Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion

Elinor J. GRIFFITHS* and Andrew P. HALESTRAP†
Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

1. The yield of mitochondria isolated from perfused hearts subjected to 30 min ischaemia followed by 15 min reperfusion was significantly less than that for control hearts, and this was associated with a decrease in the rates of ADP-stimulated respiration. 2. The presence of 0.2 μM cyclosporin A (CsA) in the perfusion medium during ischaemia and reperfusion caused mitochondrial recovery to return to control values, but did not reverse the inhibition of respiration. 3. A technique has been devised to investigate whether the Ca^{2+}-induced non-specific pore of the mitochondrial inner membrane opens during ischaemia and/or reperfusion of the isolated rat heart. The protocol involved loading the heart with 2-deoxy[^{3}H]glucose ([^{3}H]DOG), which will only enter mitochondria when the pore opens. Subsequent isolation of mitochondria demonstrated that [^{3}H]DOG did not enter mitochondria during global isothermic ischaemia, but did enter during the reperfusion period. 4. The amount of [^{3}H]DOG that entered mitochondria increased with the time of ischaemia, and reached a maximal value after 30–40 min of ischaemia. 5. CsA at 0.2 μM did not prevent [^{3}H]DOG becoming associated with the mitochondria, but rather increased it; this was despite CsA having a protective effect on heart function similar to that shown previously [Griffiths and Halestrap (1993) J. Mol. Cell. Cardiol. **25**, 1461–1469]. 6. The non-immunosuppressive CsA analogue [MeAla^6]cyclosporin was shown to have a similar K_i to CsA on purified mitochondrial peptidyl-prolyl *cis–trans*-isomerase and mitochondrial pore opening, and also to have a similar protective effect against reperfusion injury. 7. Using isolated heart mitochondria, it was demonstrated that pore opening could become CsA-insensitive under conditions of adenine nucleotide depletion and high matrix [Ca^{2+}] such as may occur during the initial phase of reperfusion. The apparent increase in mitochondrial [^{3}H]DOG in the CsA-perfused hearts is explained by the ability of the drug to stabilize pore closure and so decrease the loss of [^{3}H]DOG from the mitochondria during their preparation.

INTRODUCTION

It is now well established that a non-specific pore can open in the inner membrane of mammalian mitochondria when they become overloaded with Ca^{2+}. This process is enhanced under conditions of oxidative stress, phosphate and adenine nucleotide depletion, and is inhibited by sub-micromolar concentrations of cyclosporin A (CsA) [1–5]. When tissues such as the heart become ischaemic, the resulting hypoxia prevents oxidative phosphorylation, and consequently tissue ATP concentrations rapidly decrease and AMP and phosphate concentrations rise. Further degradation of the AMP to adenosine, inosine and xanthine leads to depletion of tissue adenine nucleotides [4,6]. The much decreased tissue ATP concentration also inhibits the Na^+/K^+ ATPase and leads to a progressive increase in intracellular [Na^+], which in turn causes intracellular [Ca^{2+}] to rise [2,4,6]. A rise in mitochondrial [Ca^{2+}] may also occur under such conditions, but is only modest, since the mitochondria are largely de-energized in the absence of oxygen [7,8]. Although several conditions now prevail that might encourage pore opening (high [P_i] and [Ca^{2+}] and depleted adenine nucleotides), other factors probably constrain the process. Thus the de-energized mitochondria are unable to accumulate Ca^{2+} to high concentrations, while the low intracellular pH, caused by the large increase in glycolytic lactic acid production, causes powerful inhibition of pore opening [9–11]. Indeed, low pH is known to protect myocytes from the effects of reoxygenation following hypoxia [12,13]. However, when the tissue is reperfused these constraints are removed. Lactic acid rapidly leaves the cell by means of the very active lactate/H^+ transporter [14], whereas the presence of oxygen allows mitochondria to become energized and accumulate Ca^{2+} in preference to ATP synthesis [1,2,7,8]. In addition, the sudden exposure of the mitochondria to oxygen leads to the production of oxygen free radicals, which induce oxidative stress. Thus conditions are now optimal for mitochondrial pore opening.

Hearts reperfused after a period of ischaemia will only recover their function if the period of ischaemia is short (normally approx. 30 min for the rat heart). After this time the cells become irreversibly damaged, and electron-microscopic studies show this to be characterized by the appearance of swollen mitochondria and amorphous densities in the matrix [2,4,6,15]. Isolation of mitochondria after ischaemia and reperfusion shows them to be functionally impaired with, among other things, decreased respiratory-chain and ATPase activity, decreased adenine nucleotide content and increased Ca^{2+} content [4,16,17]. These data are all consistent with the opening of the mitochondrial non-specific pore playing an important role in the mitochondrial damage that occurs in reperfusion injury. If this were the case, it would be predicted that CsA should provide hearts with some protection from the damage that occurs under such conditions, and we have demonstrated that this is the case [18]. Isolated perfused rat hearts pretreated with 0.2 μM CsA before 30 min of global ischaemia and subsequent reperfusion recovered both their functional properties [beat, end-diastolic pressure (EDP) and left-ventricular developed pressure (LVDP)] and their [ATP]/[ADP][AMP] ratio significantly better than did control hearts

Abbreviations used: EDP, end diastolic pressure; LVDP, left-ventricular developed pressure; CsA, cyclosporin A; CyP, cyclophilin; PPIase, peptidyl-prolyl *cis–trans*-isomerase; TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine; DOG, 2-deoxyglucose.

* Present address: Division of Cardiology, John Hopkins Hospital, 600N Wolfe Street, Baltimore, MD 21205, U.S.A.
† To whom correspondence should be addressed.

[18]. Similarly Nazareth et al. [19] have shown that isolated heart cells made anoxic and then reoxygenated were also protected from damage by 0.2 μM CsA.

These data provide indirect evidence that pore opening occurs during reperfusion after a period of ischaemia, but a direct demonstration is lacking. For this purpose it is necessary to introduce into the cytosol of the myocytes of the perfused heart an intracellular marker molecule whose entry into the mitochondria can only occur when the pore is opened. In this paper we describe how this can be achieved, using 2-deoxy[^{3}H]glucose ([^{3}H]DOG). This enters the cell on the glucose transporter and is phosphorylated to 2-deoxyglucose 6-phosphate, which cannot be metabolized further [20,21]. It therefore accumulates within the cytosol, but cannot cross the mitochondrial inner membrane [22]. However, its molecular size is such that upon pore opening it should enter mitochondria. Preparation of mitochondria from the tissue in the presence of EGTA to chelate Ca^{2+} will lead to rapid closure of the pore [23] and entrapment of the 2-deoxyglucose 6-phosphate within the matrix.

EXPERIMENTAL

Materials

2-Deoxy-D-[1-^{3}H]glucose (30 mCi/mmol) was obtained from Amersham International (Aylesbury, Bucks., U.K.) and DOG was from Sigma Chemical Co., Poole, Dorset, U.K. [MeAla^6]Cyclosporin was generously given by Merck, Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ, U.S.A. The source of all other chemicals and biochemicals was as described previously [18].

Protocol for heart perfusion

This was performed essentially as described previously [18]. Hearts were removed from female Wistar rats (200–250 g) and immediately perfused (at a flow rate of 10 ml/min) with Krebs/Henseleit bicarbonate-buffered saline, containing 11 mM glucose, 118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 1.2 mM $CaCl_2$, by a modified Langendorff procedure [24]. Aortic pressure was monitored via a pressure transducer, and ventricular pressure by a separate transducer connected to a balloon catheter inserted into the left ventricle via the left atrium [25]. The balloon was inflated (with double-distilled water) to give an initial end diastolic pressure of 2.5–5 mmHg. This allowed continuous measurement of end diastolic and systolic pressure changes during ischaemia and reperfusion. Global isothermic ischaemia was induced by switching off the pump and clamping the aorta, temperature being maintained by immersing the heart in perfusion medium at 37 °C. Reperfusion was initiated after the required ischaemic period and was usually continued for 15 min before homogenization of the heart for mitochondrial preparation. In control perfusions the ischaemic period was replaced by an equal period of flow-through perfusion. When present, CsA (dissolved in DMSO) was usually added to the perfusate 3 min before the induction of ischaemia (final concn. of DMSO $< 0.02\%$). Perfusion with DMSO alone had no effect on any of the parameters measured.

Loading of cells with [^{3}H]DOG

After an initial 15 min flow-through perfusion as described above, hearts were perfused in recirculating mode with 40 ml of standard medium supplemented with 0.5 mM [^{3}H]DOG (0.5 mCi/ml). Perfusion was then returned to flow-through mode (no DOG) in the presence or absence of 0.2 μM CsA and continued for 3 min before induction of ischaemia as outlined above.

Homogenization of hearts and preparation of mitochondria

At the end of the perfusion, the ventricles were rapidly removed from the heart, weighed (usually about 0.5 g) and homogenized in 2.5 ml of ice-cold sucrose buffer (300 mM sucrose, 10 mM Tris/HCl, 2 mM EGTA, 5 mg/ml BSA; pH 7.4) with a Polytron homogenizer (setting 3 for 5 s). A sample (0.5 ml) of the total homogenate was retained and used for measurement of citrate synthase activity and, after protein precipitation by addition of an equal volume of 5% (w/v) $HClO_4$, total heart ^{3}H d.p.m. The remainder of the homogenate was used to prepare mitochondria by using a 2 min centrifugation at 2000 *g* in a bench-top centrifuge to remove cell debris, followed by centrifugation of the supernatant at 10000 *g* for 5 min to sediment the mitochondria. The mitochondrial pellet was then washed with 2 × 30 ml of sucrose isolation buffer lacking BSA and resuspended in a final volume of 0.5 ml of isolation buffer. A 100 μl portion of mitochondrial suspension was retained for assay of citrate synthase and protein, and to the remainder was added 0.4 ml of 5% $HClO_4$ to release the entrapped ^{3}H d.p.m. Protein was precipitated by centrifugation at 8000 *g* for 1 min, and the resulting supernatant was counted for radioactivity in 10 ml of scintillant (Packard Emulsifier-Safe). In those experiments in which mitochondrial respiratory-chain activity was to be measured, the procedure for mitochondrial preparation was the same, except that all of the homogenate was used for mitochondrial preparation and the mitochondrial pellet was purified by Percoll-gradient centrifugation, followed by a subsequent wash with 30 ml of sucrose isolation buffer lacking BSA [26]. When mitochondria were required for measurement of pore opening *in vitro*, they were prepared from hearts removed directly from rats as described previously [10]. In some experiments mitochondria were depleted of their endogenous adenine nucleotides by incubation in sucrose isolation medium supplemented with 2 mM pyrophosphate for 15 min at 25 °C. We have demonstrated that this procedure removes more than 90% of the endogenous ATP and ADP (results not shown).

Other procedures

Pore opening in isolated de-energized heart mitochondria was studied at 25 °C and pH 7.2 by measuring the decrease in light-scattering on addition of Ca^{2+} as described previously [10,27]. The medium contained 150 mM KSCN, 20 mM Mops, 10 mM Tris, 2 mM nitrilotriacetate (to buffer Ca^{2+}), 2 μM A23187 (Ca^{2+} ionophore) and 0.2 μg/ml of both antimycin and rotenone. Swelling was initiated by addition of $CaCl_2$ to give the required [Ca^{2+}] calculated as in [28], and monitored continuously by measurement of the A_{520}. The citrate synthase content of the whole homogenate and of a sample of the mitochondrial preparation was assayed as described in [29]. Protein was measured by the method of Bradford [30], with BSA as standard. Measurement of the rate of mitochondrial respiration with a variety of substrates was performed at 30 °C with a Clark-type oxygen electrode as described previously [26]. The buffer contained 125 mM KCl, 10 mM Mops, 5 mM Tris, 2.5 mM potassium phosphate, 2.5 mM $MgCl_2$, 0.5 mM EGTA and 5 mg of defatted BSA/ml and was adjusted to pH 7.2.

Expression of results

The uptake of [^{3}H]DOG into mitochondria was calculated from the d.p.m. measured in the mitochondrial extract. In order to

correct for variation between experiments in the loading of the hearts with [^{3}H]DOG, the total homogenate ^{3}H d.p.m. per g wet wt. of tissue was also determined. In addition to correct for variations in the recovery of mitochondria between experiments, citrate synthase activity was measured in the mitochondrial fraction, and the mitochondrial ^{3}H d.p.m. was expressed per unit of this enzyme. These two corrections allowed the mitochondrial [^{3}H]DOG uptake in d.p.m./unit of citrate synthase to be expressed as a ratio with respect to the total tissue ^{3}H d.p.m. This ratio should be independent both of [^{3}H]DOG loading of the cells and of mitochondrial recovery. The theoretical maximal value of the mitochondrial [^{3}H]DOG expressed in this way occurs when all mitochondria become freely permeable to DOG, and can be calculated as follows. It is assumed that under such conditions the [^{3}H]DOG distributes evenly between the mitochondria and the cytosol. The total intracellular volume per g wet wt. is about 360 μl [31], whereas the matrix volume of heart mitochondria under physiological conditions is about 0.9 μl/mg of protein [26,29]. The citrate synthase activity of heart mitochondria was found to be 2.27 ± 0.11 units/mg of protein (mean $\pm$ S.E.M. of 15 separate mitochondrial preparations) and was unchanged after ischaemia or reperfusion. The citrate synthase activity of the total heart homogenate was found to be 85 ± 7.1 units/g wet wt. ($n = 8$) and was also unaffected by the perfusion conditions. Thus the volume of the mitochondrial matrix as a fraction of the total cell volume can be calculated as $(85 \times 0.9)/(2.27 \times 360) = 0.094$. This means that when all mitochondria become permeable to DOG the ratio of the mitochondrial d.p.m./unit of citrate synthase to the total tissue d.p.m./g wet wt. must be $0.094/85 = 1.1 \times 10^{-3}$. In Table 2 values of the ratio are given after multiplication by a factor of 10^5.

Statistics

Statistical analyses of the differences between control and experimental values were performed by two-tailed Student's t test.

RESULTS AND DISCUSSION

Effects of ischaemia/reperfusion on the yield of heart mitochondria and their respiration

The yield of mitochondria isolated from perfused hearts was determined by measuring the units of citrate synthase activity isolated in the mitochondrial pellet per g wet wt. of heart. This ensured that only mitochondria with an intact inner membrane were included. The yield from hearts subjected to 30 min of global normothermic ischaemia, followed by 15 min reperfusion, was only 67% of that from hearts perfused normally for 45 min (Table 1). However, when hearts were treated with 0.2 μM CsA for 3 min before and during ischaemia, the yield was 94% that from control hearts. This effect correlated with protection of the heart by CsA, as judged by both a decrease in EDP (a measure of heart contracture [32]) and an increase in the LVDP (a measure of functional competence). We have demonstrated such protective effects of CsA previously and taken them to reflect a decrease in mitochondrial pore opening in the presence of CsA [18]. Prolonged pore opening is known to cause massive swelling of mitochondria, and even loss of matrix proteins [1,4,33], and thus the lower yields of mitochondria after reperfusion and the beneficial effects of CsA are not unexpected.

Another well-documented effect of reperfusion injury on mitochondrial function is an inhibition of the respiratory chain and oxidative phosphorylation [4,6,16,17]. This is illustrated by the data of Table 1, where the rates of ADP-stimulated oxidation of both 2-oxoglutarate + malate and ascorbate + TMPD are presented. Reperfusion led to a substantial inhibition of both processes, with the rate of 2-oxoglutarate oxidation being inhibited more than that of ascorbate + TMPD, as indicated by the change in ratio of the rates of oxidation. This is not unexpected, since it is well documented that complex I of the respiratory chain is most sensitive to reperfusion injury [17,34,35].

In three of the experiments whose data are presented in Table 1, we also determined the rate of ADP-stimulated succinate oxidation, which decreased from 193 ± 32 nmol/min per mg of protein in mitochondria from normally perfused hearts to 71 ± 7.2 and 67 ± 9.0 nmol/min per mg of protein after reperfusion in the absence and presence of 0.2 μM CsA respectively. The ratio of the rate of succinate oxidation to that of ascorbate + TMPD remained unchanged at 0.54 for all conditions, which is consistent with the lack of a major effect of reperfusion/ischaemia on complex II observed by others [34,35]. From the data presented it appears that CsA gives little protection from the effects of reperfusion on respiratory-chain activity. This might be predicted, since the effects on the respiratory chain are thought to be mediated by oxygen free radicals [17,34,35], whereas the effects of CsA to inhibit pore opening are thought to be downstream of the production of oxygen free radicals. The decreased respiratory-chain activity that persists in CsA-protected hearts that recover

Table 1 Effects of ischaemia and reperfusion of the heart on the yield and respiration of subsequently isolated mitochondria

The protocols for heart perfusion, preparation of mitochondria and measurement of respiration are described in the Experimental section. The period of ischaemia was 30 min, followed by 15 min of reperfusion; control hearts (no ischaemia) were perfused for a total of 45 min before preparation of mitochondria. Rates of respiration were measured in the presence of 1 mM ADP and either 5 mM 2-oxoglutarate + 1 mM L-malate or 10 mM ascorbate + 0.3 mM *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD). Data are presented as means $\pm$ S.E.M. of 5 separate mitochondrial preparations for each perfusion condition. The values for the EDP and LVDP before induction of ischaemia were 2.8 ± 0.9 and 59.2 ± 10.6 mmHg respectively in the absence of CsA, and 3.9 ± 1.6 and 47.1 ± 10.1 mmHg in the presence of 0.2 μM CsA. Statistically significant differences in parameter values were calculated by Student's t test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ between reperfused and control heart; †$P < 0.05$, ††$P < 0.01$ between reperfused with or without CsA).

Heart	Rate of respiration (nmol of O/min per mg of protein)		Ratio oxoglutarate/ ascorbate	Mitochondrial yield (units of citrate synthase/g wet wt.)	EDP (mmHg)	LVDP (mmHg)
	Oxoglutarate + malate	Ascorbate + TMPD				
Control	185 ± 38	369 ± 78	0.51 ± 0.02	22.2 ± 2.5	2.8 ± 0.6	52.0 ± 5.7
Ischaemic reperfused	52 ± 10**	146 ± 27*	0.35 ± 0.03**	14.8 ± 0.8*	57.6 ± 8.3***	18.8 ± 7.9**
Ischaemic reperfused + 0.2 μM CsA	71 ± 13*	157 ± 28*	0.45 ± 0.03†	20.8 ± 2.6	14.0 ± 6.0††	46.3 ± 11.8†

Table 2 Effects of ischaemia and reperfusion of the heart on DOG uptake into mitochondria *in situ*

The protocol for heart perfusion and measurement of DOG uptake into the mitochondria is described in the Experimental section, where the rationale for the units of DOG uptake used is explained. The maximal value for this parameter when all mitochondria are permeable to DOG is about 110. Data are presented as means ± S.E.M. of the number of perfusions shown in parentheses. Control hearts (no ischaemia) were perfused after DOG loading for 45 min before preparation of mitochondria. Pressure measurements and DOG uptake were determined on the same hearts, but where discrepancies for *n* values are present, this is explained by a failure of the pressure measurement, which did not compromise the heart. Pre-ischaemic EDP (± S.E.M.) for reperfused hearts with and without 0.2 μM CsA was 3.3 ± 0.5 (18) and 3.8 ± 0.9 (10) mmHg respectively, and the corresponding LVDP values were 37.0 ± 3.1 and 42.8 ± 3.8 mmHg. Statistically significant differences were calculated by Student's *t* test between reperfused and control hearts (*$P < 0.05$, **$P < 0.02$, ***$P < 0.01$) and between hearts reperfused with or without CsA (†$P < 0.05$).

Time of ischaemia (min)	Time of reperfusion (min)	10^5 × Ratio of mitochondrial DOG d.p.m. per unit of citrate synthase to total heart DOG d.p.m./g wet wt.		EDP (mmHg)		LVDP (mmHg)	
		Without CsA	0.2 μM CsA	Without CsA	0.2 μM CsA	Without CsA	0.2 μM CsA
Control (no ischaemia)		31.0 ± 2.6 (5)	25.0 ± 4.0 (3)	6.5 ± 2.8 (5)	5.0 ± 2.5 (3)	54.4 ± 8.0 (5)	40 ± 2.9 (3)
30	0	31.5 ± 9.9 (5)	32.3 ± 12.0 (3)	–	–	–	–
10	15	42.8 ± 6.9 (5)	70.3 ± 10.5**† (3)	9.6 ± 4.2 (4)	7.8 ± 5.0 (3)	35.0 ± 9.2 (4)	46.7 ± 2.2 (3)
30	15	79.0 ± 16.6** (5)	91.8 ± 14.6** (4)	31.3 ± 3.3*** (4)	14.5 ± 7.9 (4)	13.8 ± 6.4*** (4)	24.8 ± 8.8 (4)
40	15	79.5 ± 14.8*** (4)	103.0 ± 5.0*** (3)	39.2 ± 5.8*** (3)	19.2 ± 3.6*† (3)	14.2 ± 3.3** (3)	25.8 ± 6.0 (3)

function may well be a major cause of their impaired performance or 'stunning' [6,17].

Demonstration of mitochondrial pore opening on reperfusion of hearts after ischaemia

Conditions were optimized for the uptake of [^{3}H]DOG into hearts. It was found that recirculating perfusion for 30 min in the presence of 11 mM glucose and 0.5 mM DOG gave no impairment of heart function, but was sufficient to allow accumulation of about 100000 d.p.m./g wet wt. Mitochondria prepared from such hearts contained about 50–300 d.p.m./mg of protein, depending on the extent of pore opening. In Table 2 we present data on the mitochondrial accumulation of [^{3}H]DOG in hearts subjected to various perfusion protocols. Some DOG was associated with the mitochondrial fraction even when prepared from control hearts after 45 min of normal perfusion, and amounted to about 25 % of that expected for equilibration of the matrix and cytosolic compartments. This could represent DOG entry into the mitochondria either *in situ* or during mitochondrial preparation. The former possibility is plausible, since it has been shown that sucrose introduced into hepatocytes by electropermeation enters the mitochondria over a period of several hours [36]. It is not known whether pore opening is responsible for this process, but if it were, it might be expected to be inhibited by CsA. We have investigated whether the presence of 1 μM CsA during the 30 min recirculating perfusion of hearts with [^{3}H]DOG inhibited mitochondrial DOG uptake. No effect of CsA was observed, the DOG uptake ratio (× 10^5) being 31.0 ± 2.6 ($n = 5$) in the absence of CsA and 25.0 ± 4.0 ($n = 3$) in its presence. This suggests that pore opening is unlikely to be responsible for the basal DOG uptake.

We have shown previously that the presence of [^{14}C]mannitol in the isolation medium during preparation of liver mitochondria leads to some ^{14}C d.p.m. becoming permanently associated with the mitochondrial fraction [37]. The extent of uptake is equivalent to about 10–15 % of that expected for complete equilibration. Although pore opening during homogenization and centrifugation is a possibility, the presence of EGTA in the homogenization medium makes this unlikely. The permeability of the mitochondrial inner membrane to sugars of increasing size has been studied extensively, and it is well established that glycerol enters mitochondria rapidly, and erythritol enters relatively slowly, whereas other sugars are regarded as impermeant [22]. However, this is not strictly true. Although experiments with isolated mitochondria cannot easily be continued for long periods of time at 37 °C without significant increases in the permeability of the inner membrane occurring, a slow permeation of mannitol relative to sucrose does occur [37]. This probably reflects very slow diffusion of the sugar through the phospholipid bilayer and represents another possible pathway for DOG entry into the mitochondria of normally perfused hearts.

The data in Table 2 show that 30 min ischaemia alone was without effect on the mitochondrial DOG uptake, whereas subsequent reperfusion for 15 min caused the uptake to more than double. This was accompanied by a 5-fold rise in EDP and a decrease in LVDP to 25 % of control values. If the period of ischaemia was increased to 40 min, no additional DOG uptake was observed, and the EDP and LVDP were not significantly different from the 30 min values. However, if the ischaemic period was decreased to 10 min, the increase in DOG uptake was only about 40 % above the control value. The accompanying increase in EDP and decrease in LVDP were both 40–50 % and not statistically significant. These data are entirely consistent with the mitochondrial pore remaining closed during the ischaemic period and opening upon reperfusion, the extent of opening being dependent on the time of ischaemia. Entry of DOG into the mitochondria as a result of a non-specific increase in membrane permeability, as might be caused by phospholipase A_2 activation, is unlikely, since the DOG remains trapped inside the mitochondria during their subsequent isolation and washing. These procedures were performed in the presence of EGTA, which should have fully blocked the pore [1,2,4] and so prevented loss of DOG that had entered through this route.

Effects of CsA on mitochondrial uptake of DOG during reperfusion

In an attempt to prove that the uptake of DOG during reperfusion was the result of pore opening, we perfused hearts with 0.2 μM CsA for 3 min before the start of ischaemia and maintained the CsA in the medium during the subsequent reperfusion. Measurement of EDP and LVDP confirmed the protective effects of CsA on heart function seen in previous experiments (Table 1, and [18]), yet the mitochondria from CsA-treated hearts contained slightly more DOG, rather than less, as had been expected. Thus either the entry of DOG is not through the CsA-sensitive non-specific

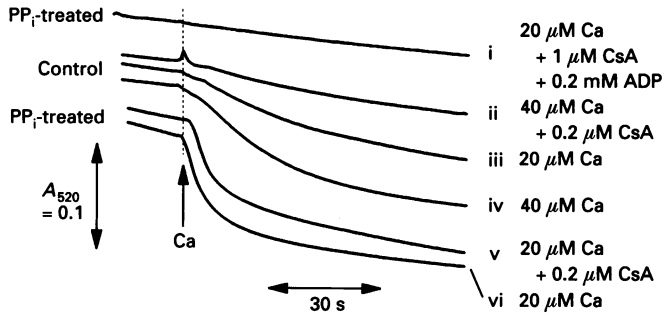

Figure 1 CsA-insensitive pore opening in adenine-nucleotide-depleted heart mitochondria

Control and PP_i-treated heart mitochondria were prepared as described in the Experimental section and incubated at 1.4 mg of protein/ml in KSCN medium. Pore opening was initiated by addition of $CaCl_2$ where indicated to give the free $[Ca^{2+}]$ shown and was monitored by measuring the light scattering as described in the Experimental section. The experiment shown is one of two experiments giving very similar results. Where indicated, CsA was present from the start of the incubation.

pore, or CsA is not totally inhibiting DOG entry through the pore. If the latter were the case, and yet CsA prevented some loss of DOG through the pore during mitochondrial preparation, this might explain the increased DOG content of the mitochondria from CsA-treated hearts. This explanation is plausible, since CsA inhibition of the pore has been reported to be reversed at high $[Ca^{2+}]$, especially when other factors such as decreased adenine nucleotides and oxidative stress co-operate to enhance the sensitivity of pore opening to $[Ca^{2+}]$ [4,28,38,39]. At the end of the ischaemic period, mitochondrial adenine nucleotides are already depleted and phosphate concentrations increased. Upon reperfusion the availability of oxygen leads to a burst of oxidative stress and rapid mitochondrial energization, which drives the accumulation of Ca^{2+} from the cytosol into the matrix [7,8,40–42]. Under such conditions data obtained with liver mitochondria would suggest that pore opening might not be inhibited by CsA [28,38,39]. Our data of Figure 1 confirm that this may also be the case with heart mitochondria.

Insensitivity of heart mitochondrial pore opening to CsA after adenine nucleotide depletion

Mitochondrial pore opening was measured by monitoring the light-scattering by de-energized mitochondria in a KSCN medium as described previously [27]. The medium was supplemented with the ionophore A23187 to ensure rapid equilibration of Ca^{2+} across the mitochondrial inner membrane [10]. Under such conditions, heart mitochondria swell as a result of pore opening with a rate that increases as $[Ca^{2+}]$ increases (Figure 1). Swelling induced by 20 μM $[Ca^{2+}]$ (trace iii) was blocked totally by addition of 0.2 μM CsA (results not shown), whereas the faster swelling seen at 40 μM $[Ca^{2+}]$ (trace iv) was not (trace ii). The sensitivity of swelling to $[Ca^{2+}]$ was greatly increased by treatment of the mitochondria with 2 mM PP_i at 25 °C for 10 min (trace vi). After such treatment CsA was a very poor inhibitor of swelling (trace v), whereas 0.2 mM ADP was still able to block the process totally (trace i). Higher concentrations of CsA were no more effective than 0.2 μM CsA (results not shown), and this would be predicted, since the K_i of CsA for inhibition of pore opening is about 5 nM [27,43].

Effect of [MeAla^6]cyclosporin on mitochondrial pore opening and reperfusion injury

We have demonstrated that the relative potency of the CsA analogues cyclosporin G and cyclosporin H as inhibitors of mitochondrial pore opening *in vitro* and their protective effect against reperfusion injury of the isolated perfused heart both correlate with their K_i values for inhibition of mitochondrial peptidyl-prolyl *cis–trans*-isomerase (PPIase) activity [18,43]. However, these analogues are immunosuppressive because, like CsA, their complex with cyclophilin (CyP) binds to and inhibits calcineurin, a calmodulin-dependent Ca^{2+}-activated protein phosphatase [44]. In order to confirm that the protective effects of CsA and its analogues do not involve inhibition of calcineurin, we have studied the effects of the analogue [MeAla^6]cyclosporin, which inhibits the PPIase activity of CyP, but whose complex with CyP does not inhibit calcineurin [44]. Using the purified mitochondrial-matrix CyP [45] and the protocol described previously [27,43], we determined the K_i ($\pm$S.E.M.) for inhibition of PPIase activity to be 14.5 ± 5.5 nM. The K_i of [MeAla^6]-cyclosporin for inhibition of mitochondrial pore opening was also determined by monitoring Ca^{2+}-induced swelling as described previously [27,43]. The mean value ($\pm$S.E.M.) for three separate experiments was 33.4 ± 7 nM, similar to the value for PPIase inhibition. This is consistent with a role for this enzyme, but not for calcineurin, in pore opening. A similar conclusion has been reached by Schweizer et al. [46], who used a different set of CsA analogues. In the perfused heart, exposure to 0.2 μM [MeAla^6]cyclosporin during pre-perfusion, followed by 30 min ischaemia and then 15 min reperfusion, improved recovery of the heart. In the presence of [MeAla^6]cyclosporin the LVDP after reperfusion was 31.5 ± 9.8 mmHg (mean $\pm$ S.E.M. of 5 separate perfusions), compared with 24.8 ± 8.8 mmHg ($n = 4$) in the presence of 0.2 μM CsA and 13.8 ± 6.4 mmHg ($n = 4$) in the absence of drug. In the same experiments, the uptake of [^{3}H]DOG into the mitochondrial fraction, measured as a ratio of the total d.p.m. taken up (see the Experimental section and Table 2) was 79.0 ± 16.6, 91.8 ± 14.6 and 87.2 ± 10.3 respectively for the three conditions.

General discussion and conclusions

Reperfusion of the ischaemic heart produces mitochondrial Ca^{2+} overload and an intracellular environment ideally suited for the opening of the mitochondrial CsA-sensitive non-specific pore (see the Introduction). Indeed, recovery of hearts has been inversely correlated with mitochondrial Ca^{2+} accumulation [7] and with leakage of mitochondrial enzymes from the heart [47]. Furthermore, we have shown previously that CsA can protect the heart from irreversible damage during reperfusion [18] and confirm our observations here. Others have reported protective effects of CsA on the ischaemic/reperfused liver [48,49] and in cardiomyocytes that are reoxygenated after a prolonged period of anoxia [19], a process accompanied by mitochondrial depolarization indicative of pore opening [50]. In addition, hepatocytes subjected to oxidative stress or 'chemical anoxia' (respiratory-chain inhibition) may also be protected against loss of mitochondrial membrane potential and irreversible damage by CsA [51–54]. However, direct evidence for mitochondrial pore opening *in situ* under any of these conditions has been lacking, but is provided by the use of [^{3}H]DOG described in this paper. Our data confirm that pore opening occurs during reperfusion of the heart after ischaemia, but not during ischaemia itself. Furthermore, the extent of DOG uptake increases as the period of ischaemia preceding reperfusion increases, and approaches the theoretical maximum (the matrix of all mitochondria equilibrated

with the cytosol) after 30–40 min. This correlates with the greater damage to the heart that occurs as the ischaemic period is prolonged.

The inability of CsA to prevent the entry of DOG into the mitochondria during reperfusion might appear at first glance to argue against the involvement of the CsA-sensitive non-specific pore in this process. However, we have shown that this is not the case, since, when isolated mitochondria are incubated under the conditions prevailing at the point of reperfusion, the pore cannot be totally blocked by CsA (Figure 1). Thus, even in the presence of CsA, enough pores may be open for sufficient time to allow cytosolic DOG to equilibrate with the mitochondrial matrix during reperfusion, but they could then re-seal, entrapping the DOG within. Once resealed they could continue to support ATP production, enabling the heart to recover ionic homoeostasis rapidly, and, more slowly, to re-establish normal metabolic and contractile function as cellular damage is repaired. This would contrast with hearts perfused without CsA, in which pores would remain open as reperfusion continues, preventing the mitochondria from generating sufficient ATP to maintain ionic homoeostasis. This would lead to a spiral of further damage and ultimately irreversible death. The mitochondria of such irreversibly damaged hearts could be so disrupted that some non-specific leakage of DOG out of the mitochondria might occur. This would not be blocked by EGTA, and so would lead to the lower amounts of DOG observed in the mitochondrial fraction of control hearts compared with that from CsA-treated hearts. Indeed it has been demonstrated with isolated liver mitochondria that prolonged exposure to Ca^{2+} leads to an increase in permeability that can only be totally blocked by the presence of both CsA and a phospholipase A_2 inhibitor such as trifluoperazine [55]. This has been confirmed with hepatocyte mitochondria *in situ*, where protection from mitochondrial depolarization and irreversible cell death caused by oxidative stress is much greater when trifluoperazine (an inhibitor of phospholipase A_2) is added in conjunction with CsA than when CsA is used alone [54]. It was not possible to use trifluoperazine in the present studies, since it is a very potent calmodulin antagonist, which is inappropriate for use in the heart.

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