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The involvement of cyclosporin A binding proteins in regulating and uncoupling mitochondrial energy transduction

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The uncoupling of mitochondrial energy transduction by excess Ca^{2+} may be a factor in the pathogenesis of tissue injury brought about by energy deprivation, for example, in ischaemia. In isolated mitochondria the lesion appears as a large, 20 Å, pore in the inner membrane. The pore is blocked potently by the immunosuppressant cyclosporin A. Cyclosporin A also markedly retards collapse of the mitochondrial inner membrane potential in energy-deprived (respiration-inhibited) cardiomyocytes as judged by changes in rhodamine 123 fluorescence, and prolongs cell viability. A potential mitochondrial target for cyclosporin A is the matrix protein cyclophilin. Purified cyclophilin activates the respiratory chain of submitochondrial particles. This might reflect not only a physiological function of this protein, but also a component involved in the generation of the 20 Å pore under pathological conditions.

It has long been known that excessive amounts of Ca^{2+} induce uncoupling of mitochondrial energy transduction. Much of the current interest in this phenomenon stems from the fact that cellular Ca^{2+} overload is a critical element in certain types of cell injury. Of these, myocardial reperfusion injury is of major clinical importance. Although the heart can recover completely on reperfusion after relatively short ischaemic insults, restoration of blood flow after prolonged ischaemia markedly accelerates, rather than halts or delays, the onset of cell death (reperfusion injury). Evidently, changes occur during ischaemia that render the tissue adversely sensitive to oxygenated blood flow when reintroduced. Among the changes that are believed to be critical are ATP depletion and the net loss of adenine nucleotides as they are degraded to nucleosides and bases, increases in cellular $[P_i]$, $[H^+]$, $[Na^+]$ and $[Ca^{2+}]$, and on reperfusion, inadequate removal of reactive oxygen species, i.e., oxidative stress [1,2]. Clearly, an understanding of the sequence and interdependence of these changes and, in particular, the point at which they become irreversible is crucial to the design of cardioplegic solutions that are used in the maintenance of the underperfused heart during bypass surgery and in the preservation of donor hearts for transplantation, and in minimising tissue injury after removal of a coronary obstruction (angioplasty, thrombolysis). In particular, we need to identify critical processes that need to be blocked in order that irreversible injury may be retarded – processes that would provide targets for pharmacological intervention.

Cellular Ca^{2+} overload is widely believed to be a major contributor to the pathogenesis of the injury [2]; recent studies have confirmed that Ca^{2+} overload precedes irreversible injury in both global ischaemia [1] and anoxia (isolated myocytes [3]). Among numerous potentially adverse consequences, excess Ca^{2+} uncouples mitochondrial energy transduction. Recently, the mechanism of Ca^{2+}-induced uncoupling has come under close scrutiny following the realisation that the particular metabolic constraints under which Ca^{2+} uncouples relate closely to the specific metabolic disturbances associated with ischaemia/reperfusion [4–7]. In particular. following earlier indications [8], the mitochondrial lesion has been identified as a latent, inner membrane pore that opens reversibly when triggered by Ca^{2+}, P_i and oxidative stress [5,6]. The pore is blocked by ATP, but more than 1 mM ATP is required [9]. As a working hypothesis. we suggested that the initiation of respiration on reperfusion after prolonged ischaemia, when resting cytosolic free Ca^{2+} is high, would induce mitochondrial Ca^{2+} overload which, with

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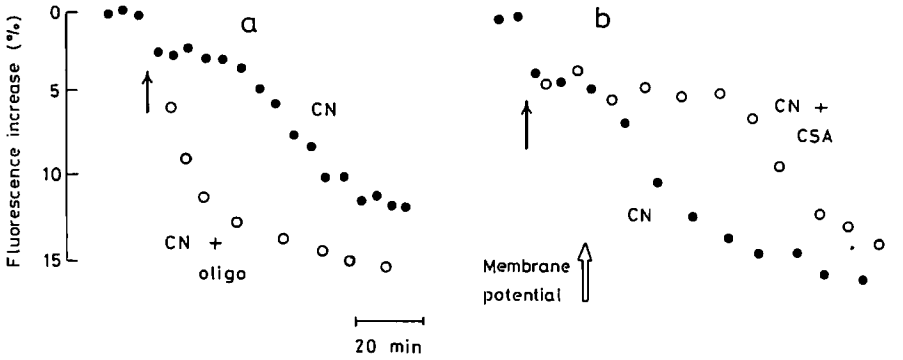

Fig. 1. The effect of cyclosporin A on the dissipation of the mitochondrial inner membrane potential in CN^--treated cardiomyocytes. Rat cardiomyocytes were prepared [11], loaded with 3–6 μM rhodamine 123, and suspended at a density of 10^5 cells/ml in medium (pH 7.4) containing 118 mM NaCl, 4.8 mM KCl, 25 mM Hepes, 1.2 mM KH_2PO_4, 1.2 mM Mg_2O_4 and 1 mM $CaCl_2$. When added, 300 nM cyclosporin A (CSA) was introduced at the beginning of the incubation. After incubation for 20 min at 35°C, further additions of 1 mM NaCN (CN) and 5 μg/ml oligomycin (oligo) were made as indicated by the arrow. The increase in rhodamine fluorescence, which corresponds to a decrease in inner membrane potential, was measured at 490 nm (excitation) and 530 nm (emmission).

the prevailing low ATP, high P_i and oxidative stress would trigger pore opening. Since pore opening prevents oxidative phosphorylation and allows high uncoupled F-ATPase activity [5], pore activation would effectively enter the cell into a vicious cycle of irreversible injury – lowered cytosolic phosphorylation potential and, as a consequence. further Ca^{2+} entry to the cytosol, further pore opening, further ATP dissipation.

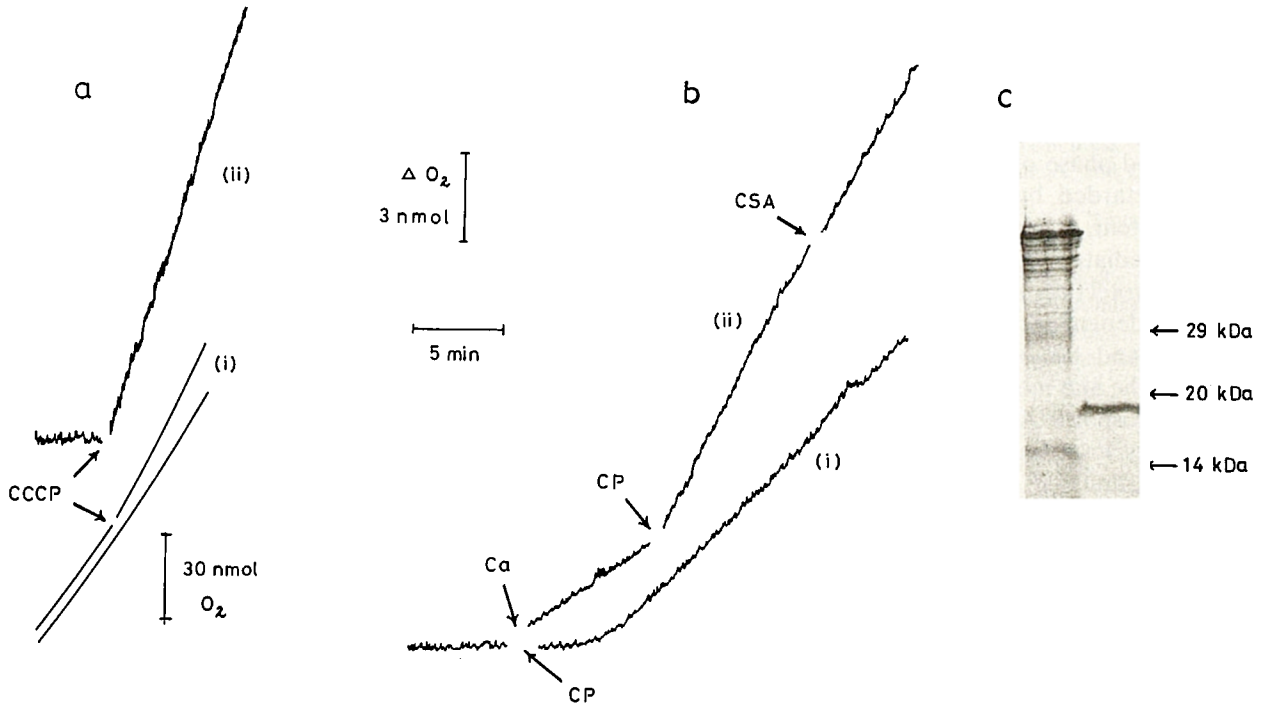

Fig. 2. Cyclophilin- and Ca^{2+}-induced stimulation of respiration in liver submitochondrial particles (SMP), Rat liver mitochondria (prepared as in Ref. 13) were incubated at 25°C at a concentration of 4 mg protein/ml in medium (pH 7.4) containing 250 mM sucrose, 10 mM Tris-HCl and 50 μM EGTA. Incubations were performed in parallel in two O_2 electrode chambers, i.e., test and control, further additions (below) being made to the test chamber alone. a(i) Conventional O_2 electrode traces with the addition of 1 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to the test (upper trace) incubation. a(ii) The amplified difference between the traces of (i). (b) Difference traces obtained by addition of 100 pmol cyclophilin (CP) alone in trace (i), and by addition of 100 μM Ca^{2+} (Ca), 100 pmol CP and 1 μM cyclosporin A (CSA) in trace (ii). Mitochondrial cyclophilin was purified from the water-soluble fraction of rat liver mitochondria by FPLC (Pharmacia) using cation-exchange (mono-S) and gel-filtration (Superdex-75) columns. (c) SDS-PAGE of the soluble fraction (left) and purified cyclophilin (right). The molecular weight positions were determined with carbonic anhydrase, trypsin inbibitor and α-lactalbumin.

and so forth [4,5,7]. Consistent with this, the rise in resting cytosolic free Ca^{2+} in ischaemia (> 1 μM) occurs after ATP depletion to levels (< 500 nM) that would be insufficient to prevent pore activation [1]. This article focuses on the use of the immunosuppressant cyclosporin A in investigating this topic and how it has exposed a possible wider involvement of the protein that binds cyclosporin A in regulating energy transduction.

The discovery that cyclosporin A is a potent inhibitor of pore opening in intact mitochondria [7], recently confirmed by patch clamp of the inner membrane of giant mitochondria [10], has allowed testing of the hypothesis. In general agreement, low concentrations of cyclosporin A substantially inhibited necrosis of cardiomyocytes subjected to anoxia/reoxygenation according to a number of parameters [11]. Rather unexpectedly, it appeared that cyclosporin A protection was exerted, at least in part, during the anoxic phase. This has now been confirmed by following changes in the mitochondrial inner membrane potential of cardiomyocytes during chemical anoxia (CN^-), as reported in Fig. 1. The inner membrane potential was monitored using Rhodamine 123 fluorescence, which increases on membrane depolarization [12]. Exposure to CN^- alone yielded a biphasic depolarization (Fig. 1a) which may be interpreted to reflect initial inhibition of respiration followed by ATP-dependent generation of the potential, decaying as ATP was dissipated. in agreement with this, coaddition of CN^- and oligomycin abolished the second phase. Fig 2b shows that the delayed phase of CN^--induced depolarization is markedly retarded by inclusion of cyclosporin A, which is consistent, at least, with cyclosporin A-inhibition of pore-mediated ATP dissipation. As in previous studies [11], protection by cyclosporin A was acutely concentration dependent, being maximal with 300 nM cyclosporin A and 10^5 cells/ml. Cyclosporin A is extremely lipophilic and most was bound to the cells. The use of [^{3}H]cyclosporin A to estimate the optimal free extracellular [cyclosporin A] yielded values of 10–20 nM, which are similar to the concentration required to inhibit cyclophilin (below).

The mechanisms of pore activation and its blockade by cyclosporin A have not yet been resolved. Mitochondria contain two (at least) high-affinity cyclosporin A binding components (K_d values approx. 5 nM and 50 nM [13]). Inhibition/binding titrations suggested that binding of cyclosporin A to the highest affinity component was responsible for pore blockade [13]. This component was tentatively identified as cyclophilin [13,14], a ubiquitous water-soluble protein located in the cytosol and mitochondrial matrix. Thus, cyclosporin A may block the pore, not by interacting with the pore itself, but rather with the matrix protein cyclophilin which is somehow involved in the regulation of pore state. The cellular function of cyclophilin is unknown, but in vitro it exhibits peptidyl prolyl *cis-trans* isomerase activity towards test peptides, and accelerates protein folding, presumably by catalyzing isomerization of surface exposed prolylpeptide bonds important for the conformational change [15]. Peptidyl-prolyl *cis-trans* isomerase activity is blocked by cyclosporin A.

The involvement of cyclophilin has been examined further from the effects of cyclophilin on the permeability properties of submitochondrial particles, as reported in Fig 2. Cyclophilin was purified (approx. 1000-fold) from the soluble fraction of rat liver mitochondria to a fraction yielding a single 18 kDa band on SDS-PAGE (Fig. 2c). Initial experiments examined the capacity of cyclophilin to stimulate respiration. In order to facilitate these measurements, two O_2 electrodes were set up in parallel (test and control; Fig 2a, traces (i)) and the difference between the O_2 consumption of each was amplified as shown in Fig 2a, traces (ii), where CCCP was used as uncoupling agent to demonstrate the procedure. Difference traces of the type in Fig. 2a(ii) were then obtained with cyclophilin, as reported in Fig. 2b. It is evident that cyclophilin stimulated respiration in the absence of Ca^{2+} after a lag phase of 2–3 min (Fig. 2b(i)). Ca^{2+} alone induced some stimulation (Fig. 2b(ii)), but subsequent introduction of cyclophilin stimulated respiration further, and in this case, the response was immediate. However, these effects of cyclophilin cannot be attributed to uncoupling, since essentially the same difference traces were obtained with cyclophilin and Ca^{2+} when 1 μM CCCP was added to both control and test chambers (not shown). In addition, measurements of the membrane potential in submitochondrial particles (using assay media as in Fig. 2 with anilinenaphthalene sulphonate as potential indicator) revealed no detectable effect of cyclophilin on this parameter in the presence or absence of Ca^{2+}. Since any pore activation would be expected to induce uncoupling, it appears that pore activation was not obtained. This conclusion was examined further. In whole mitochondria, Ca^{2+}-dependent activation produces a very large pore, 20 Å internal diameter, that allows permeation of sucrose and most metabolites [6] and activation to the 20 Å state is prevented by cyclosporin A [13]. Rapid pulsed flow measurements have indicated that, once activated, the pore opens and closes continuously [6], a behaviour corroborated by patch clamp experiments demonstrating rapid pore flicker between closed and open states [10,16]. It is possible that occasional flicker may allow maintenance of the inner membrane potential. Yet, in parallel experiments (to those of Fig. 2), in which submitochondrial particles were prepared with entrapped 10 mM [^{14}C]sucrose (as in Ref. 13), the addition of cyclophilin and Ca^{2+} led to no detectable loss

of the entrapped [^{14}C]sucrose (i.e., $< 8\%$ release after 20 min incubation as in Fig. 2). This confirms that cyclophilin and Ca^{2+} did not induce pore opening in submitochondrial particles. Possibly, the process may require additional proteins that are lost on particle preparation.

From the present data it is clear that cyclophilin interacts with the respiratory chain, and that this interaction is promoted by Ca^{2+}: Evidently, Ca^{2+} is not obligatory, but Ca^{2+} nevertheless increases the rate by which cyclophilin brings about respiratory chain stimulation (Fig. 2b). It is also noteworthy that respiratory stimulation by cyclophilin was not reversed in the presence of sufficient cyclosporin A to inhibit peptidylprolyl isomerase activity completely (although boiled cyclophilin was shown to be ineffective). It seems, therefore, that the activity of a component of the respiratory chain may be modified by association with cyclophilin or the cyclophilin-cyclosporin A complex. Whether or not these observations reflect a physiological function of mitochondrial cyclophilin remains speculative, but numerous examples are known in which hormonal pretreatment of a tissue (e.g., the action of glucagon and α_1-adrenergic agonists on liver) leads to enhanced respiratory chain activity in subsequently isolated mitochondria (e.g., Ref. 17), and a possible involvement of cyclophilin in the intramitochondrial signal transduction pathway might be considered. Activation of the respiratory chain in whole mitochondria has been correlated with increases in matrix volume [17], but it is not obvious how the effects of cyclophilin on respiration in submitochondrial particles might be mediated via changes in particle volume and, in this case at least, a more direct mechanism involving the binding of cyclophilin to a particular component of the respiratory chain is indicated. It is intriguing that the same hormonal pretreatments also increase the tolerance of subsequently isolated mitochondria to high Ca^{2+} load [17], i.e., they depress pathological generation of the pore, which suggests the participation of common elements in respiratory stimulation and pore generation. From this and previous studies [13,14], it is concluded that one potential Component common to both is cyclophilin.

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References

1 Murphy, E., Perlman, M., London, R.E. and Steenbergen, C. (1991) Circ. Res. 68, 1250–1258.
2 Chien, K.R. and Engler, R. (1990) in Calcium and the Heart (Langer, G.A., ed.), pp. 333–354. Raven Press, New York.
3 Allshire, A., Piper, M.H., Cuthbertson, K.S.P. and Cobbold, P. (1987) Biochem. J. 244, 381–385.
4 Crompton M. (1990) in Calcium and the Heart (Langer, G.A., ed.), pp. 167–198,Raven Press, New York.
5 Crompton, M. and Costi, A. (1988) Eur. J. Biochem. 178, 489–501.
6 Crompton, M. and Costi, A. (1990) Biochem. J. 266, 33–39.
7 Crompton, M., Ellinger, H. and Costi, A. (1988) Biochem. J. 255, 357–360.
8 Hunter, D.R. and Haworth, R.W. (1979) Arch. Biochem. Biophys. 195, 468–477.
9 McGuinness, O. and Crompton, M. (1990) Biochem. Soc. Tran. 18, 883–4.
10 Szabo, I. and Zovatti, M. (1991) J. Biol. Chem. 266, 3376–3379.
11 Nazareth, W., Yafe, N. and Crompton, M. (1992) J. Mol. Cell Cardiol., in press.
12 Duchen, M.R. (1992) Biochem. J., in press.
13 McGuinness, O., Yafei, N., Costi, A. and Crompton, M. (1990) Eur. J. Biochem. 194, 671–679.
14 Halestrap, A.P. and Davidson, A.M. (1990) Biochem. J. 268, 153–160.
15 Fischer, G. and Schmid, F.X. (1990) Biochemistry 29, 2205–2212.
16 Moran, O., Sandri, G.., Panfili, E., Stuhmer, W., and Sorgato, M.C. (1990) J. Biol. Chem. 265, 908–913.
17 Halestrap, A.P., Quinlan, P.T., Armston, A.E. and Whipps, D.E. (1985) Biochem. Soc. Trans. 13, 659–665.