropschug et al., 1988). In this paper we show that in both heart and liver mitochondria the K_i for cyclosporin inhibition of the matrix peptidyl-prolyl cis–trans isomerase and the calculated number of binding sites are similar to the K_i and number of binding sites for the inhibition of Ca^{2+}-mediated swelling of de-energized mitochondria in KSCN medium. Furthermore, the conformational change induced by sub-micromolar $[Ca^{2+}]$ in energized heart mitochondria (Halestrap, 1987) is shown to be reversed by ADP, ATP or bongkrekic acid, all of which inhibit large-amplitude swelling. Similarly, the effects of ADP and ATP on the conformational change are blocked by carboxyatractyloside. These results are interpreted in terms of a model in which pore formation results from an interaction of

peptidyl-prolyl *cis–trans* isomerase with the adenine nucleotide carrier when it is in the 'c' conformation and binding Ca^{2+} but not adenine nucleotides. The model also incorporates a mechanism by which PP_i may interact with the adenine nucleotide carrier and induce an increase in K^+ permeability.

EXPERIMENTAL

Materials

N-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was obtained from Sigma Chemical Co., Poole, Dorset, U.K., and chymotrypsin A_4 was from Boehringer Corp., Lewes, Sussex, U.K. The sources of all other chemicals and biochemicals were as given in the preceding paper (Davidson & Halestrap, 1990). Rat liver and heart mitochondria were prepared by Percoll-gradient centrifugation as described previously (Halestrap, 1987, 1988; Davidson & Halestrap, 1987).

Methods

Ca^{2+}-induced swelling of de-energized mitochondria. Mitochondria (5–15 mg of protein) were added to 7.4 ml of buffer containing 150 mM-KSCN, 10 mM-Mops, 5 mM-Tris, 0.5 μg of rotenone/ml and 0.5 μg of antimycin/ml at pH 7.2 and 30 °C. After mixing, 3.6 ml portions of the suspension were added to both sample and reference cuvettes of a split-beam spectrophotometer. Swelling was initiated by addition of Ca^{2+} (usually 250 μM) to the sample cuvette, and the A_{520} was monitored with an on-line computer. In some experiments KSCN was replaced by 150 mM-sucrose. In order to see swelling of heart mitochondria upon addition of Ca^{2+} alone, it was necessary to store the mitochondria overnight at 0 °C.

Assay of peptidyl-prolyl *cis–trans* isomerase in mitochondrial-matrix fractions. Matrix fractions were prepared from both heart and liver mitochondria by digitonin treatment and sonication as described previously (Davidson & Halestrap, 1989). No detectable succinate dehydrogenase activity was present in this fraction, which was therefore devoid of inner-membrane contamination, and assay of lactate dehydrogenase and adenylate kinase activity was used to demonstrate that there was no significant contamination by cytosolic or intermembrane proteins (Vargas, 1982; Davidson & Halestrap, 1989). Citrate synthase activity was measured per mg of mitochondrial protein before subfractionation and per mg of protein in the final matrix preparation. This allowed the activity of peptidyl-prolyl *cis–trans* isomerase in the matrix fraction to be expressed per mg of total mitochondrial protein. No detectable activity of this isomerase was found in the inner-membrane fraction, suggesting that all the isomerase inside the mitochondria was free in the matrix. This was confirmed by demonstrating that the ratio of isomerase to citrate synthase activities in the matrix fraction was the same as that for mitoplasts. Assay of the isomerase was performed at 10 °C essentially as described by Fischer *et al.* (1989). A 3.5 ml portion of buffer (35 mM-Hepes, pH 7.8, containing 0.26 mg of chymotrypsin/ml A_4) was added to the sample cuvette of a split-beam spectrophotometer. In order to balance the signal, the reference cuvette contained a previous assay that had reached completion. Where required, mitochondrial-matrix extract (50–300 μg of protein) and cyclosporin were added to the sample cuvette, which was constantly stirred. The A_{390} was constantly monitored by an on-line computer (14 data points/s). After 30 s 3.5 μl of a solution of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (25 mM in dimethyl sulphoxide) was added to the sample cuvette through an injection port, and data acquisition continued until the reaction was complete. The reaction proceeds through a burst phase taking 3–5 s, which is due to the rapid hydrolysis of
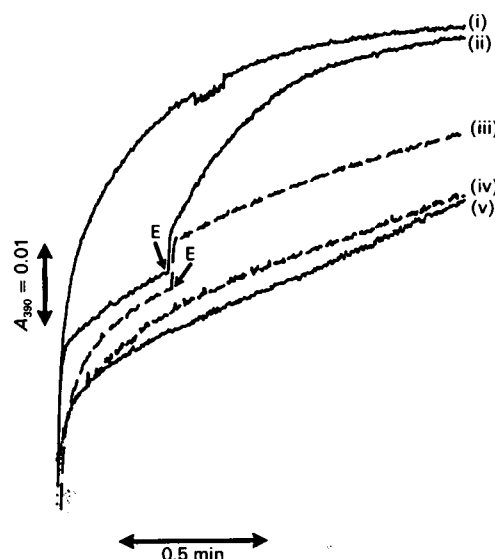

Fig. 1. Assay of peptidyl-prolyl *cis–trans* isomerase and its inhibition by cyclosporin

The assay involves measurement of the increase in A_{390} resulting from hydrolysis by chymotrypsin of an artificial peptide as described in the Experimental section. In traces (i) and (iv) liver mitochondrial-matrix extract was present at 25 μg of protein/ml from the start of the assay, whereas in traces (ii) and (iii) the extract (E) was added as indicated on the trace. In trace (v) no extract was present. The broken traces (ii and iv) represent experiments where 1 μM-cyclosporin was present in the medium. In all cases the initial burst phase of the hydrolysis of the substrate has been omitted.
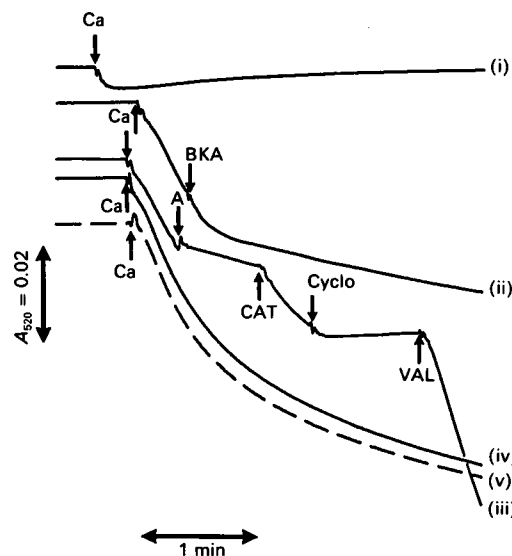

Fig. 2. Ca^{2+}-induced swelling of de-energized liver mitochondria in 150 mM-KSCN

Mitochondria were incubated in buffered KSCN medium containing respiratory-chain inhibitors in both sample and reference cuvettes of a split-beam spectrophotometer as described in the Experimental section. A decrease in light-scattering is shown as a decrease in A_{520}. Where indicated, additions were made to the sample cuvette alone: 250 μM-CaCl$_2$ (Ca), 10 μM-bongkrekic acid (BKA), 10 μM-carboxyatractyloside (CAT), 0.2 mM-ADP (A), 1 μM-cyclosporin (Cyclo) and 0.1 μM-valinomycin (VAL). The broken trace (v) shows an experiment where 2 mM-phosphate was present in the medium.

the *trans* isomer of the substrate (85 %), followed by a slower phase which is dependent on the isomerization of the *cis* isomer, which is not a substrate for the isomerase, to the *trans* isomer, which is. It is this second phase which is stimulated by peptidyl-prolyl *cis–trans* isomerase in a cyclosporin-sensitive manner, as shown in Fig. 1.

RESULTS

Swelling of de-energized liver and heart mitochondria induced by Ca^{2+} in KSCN and sucrose media

The data of Fig. 2 show that, when liver mitochondria are incubated in 150 mM-KSCN in the presence of rotenone and antimycin and exposed to 250 μM-Ca^{2+}, there is a decrease in light-scattering (A_{520}) caused by their swelling (trace iv). Since

the mitochondria are freely permeable to the SCN$^-$ anion, this indicates that Ca^{2+} increases the permeability of the mitochondrial inner membrane to K$^+$, whose rate of entry into the mitochondria determines the rate of swelling under these conditions (see Davidson & Halestrap, 1987). Thus addition of valinomycin also produced rapid swelling (trace iii). The effect of Ca^{2+} was almost totally inhibited by 1 μM-cyclosporin (traces i and iii), by 10 μM-bongkrekic acid (trace ii) and by 0.2 mM-ADP (trace iii) or -ATP (results not shown). The effect of ADP was reversed by 10 μM-carboxyatractyloside (trace iii). In these respects the swelling has the same characteristics as that induced in energized mitochondria upon loading with excess Ca^{2+} (see the Introduction), but with the exception that phosphate is not required (trace v compared with trace iv). We have also demonstrated that Mn^{2+} and Sr^{2+} cannot substitute for Ca^{2+} (results not shown); this is also the case in energized mitochondria (Pfeiffer et al., 1979; Beatrice et al., 1980).

In Fig. 3 we demonstrate that swelling of de-energized mitochondria can also be induced by Ca^{2+} under similar conditions in sucrose medium. It was found that hypo-osmotic sucrose media (150 mM) gave better results than in iso-osmotic media (Fig. 3a, trace i compared with trace iv) and that phosphate greatly stimulated under these conditions (Fig. 3a, trace ii compared with trace iv). Once again cyclosporin totally inhibited swelling (Fig. 3a, trace iii). However, swelling was stimulated by ATP (Fig. 3b, trace i) or ADP (Fig. 3b trace, iii); this stimulation could be inhibited by bongkrekic acid (Fig. 3b, trace i), but not by carboxyatractyloside (Fig. 3b, trace iii). A possible explanation for the opposite effects of ATP and ADP in the two media is offered in the Discussion section. In both media, heart mitochondria showed Ca^{2+}-induced swelling similar to that observed for liver mitochondria. However, it was found necessary to leave heart mitochondria for 12 h at 0 °C before Ca^{2+} alone could induce swelling. This technique has been shown by others to increase the sensitivity of mitochondria towards Ca^{2+}-induced swelling (Pfeiffer et al., 1979), and it is known that fresh heart
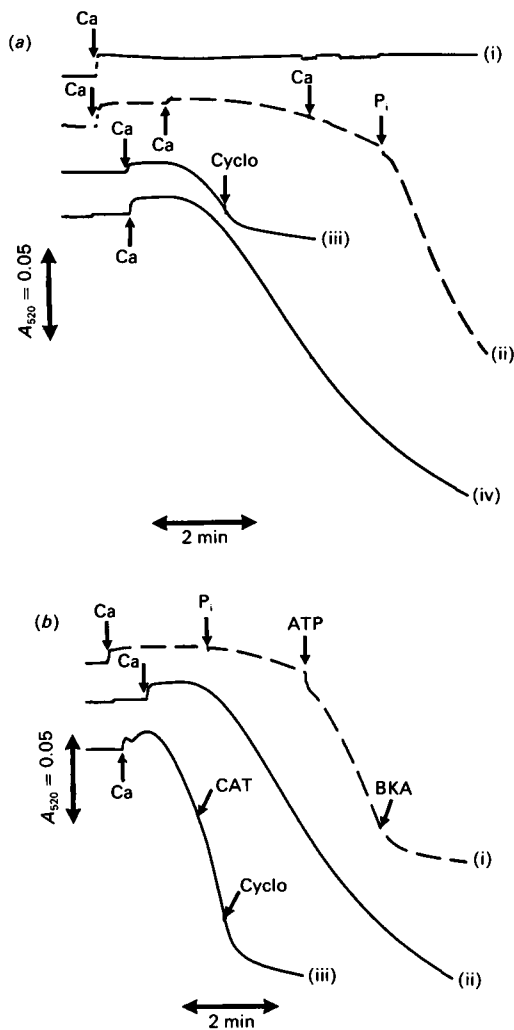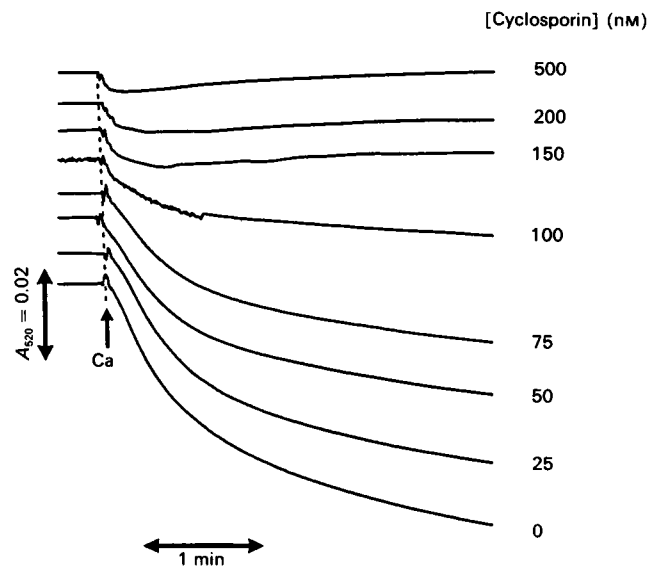
Fig. 3. Ca^{2+}-induced swelling of de-energized liver mitochondria in sucrose medium

Mitochondria were incubated in buffered sucrose medium (150 mM, except for trace i in a, which was 300 mM-sucrose) containing respiratory-chain inhibitors as described in the Experimental section and the legend to Fig. 2. Phosphate (P_i; 2 mM) was present in all cases except the broken traces (a,ii and b,i) where it was added as indicated at 2 mM and 0.25 mM respectively. Further additions were made to the sample cuvette alone as shown: 500 μM-$CaCl_2$ (Ca), 5 μM-bongkrekic acid (BKA), 5 μM-carboxyatractyloside (CAT), 1 mM-ATP, 0.5 μM-cyclosporin (Cyclo). For trace b,iii, 0.2 mM-ADP was present from the start of the experiment.

Fig. 4. Inhibition of Ca^{2+}-induced swelling of de-energized liver mitochondria by cyclosporin

Experimental details were the same as for Fig. 2, but the buffer contained cyclosporin at the concentration shown. Rat liver mitochondria were present at 1.18 mg/ml and swelling was initiated with 250 μM-Ca^{2+}.
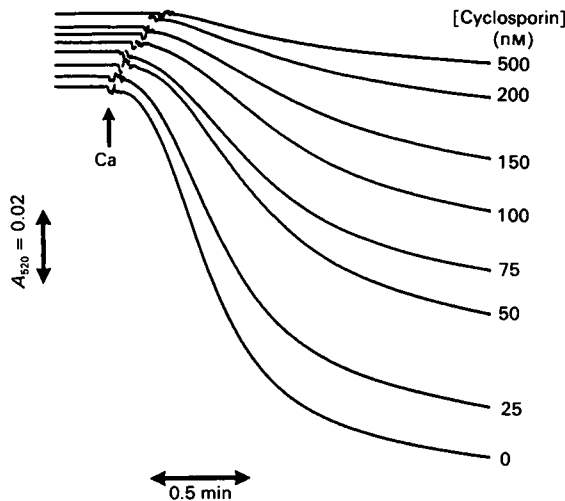
Fig. 5. Inhibition of Ca^{2+}-induced swelling of de-energized heart mitochondria by cyclosporin

Experimental details were the same as for Fig. 4. Rat heart mitochondria, aged overnight at 0 °C, were present at 0.86 mg/ml and swelling was initiated with 250 μM-Ca^{2+}.
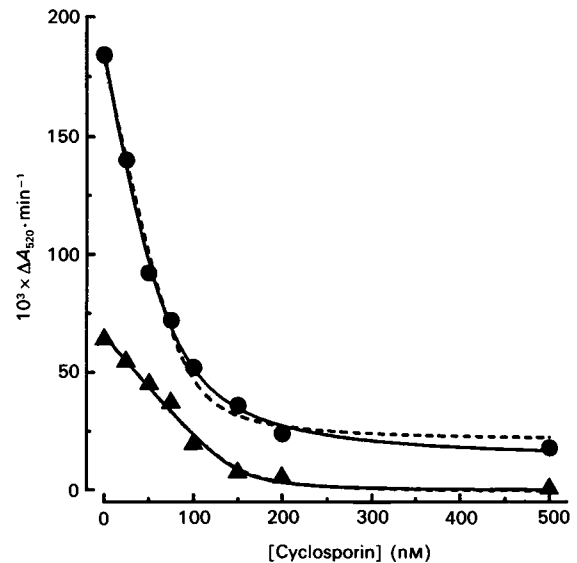

Fig. 6. Concentration-dependence of the inhibition of Ca^{2+}-induced swelling of liver and heart mitochondria by cyclosporin

The traces of Figs. 4 and 5 were differentiated and the maximal rates of swelling occurring after the first 10 s were calculated. The data were fitted mathematically to the equation for a very tight-binding inhibitor as described in the text. The continuous lines represent the theoretical curves for the derived parameter values where the computer was allowed to estimate both the K_i and the total number of binding sites. For the data shown, the values for K_i and concentration of cyclosporin-binding sites ($\pm$S.E.) for liver mitochondria ($\blacktriangle$) were 3.79 ± 5.19 nM and 147 ± 18 nM respectively, and the values for heart mitochondria ($\bullet$) were 13.8 ± 4.7 nM and 71 ± 10 nM. The dashed lines are for the fit when K_i was fixed at 5 nM, as discussed in the text; the derived concentrations of cyclosporin-binding sites for liver and heart mitochondria were 146 ± 14 and 89 ± 7 nM respectively. Values from several experiments are given as means $\pm$ S.E.M. in Table 1.

mitochondria are much less sensitive to Ca^{2+}-induced swelling than are those from liver (Palmer & Pfeiffer, 1981). Aging was used in preference to treatment of mitochondria with t-butyl hydroperoxide, which also sensitizes mitochondria to Ca^{2+}-induced permeability changes (Beatrice et al., 1982; Crompton et al., 1987, 1988; Crompton & Costi, 1988; Broekemeier et al., 1989).

The data of Figs. 4 and 5 show the progressive inhibition of the Ca^{2+}-induced swelling of de-energized liver and heart mitochondria respectively by increasing concentrations of cyclosporin. Differentiation of the time course of swelling was performed in order to measure the maximal rate of swelling (excluding the first 10 s, where addition artefacts obscured the true rate). In Fig. 6 the maximal rates of swelling are plotted against the cyclosporin concentration for the data from both Figs. 4 and 5. It is clear that the inhibition of swelling was linear with respect to the cyclosporin concentration until 50 % inhibition or more had been reached. Linear extrapolation of the data of Fig. 6 to 100 % inhibition yields values for the number of cyclosporin-sensitive sites of about 125 pmol/mg of protein for both liver and heart mitochondria. It was found that the concentration of cyclosporin giving half-maximal inhibition was dependent on the mitochondrial protein concentration used (results not shown). This suggests stoichiometric binding of cyclosporin to a binding protein with a K_i considerably less than the concentration of binding sites. Mathematical analysis of these binding curves can be performed as described in the preceding paper (Davidson & Halestrap, 1990), and values for the K_i and total number of binding sites can be calculated. Results for the experiment shown are given in the legend to Fig. 6, and a summary of results for several preparations of heart and liver mitochondria performed at different protein concentrations is given in Table 1. One problem with this mathematical analysis is that the derived values for the K_i and total number of binding sites are mutually inter-related. Thus it is possible to achieve quite good fits to the data by either increasing K_i and decreasing the total number of binding sites, or vice versa. Thus we have also fitted the data by fixing the K_i at 5 nM, an average value of the derived K_i values, since it can be argued that K_i must be the same in all experiments. This procedure, by removing one variable

Table 1. Summary of the derived values for the K_i for cyclosporin and the number of cyclosporin-binding sites involved in the inhibition of Ca^{2+}-induced swelling of energized and de-energized liver and heart mitochondria and of their peptidyl-prolyl cis–trans isomerase

Data for the energized liver mitochondria are taken from experiments described in the preceding paper (Davidson & Halestrap, 1990), and the remaining data were obtained as described in the present paper. Values are expressed as means $\pm$ S.E.M. for n experiments, each involving a separate preparation of mitochondria or matrix extract. The two values for the number of cyclosporin-binding sites represent those derived with the K_i fixed at 5 nM or with the K_i calculated from the best data fit as described in the legend to Fig. 6. The mean of the latter K_i value is given in the Table.

		Cyclosporin-binding sites (pmol/mg of mitochondrial protein)		
Conditions	K_i (nM)	Variable K_i	K_i at 5 nM	n
Liver				
KSCN swelling	4.1 ± 2.5	124.1 ± 8.7	113.7 ± 5.0	9
Energized swelling	5.3 ± 1.65	127.9 ± 13.9	122.3 ± 10.7	10
Isomerase	2.3 ± 0.77	110.6 ± 10.1	87.7 ± 11.4	5
Heart				
KSCN swelling	14.2 ± 2.45	103.7 ± 12.2	124.3 ± 11.2	10
Isomerase	2.6 ± 1.63	165.4 ± 15.0	131.3 ± 12.2	3

parameter from the analysis, simplifies the fit and thus improves the confidence limits of the derived parameter values, including that for the number of binding sites. The dashed lines in Fig. 6 represent these fits, and combined data are given in Table 1.

Inhibition of mitochondrial-matrix peptidyl-prolyl *cis–trans* isomerase by cyclosporin

The presence of peptidyl-prolyl *cis–trans* isomerase in liver mitochondrial matrix was demonstrated in Fig. 1, where it was also shown to be inhibited by cyclosporin. It should be stressed that the contamination of the matrix extract by cytosolic, membrane or intermembrane enzymes was negligible, as judged by lactate dehydrogenase, succinate dehydrogenase and adenylate kinase activities (see Davidson & Halestrap, 1989). Thus the peptidyl-prolyl *cis–trans* isomerase activity measured does represent matrix activity. In order to calculate the amount of enzyme present in both heart and liver matrix, the activity of the enzyme was titrated with different cyclosporin concentrations as shown in Fig. 7. At least two different protein concentrations were used within each experiment. By incorporating the protein concentration as a second x variable in the analysis, the confidence limits on the derived values for K_i and total number of binding sites were improved. Mean values from several experiments are given in Table 1, and are similar to the values obtained for the de-energized swelling. We have been unable to show any significant effect of either EGTA (0.5 mM) or Ca^{2+} (0.01–1 mM) on the enzyme activity.

Effect of substrates and inhibitors of the adenine nucleotide translocase on the conformational change induced in heart mitochondria by Ca^{2+}

We propose a model in the Discussion section which involves peptidyl-prolyl *cis–trans* isomerase interacting with the adenine nucleotide carrier in the presence of Ca^{2+}. This requires that Ca^{2+} interacts with the carrier. In heart mitochondria we have shown previously that Ca^{2+} causes a large and rapid decrease in light-
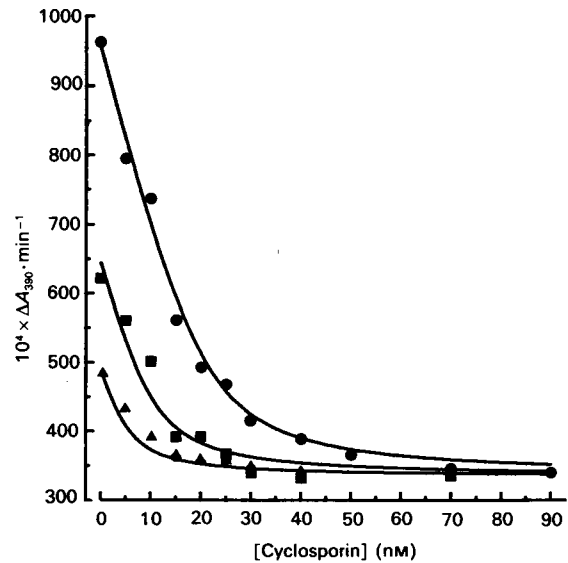

Fig. 7. Concentration-dependence of the inhibition of liver mitochondrial-matrix peptidyl-prolyl *cis–trans* isomerase by cyclosporin

Assay of the isomerase was performed as described in the Experimental section and the legend to Fig. 1. Liver mitochondrial-matrix extract was present from the start of the assay at 13.5 (▲), 27 (■) and 54 (●) μg of protein/ml. The rate of change of A_{390} was calculated 10 s after substrate addition, at which time the burst phase of the reaction was essentially complete. The lines shown represent the fit of the data to the equation for inhibition of an enzyme by a very tight-binding inhibitor, but including the protein concentration as a second x variable as described in the text. The derived values ($\pm$S.E.) for the K_i and number of cyclosporin-binding sites were 2.10 ± 0.78 nM and 384 ± 46 pmol/mg of matrix protein respectively. Mean values from several experiments are shown in Table 1, where results are expressed in terms of total mitochondrial protein to allow comparison with the data from the mitochondrial-swelling experiments.
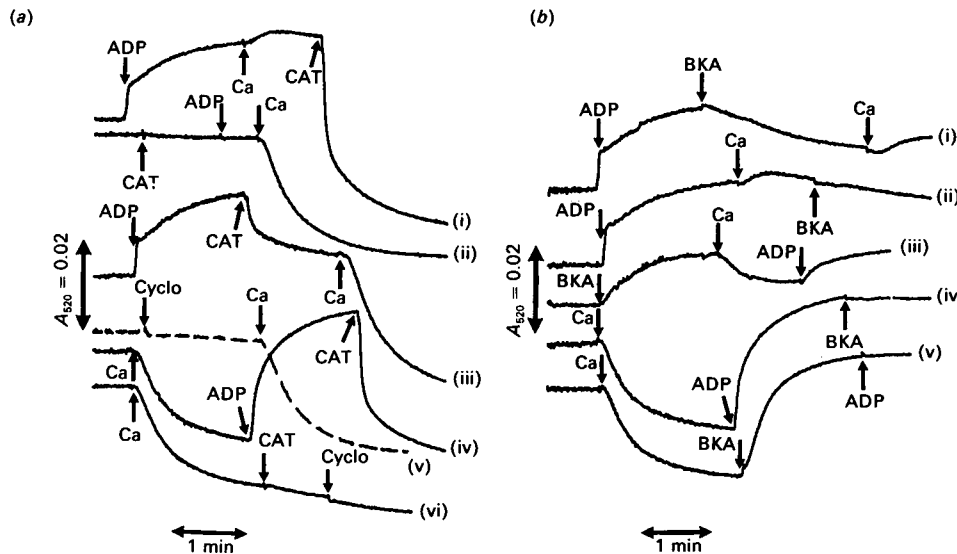

Fig. 8. Effects of ADP, carboxyatractyloside, bongkrekic acid and cyclosporin on the conformational change of isolated rat heart mitochondria induced by Ca^{2+}

Heart mitochondria (final concn. approx. 2 mg of protein/ml) were incubated in a split-beam spectrophotometer at 30 °C in buffer containing 125 mM-KCl, 10 mM-Mops, 5.5 mM-Tris, 0.5 mM-EGTA, 5 mM-sodium glutamate and 1 mM-L-malate, pH 7.2. Conformational changes were detected as changes in light-scattering (A_{520}), which were shown previously to occur in the absence of any change in mitochondrial volume (Halestrap, 1987). Where indicated, additions were made to the sample cuvette as follows: 1 μM-Ca^{2+} (Ca), 10 μM-bongkrekic acid (BKA), 10 μM-carboxyatractyloside (CAT), 0.2 mM-ADP and 1 μM-cyclosporin (Cyclo).

scattering (A_{520}), with a $K_{0.5}$ of 0.3 μM. However, unlike the light-scattering change associated with swelling, this was not accompanied by a change in matrix volume, and thus probably reflects a conformational change of the mitochondria (Halestrap, 1987). It has been shown by others that ATP, ADP, carboxyatractyloside and bongkrekic acid also produce substantial light-scattering changes in heart mitochondria, which are associated with changes in the conformation of the adenine nucleotide translocase (Klingenberg et al., 1971; Stoner & Sirak, 1973a,b; Scherer & Klingenberg, 1974). The results presented in Fig. 8 confirm these observations, but in addition illustrate the interaction between these substrates and inhibitors of the carrier and the conformational change induced by Ca^{2+}. The decrease in light-scattering induced by 1 μM-Ca^{2+} was prevented by ADP, but this effect was reversed by the presence of carboxyatractyloside (Fig. 8a, traces i–iii). If ADP was added after Ca^{2+}, the light-scattering increased again, reaching the same level as observed in the absence of Ca^{2+}. Subsequent addition of carboxyatractyloside after the ADP caused a decrease in light-scattering, to give a value equivalent to that observed with Ca^{2+} alone (Fig. 8a, trace iv). In the absence of ADP, carboxyatractyloside had little or no effect on light-scattering (Fig. 8a, traces iv and vi). Fig. 8(b) contains data to illustrate the effects of bongkrekic acid. This inhibitor caused an increase in light-scattering as found by previous workers, but subsequent addition of Ca^{2+} gave little change in light scattering (Fig. 8b, trace iii). Furthermore, addition of bongkrekic acid after the decrease in light-scattering induced by Ca^{2+} caused a reversal of this effect and an increase in light-scattering to values similar to those seen with bongkrekic acid added in the absence of Ca^{2+} (Fig. 8b, trace v). This effect of bongkrekic acid was similar to that of ADP (Fig. 8b, trace iv) and these two agents had no additive effect in the presence of Ca^{2+} (Fig. 8b, traces iii and iv). All the above data imply that the light-scattering change induced by Ca^{2+} is the consequence of an interaction of Ca^{2+} with the 'c' conformation of the adenine nucleotide carrier. This is stabilized by carboxyatractyloside and reversed by bongkrekic acid or ADP, which stabilize the 'm' conformation of the carrier. In Fig. 8(a) we also illustrate the lack of any substantial effect of cyclosporin on the conformational change induced by Ca^{2+} (traces v and vi). Nor was there any effect of this agent on the light-scattering changes observed in the presence of ADP, bongkrekic acid or carboxyatractyloside (results not shown). There was consistently a very slow decrease in light-scattering induced by cyclosporin itself, however, as illustrated in Fig. 8(a), trace v.

DISCUSSION

Nature of the cyclosporin-sensitive and -insensitive mechanisms of mitochondrial swelling

In the preceding paper (Davidson & Halestrap, 1990) we provided evidence that Ca^{2+} at physiological concentrations causes swelling of energized liver mitochondria in vitro and in situ, primarily through a cyclosporin-insensitive mechanism involving PP_i, but that an additional cyclosporin-sensitive pathway also operates and becomes dominant at a higher (supraphysiological) [Ca^{2+}]. We have also suggested previously that the swelling of mitochondria caused by an increase in matrix [PP_i] is due to an interaction of PP_i and P_i with the adenine nucleotide carrier (Davidson & Halestrap, 1987; Halestrap & Davidson, 1989). Recent evidence supports a sequential model for the adenine nucleotide carrier with the simultaneous occupation by ATP and ADP of both an inner and an outer nucleotide-binding site (Sluse et al., 1989; Vignais et al., 1989). However, phosphate and PP_i can both act as weak substrates for the adenine nucleotide carrier (Asimakis & Aprille, 1980;
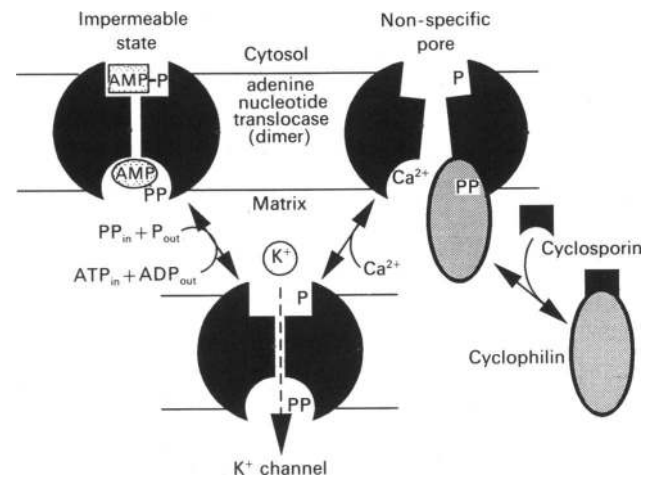
Scheme 1. **Possible involvement of the adenine nucleotide translocase in the PP_i-mediated cyclosporin-insensitive K^+-channel formation and the cyclosporin-sensitive Ca^{2+}-mediated non-specific pore formation**

AMP-P, AMP-PP, P and PP represent ADP, ATP, P_i and PP_i respectively. In all cases the carrier is assumed to be in the 'c' conformation, although this is not essential for the cyclosporin-insensitive mechanism, since bongkrekic acid-induced swelling is not prevented by cyclosporin. A detailed explanation of the scheme is given in the text.

Krämer, 1985; Halestrap & Davidson, 1989). We propose that, when both PP_i and phosphate occupy these inner and outer substrate-binding sites respectively, a channel through the transporter is exposed which is normally blocked by the presence of the nucleotide moiety of the substrates. This channel is of sufficient diameter to allow the electrogenic uptake of K^+ ions, as illustrated in Scheme 1. Since the affinity of the carrier for PP_i and phosphate is substantially less than that for ADP and ATP (see Halestrap & Davidson, 1989), the K^+ channel will only open relatively infrequently, so preventing a massive increase in K^+ permeability and catastrophic swelling. This PP_i/phosphate-mediated swelling is insensitive to cyclosporin, and can occur in the presence of either bongkrekic acid or carboxyatractyloside. The latter observation implies that such swelling is insensitive to whether the carrier is in the 'm' or the 'c' conformation.

However, our present data indicate that Ca^{2+} must have an additional effect on K^+ permeability that cannot be explained by increases in matrix [PP_i] alone, but which is inhibited by cyclosporin. This process was studied in the present paper by measuring the swelling (decrease in light-scattering at 520 nm) of de-energized mitochondria exposed to 250 μM-Ca^{2+} in KSCN medium. The responses seen under such conditions were highly reproducible and appeared to mimic those seen in energized mitochondria loaded with large quantities of Ca^{2+}. The opposing effects of carboxyatractyloside and adenine nucleotides or bongkrekic acid on this large-amplitude swelling and the requirement for phosphate again argue for an involvement of the adenine nucleotide translocase in this process, perhaps when the carrier is in the 'c' conformation with phosphate and PP_i replacing ADP and ATP. The data of Fig. 8 add weight to this proposal, since they show more directly that Ca^{2+} may interact with the adenine nucleotide translocase in the 'c' conformation. Thus Ca^{2+} induces a conformational change that is reversed by ADP or bongkrekic acid, but not by carboxyatractyloside, which prevents the effect of ADP.

Two features of the Ca^{2+}-induced swelling of de-energized mitochondria in KSCN medium are not the same as that seen in

sucrose medium. Firstly, phosphate is required to obtain swelling in sucrose medium, but not in KSCN medium. This could be explained if SCN^- could substitute for phosphate binding to the external substrate-binding site at the high concentration (150 mM) of KSCN used. Secondly, the inhibitory effect of ADP on the Ca^{2+}-induced swelling of de-energized mitochondria in KSCN medium is similar to the effect seen in energized mitochondria, but is replaced by a stimulatory effect of ADP in the sucrose medium under de-energized conditions. This might be explained by the generation of a membrane potential by the entry of SCN^- into the mitochondria, similar to that seen in energized mitochondria but not possible under de-energized conditions in sucrose medium. The re-orientation of the carrier from the 'm' to the 'c' conformation and the relative binding affinities of the inner and outer binding sites for adenine nucleotides are influenced by the membrane potential (see Klingenberg, 1976). Thus it is possible that in the absence of a membrane potential ADP and ATP can allow interconversion of any carrier trapped in the 'm' conformation into the 'c' conformation, which then activates swelling. It is noteworthy that Crompton & Costi (1990) also report that ADP is able to increase the permeability coefficient of de-energized mitochondria made permeable with Ca^{2+} treatment.

Peptidyl-prolyl cis–trans isomerase involvement in Ca^{2+}-mediated swelling and its inhibition by cyclosporin

It remains to be explained how cyclosporin might interact with this system to inhibit swelling. Four observations are significant. Firstly, cyclosporin is known to interact with the enzyme peptidyl-prolyl cis–trans isomerase, otherwise known as cyclophilin (Fischer et al., 1989; Takahashi et al., 1989). In the present paper we have shown that this enzyme is present in liver and heart mitochondria, as it is in mitochondria from Neurospora crassa (Tropschug et al., 1988). Secondly, we have shown that the K_i for inhibition of swelling by cyclosporin is similar to the K_i value of about 5 nM for inhibition of the mitochondrial-matrix isomerase (see Table 1). A value of 2.6 nM for the K_i of cyclosporin for the cytosolic enzyme has been reported by others (Tropschug et al., 1988). Thirdly, the calculated number of cyclosporin-binding sites involved in the inhibition of swelling of both liver and heart mitochondria (about 125 pmol/mg of protein) is very close to the amount of the isomerase present in the matrix calculated from the cyclosporin data (see Table 1). These values are slightly higher than the values of about 40 and 80–90 pmol/mg of heart and liver mitochondrial protein respectively that have been reported by others (Crompton et al., 1988; Broekemeier et al., 1989), but this may be explained by the use of Percoll-gradient centrifugation in the preparation of mitochondria in the present studies. This has the effect of removing substantial membrane contamination from the mitochondria and thus enriching the true mitochondrial protein as a percentage of the total protein content of the mitochondrial preparation (see Halestrap, 1988). Fourthly, the proposed structure for the adenine nucleotide carrier places highly conserved proline residues at the end of three of the transmembrane α-helices as they emerge on the matrix surface of the membrane (Aquila et al., 1987; Klingenberg, 1989). Furthermore, an additional proline residue (residue 61) on the matrix side, but not thought to be membrane-associated, is adjacent to a lysine (residue 62) which can only be covalently modified by pyridoxal phosphate when the carrier is in the 'c' conformation with carboxyatractyloside bound (Bogner et al., 1986).

In the light of these observations we have proposed a model for the swelling of mitochondria observed under conditions of high Ca^{2+} loading, which is summarized in Scheme 1. It is suggested that when the adenine nucleotide translocase is in 'c' conformation it can bind Ca^{2+}, as indicated by the data of Fig.

8. Under these conditions, when PP_i and phosphate, rather than adenine nucleotides, are bound to the substrate-binding sites, it is proposed that one of the proline residues is able to bind to the matrix peptidyl-prolyl cis–trans isomerase. This interaction might cause disruption of the carrier structure sufficient to create a pore. Cyclosporin, by binding to the enzyme, would cause it to dissociate from the carrier and so block the pore. It would seem unlikely that the enzyme is able to catalyse isomerization of a peptidyl-prolyl bond within the carrier, since cyclosporin is able to stop further swelling immediately, as shown in Fig. 2. If isomerization had taken place, inhibition of swelling would occur slowly only as spontaneous cis–trans isomerization reformed the carrier's native conformation. In addition if isomerization were involved, the effect of the enzyme would be strictly catalytic and the maximal rate of swelling observed would be related more to the amount of adenine nucleotide carrier present than to the activity of the isomerase. Thus it would be hard to explain how the swelling of heart mitochondria, which possess at least 5 times the amount of the adenine nucleotide translocase as do liver mitochondria (Klingenberg, 1976), shows a similar inhibition profile with respect to cyclosporin concentration as do liver mitochondria. Nor would such a mechanism be expected to give linear relationships between the inhibition of swelling and the cyclosporin concentration.

An additional effect of Ca^{2+} that is probably a prerequisite for swelling of fresh mitochondria under energized conditions is the activation of mitochondrial phospholipase A_2 and resultant accumulation of unsaturated fatty acids (Beatrice et al., 1980). These act like carboxyatractyloside on the adenine nucleotide translocase (see Le Quoc & Le Quoc, 1988), enhancing the formation of the 'c' conformation and swelling. This explains why phospholipase A_2 inhibitors are effective inhibitors of the swelling (Broekemeier et al., 1985).

Physiological and pathological implications

At the sub-micromolar $[Ca^{2+}]$ that occurs in stimulated cells and was used in isolated energized mitochondria to mimic the effects of hormones on mitochondrial volume (Halestrap et al., 1986; Davidson & Halestrap, 1987, 1988, 1990), most of the swelling seen appears to occur through the PP_i-dependent cyclosporin-insensitive pathway. The small portion of the swelling that is cyclosporin-sensitive will represent the disruption of the carrier structure for very short periods of time. This could allow significant electrogenic uptake of K^+ to occur, driven by the membrane potential and the high $[K^+]$, but uptake of sucrose would be minimal before the pore closed again. It would also explain why little swelling is seen in sucrose buffer compared with KCl buffer (Halestrap et al., 1986). However, it is of interest that [^{14}C]sucrose introduced into hepatocytes by electropermeabilization does enter the mitochondria over a period of hours (Tolleshaug & Seglen, 1985). This would be predicted if occasional transient opening-up of the carrier structure occurred. It is possible that such occasional opening of a pore in the inner membrane is important for the mitochondria to avoid build-up of waste products for which there are no specific transporters. A major risk of cyclosporin therapy is liver and kidney damage, which appears to be associated with mitochondrial dysfunction (Aupetit et al., 1988; Boelsterli et al., 1988; Borel, 1989). Prevention of pore opening and loss of such waste products might account for this damage. The new immunosuppressant drug FK506 has fewer such side-effects, and we have demonstrated recently that it does not protect the mitochondria against Ca^{2+}-induced damage, nor does it inhibit mitochondrial peptidyl-prolyl cis–trans isomerase (Kay et al., 1990).

When mitochondrial $[Ca^{2+}]$ increases, especially when adenine nucleotides are depleted, as occurs in hypoxia and subsequent re-

oxygenation (Sordahl & Stewart, 1980; Asimakis & Sordahl, 1981; LaNoue et al., 1981; Watanabe et al., 1983), the statistical probability of the pore being open would increase sufficiently to allow massive swelling to occur. The effects would be exacerbated by stimulation of phospholipase A_2 as outlined above. Fatty acids are known to accumulate in ischaemic tissue (Smith et al., 1980). Furthermore, lipid peroxides can increase in hypoxia and re-oxygenation as the result of free-radical formation (de Groot & Noll, 1987; Bindoli, 1988), and these are excellent substrates for the mitochondrial phospholipase A_2 (Van Kuijk et al., 1987). Re-esterification, which would normally occur, will be inhibited by the lack of ATP (Pfeiffer et al., 1979; Beatrice et al., 1980).

Consideration of all the above factors suggests that opening of the mitochondrial pore may well be the basis of the mitochondrial damage occurring in reperfusion injury, as has been suggested by others (Crompton & Costi, 1988; Broekemeier et al., 1989). It is of interest that pretreatment of dogs with cyclosporin before their livers were subjected to ischaemia was found to increase greatly the chances of complete recovery (Hayashi et al., 1988).

Conclusion

We believe that the adenine nucleotide translocase is involved in the mechanism of both physiological and pathological forms of mitochondrial swelling. The mechanism that we have outlined would give the appearance of an electrogenic K^+ pore at low $[Ca^{2+}]$ but a non-specific pore at high $[Ca^{2+}]$. This is what is observed. The model, summarized in Scheme 1, also explains why both processes require phosphate, and are inhibited by cyclosporin, bongkrekic acid and adenine nucleotides and activated by carboxyatractyloside and oxidative stress. It is presented as a plausible hypothesis which requires further testing.

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