Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart

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Opening of the mitochondrial permeability transition pore (MPTP) is thought to be a critical event in mediating the damage to hearts that accompanies their reperfusion following prolonged ischaemia. Protection from reperfusion injury occurs if the prolonged ischaemic period is preceded by short ischaemic periods followed by recovery. Here we investigate whether such ischaemic preconditioning (IPC) is accompanied by inhibition of MPTP opening. MPTP opening in Langendorff-perfused rat hearts was determined by perfusion with 2-deoxy[3H]glucose ([3H]DOG) and measurement of mitochondrial [3H]DOG entrapment. We demonstrate that IPC inhibits initial MPTP opening in hearts reperfused after 30 min global ischaemia, and subsequently enhances pore closure as hearts recover. However, MPTP opening in mitochondria isolated from IPC hearts occurred more readily than control mitochondria, implying that MPTP inhibition by IPC in situ was secondary to other factors such as decreased calcium overload and oxidative stress. Hearts perfused with cyclosporin A or sanglifehrin A, powerful inhibitors of the MPTP, also recovered better from ischaemia than controls (improved haemodynamic function and less lactate dehydrogenase release). However, the mitochondrial DOG entrapment technique showed these agents to be less effective than IPC at preventing MPTP opening. Our data suggest that protection from reperfusion injury is better achieved by reducing factors that induce MPTP opening than by inhibiting the MPTP directly.

In order to salvage the ischaemic heart it must be reperfused, yet the very act of reperfusion exacerbates the damage inflicted by ischaemia. This ‘reperfusion injury’ is associated with hallmarks of necrotic cell death such as the release of intracellular proteins and major morphological changes including grossly swollen mitochondria. Mitochondrial swelling is one consequence of the opening of the mitochondrial permeability transition pore (MPTP), a non-specific pore that opens in the mitochondrial inner membrane under conditions of high matrix [Ca2+], especially when this is accompanied by adenine nucleotide depletion, elevated inorganic phosphate and oxidative stress (Halestrap et al. 1998; Crompton, 1999; Bernardi, 1999; Kroemer & Reed, 2000). These are exactly the conditions that occur during reperfusion after a period of ischaemia. Indeed, hearts may be protected from reperfusion injury by cyclosporin A (CsA) and sanglifehrin A (SfA) both of which inhibit the MPTP directly (Nazareth et al. 1991; Griffiths & Halestrap, 1993; Weinbrenner et al. 1998; Minners et al. 2000; DiLisa et al. 2001; Hauserloy et al. 2002; Clarke et al. 2002). Furthermore, we have developed a technique to measure MPTP opening in the perfused heart that involves mitochondrial entrapment of 2-deoxy[3H]glucose ([3H]DOG), and used it to confirm that pore opening does occur during reperfusion but not during ischaemia (Griffiths & Halestrap, 1995; Halestrap et al. 1997a). The mitochondrial DOG entrapment technique has been used to demonstrate that inhibition of MPTP opening correlates with the improved haemodynamic recovery of the heart mediated by pyruvate (Kerr et al. 1999) and the free radical scavenging anaesthetic, propofol (Javadov et al. 2000).

This body of data has led us and others to argue that opening of the MPTP may be a critical event in the transition from reversible to irreversible reperfusion injury (Halestrap et al. 1998; Suleiman et al. 2001). When it opens, the MPTP allows free passage of any molecule <1500 Da across the mitochondrial inner membrane, including protons. Under such conditions the mitochondria are unable to synthesise ATP by oxidative phosphorylation; indeed by reversing the proton-translocating ATPase such uncoupled mitochondria actively hydrolyse ATP synthesised by glycolysis and remaining healthy mitochondria. As a result, the cardiac myocyte can no longer produce sufficient ATP to maintain ionic homeostasis or repair damage to...
cellular components induced by the ischaemic insult, and necrosis becomes inevitable.

One of the most effective ways of protecting hearts from reperfusion injury is by subjecting them to one or more brief (3–5 min) ischaemic periods with intervening recovery before prolonged ischaemia is initiated. Protection from ischaemia is maintained for 1–2 h (first window) and then reappears about 24 h later (second window) (Schwarz et al. 1997; Baines et al. 1999; Takeo & Nasa, 1999). The mechanisms responsible for such ischaemic preconditioning (IPC) are debated, but activation of protein kinase C (PKC) by factors released during the brief ischaemic periods (e.g. adenosine, bradykinin, noradrenaline and endorphins acting via their receptors) or intervening reperfusion (reactive oxygen species) have been strongly implicated. Thus PKC inhibitors and free radical scavengers antagonise IPC, whilst adenosine agonists and PKC activators mimic the effect (VandenHoek et al. 1998; Baines et al. 1999; Schulz et al. 2001). The ultimate target of these kinases that mediates protection is unknown, although studies using a range of openers and blockers of the mitochondrial ATP-dependent potassium channel (mitoKATP) such as diazoxide and 5-hydroxydecanoate have implicated activation of the mitoKATP (Szewczyk & Marban, 1999; Ghosh 5-hydroxydecanoate have implicated activation of the mitochondrial ATP-dependent potassium channel (mitoKATP) such as diazoxide and 5-hydroxydecanoate have implicated activation of the mitoKATP (Szewczyk & Marban, 1999; Ghosh et al. 2000; Grover & Garlid, 2000; Murata et al. 2001). However, there is a substantial body of data that casts doubt on the specificity of these agents (Schäfer et al. 1969, 1971; Grimmssman & Rustenbeck, 1998; Kowaltowski et al. 2001b; Lawrence et al. 2001; Hanley et al. 2002; Lim et al. 2002; Das et al. 2003). In view of its critical role in reperfusion injury, the MPTP is likely to be another target of IPC and indirect evidence supports this conclusion (Xu et al. 2001; Hausenloy et al. 2002). In this paper we investigate this possibility directly by using a technique involving the mitochondrial entrapment of \([^3H]DOG\) (Griffiths & Halestrap, 1995). Our data provide the first direct evidence that IPC inhibits opening of the MPTP in situ. However, induction of the MPTP in mitochondria isolated from hearts subject to IPC is not inhibited, unlike those isolated from hearts treated with the MPTP inhibitors CsA and SFA. These data suggest that the effect of IPC on the MPTP in situ is indirect.

METHODS

Heart perfusion

All procedures used conformed with the UK Animals (Scientific Procedures) Act 1986, and were essentially the same as described previously (Kerr et al. 1999; Javadov et al. 2000). Male Wistar rats (250–260 g) were killed by stunning and cervical dislocation, and hearts (about 0.85 g) were rapidly removed and immediately arrested in ice-cold buffered Krebs-Henseleit solution. The aorta was rapidly cannulated and the heart perfused at 12 ml min \(^{-1}\) in the Langendorff mode with in-line filter using Krebs-Henseleit buffer containing (mm): NaCl 118, NaHCO\(_3\) 25, KCl 4.8, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, glucose 11 and CaCl\(_2\) 1.2, gassed with 95% O\(_2\),5% CO\(_2\); at 37°C (pH 7.4). Monitoring of left ventricular developed pressure (LVDP) was performed with a water-filled balloon inserted into the left ventricle, set to give an initial end diastolic pressure (EDP) of 2.5–5 mmHg. All hearts were allowed an equilibration period of at least 15 min before any additional treatments were given. IPC was elicited by two cycles of 5 min of global ischaemia interspersed with 5 min reperfusion prior to 30 min global normothermic ischaemia. When added, SFA and CsA were present at 0.2 \(\mu\)M for 10 min and 5 min, respectively, before the induction of ischaemia and maintained in the perfusion buffer for the first 10 min of reperfusion (Clarke et al. 2002). For reasons explained below, the concentration of SFA used in the present experiments (0.2 \(\mu\)M) was less than the 1 \(\mu\)M employed in previous experiments (Clarke et al. 2002). We have found that the stock solutions of SFA in ethanol used in previous experiments had decayed by about 80% over a period of months when stored at \(-20°C\), and the present experiments used fresh SFA. Use of concentrations of SFA higher than 0.2 \(\mu\)M led to the drug coming out of solution. In some experiments, samples of perfusate were collected prior to ischaemia and every 1 min during reperfusion for the determination of lactate dehydrogenase (LDH) activity spectrophotometrically. The total LDH concentration of hearts was determined by assaying a sample of homogenate prepared as described below.

Measurement of MPTP opening in situ using mitochondrial 2-deoxy[^3H]glucose entrapment

This was performed as described previously (Griffiths & Halestrap, 1995; Kerr et al. 1999; Javadov et al. 2000). For pre-ischaemic loading, after a 15 min flow-through period for stabilisation, hearts were perfused in recirculating mode with 40 ml of Krebs-Henseleit solution containing 0.5 mmol 1\(^{-1}\) \[^3H\]DOG (0.1 \(\mu\)g ml\(^{-1}\)) for 30 min. Perfusion was then returned to flow-through (non-recirculating) mode with normal buffer for 5 min before the preconditioning protocol (or equivalent time of perfusion for control or drug-treated hearts) followed by 30 min of global ischaemia and 30 min of reperfusion. Individual protocols are shown schematically in Fig. 1A. For post-ischaemic loading, hearts were perfused for 40 min in flow-through mode and where required IPC or drug treatment was performed immediately prior to induction of ischaemia. After 30 min global ischaemia hearts were reperfused for 25 min to achieve full functional recovery, at which time they were loaded with \[^3H\]DOG for 30 min as described above. Extracellular \[^3H\]DOG was washed out by 10 min of perfusion with Krebs-Henseleit solution. Individual protocols for post-loading are shown schematically in Fig. 1B. After the appropriate perfusion protocol, hearts were homogenised with a Polytron homogeniser, mitochondria were prepared and samples of crude homogenate and mitochondria were assayed for \[^3H\] and citrate synthase activity exactly as described previously (Griffiths & Halestrap, 1995; Kerr et al. 1999; Javadov et al. 2000). Mitochondrial entrapment of 2-deoxy[^3H]glucose-6-phosphate (\[^3H\]DOG-6P) is usually expressed as 10^\(x\) (mitochondrial \[^3H\] d.p.m. per unit citrate synthase)/(total heart \[^3H\] d.p.m. (g wet weight)^\(-1\)). In this calculation, citrate synthase is used to correct for variations in the recovery of intact mitochondria and the total homogenate \[^3H\] takes into account differences in loading of hearts with \[^3H\]DOG-6P between experiments. It can be estimated that when all mitochondria have undergone MPTP opening, this value should be about 110 (Griffiths & Halestrap, 1995). It should be noted that in two previous studies from this laboratory (Kerr et al. 1999; Javadov et al. 2000) values for the \[^3H\]DOG entrapment were underestimated by a factor of 2 due to an error in the calculations. In this paper (Tables 1 and 2) we also present data in a manner that endeavours to take into account the loss of mitochondria.
predicted to occur when cells become necrotic (see Discussion). For this purpose we multiply the DOG entrapment units by the mitochondrial recovery expressed as units of citrate synthase per gram of heart.

Measurement of MPTP opening in isolated heart mitochondria

After the appropriate perfusion protocol, the heart was homogenised using a Polytron homogeniser and the preparation of mitochondria performed as above but with the addition of a Percoll density gradient purification step and final wash (Halestrap, 1987). For measurement of MPTP opening, swelling of both energised and de-energised mitochondria at a range of calcium concentrations was determined by monitoring the decrease in light scattering at 520 nm as described previously (Halestrap et al. 1997b). For de-energised conditions, mitochondria were incubated at 25°C and 0.5 mg ml⁻¹ protein in 3.5 ml buffer (pH 7.2) containing (mM): KSCN 150, Mops 20, Tris 10 and nitrilotriacetic acid 2, supplemented with 0.5 μM rotenone, 0.5 μM antimycin and 2 μM A23187. Nitrilotriacetic acid was present to allow buffered calcium concentrations of 50–250 μM to be employed, whilst the calcium ionophore A23187 was added to ensure complete equilibration of [Ca²⁺] across the mitochondrial inner membrane under de-energised conditions. For energised conditions the buffer (pH 7.2) contained (mM): KCl 125, Mops 20, Tris 10, KPi 2.5, MgCl₂ 2.5, NaCl 5, Tris-succinate 5 and 1 μM rotenone. Mitochondria were added (1 mg protein per ml) and swelling was initiated by progressive additions of CaCl₂ (50 μM).

Statistical analysis

All data are presented as mean values ± S.E.M. The statistical significance of differences between control and IPC or drug-treated hearts was calculated by Student’s unpaired t test.

RESULTS

Ischaemic preconditioning inhibits opening of the mitochondrial permeability transition pore

A series of experiments was performed to determine the extent of MPTP opening in control and preconditioned hearts. For this purpose the mitochondrial entrapment of [³H]DOG was measured using one of two protocols. In the pre-loading protocol, hearts were perfused with [³H]DOG prior to 30 min global isothermic ischaemia whilst in the post-loading technique [³H]DOG was loaded after hearts had been reperfused for 25 min and achieved their maximum functional recovery. The former protocol determines the degree of pore opening that occurs during the initial phase of reperfusion, irrespective of whether or not the pores subsequently reseal. In contrast, the latter

![Figure 1. Perfusion protocols](image)

Perfusion protocols used for measuring the effects of IPC, SfA and CsA on opening of the MPTP using mitochondrial DOG-entrapment with either pre-loading (A) or post-loading (B).
protocol only detects those mitochondria whose pores remain open once the heart has reached its maximum recovery (Kerr et al. 1999). For both protocols, $[^{3}H]$DOG entrapment was determined before the ischaemic insult and, in separate experiments, after reperfusion. Data are presented in Table 1 where corresponding data on heart haemodynamic function (left ventricular developed pressure (LVDP) and end diastolic pressure (EDP)) are also presented. It is clear that for both protocols, IPC improved the recovery of LVDP from about 50 to 100% whilst reducing the EDP by 50% or more. In parallel there was a considerable drop in the $[^{3}H]$DOG entrapment by mitochondria that occurred upon reperfusion. Baseline entrapment (ratio units) was determined in non-ischaemic control and preconditioned hearts and both gave similar values (17–19). We have demonstrated previously that this basal value may represent a combination of a slow MPTP-independent uptake of $[^{3}H]$DOG into mitochondria and contaminating vesicular components in the mitochondrial fraction (Griffiths & Halestrap, 1995; Halestrap et al. 1997a).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DOG pre-loading experiments</th>
<th>DOG post-loading experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-ischaemic</td>
<td>Reperfused</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>IPC n = 6</td>
</tr>
<tr>
<td>Pre-ischaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>7.1 ± 1.3 8.7 ± 0.9</td>
<td>8.1 ± 1.2 7.2 ± 0.9</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>89.2 ± 6.1 81.1 ± 4.7</td>
<td>83.5 ± 7.6 82.3 ± 8.1</td>
</tr>
<tr>
<td>Ischaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to IC (min)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Max IC (mmHg)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Post-ischaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LVDP recovery (%)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total $[^{3}H]$DOG uptake</td>
<td>(d.p.m. per gram heart × 10^{-3})</td>
<td>308 ± 27.0 268 ± 21.5</td>
</tr>
<tr>
<td>Recovery of mitochondria</td>
<td>(CS units per gram heart)</td>
<td>20.0 ± 0.30 20.7 ± 0.94</td>
</tr>
<tr>
<td>Mitochondrial DOG entrapment</td>
<td>DOG units per gram heart</td>
<td>17.63 ± 0.33 16.93 ± 1.11</td>
</tr>
<tr>
<td></td>
<td>DOG units per unit recovered CS</td>
<td>0.74 ± 0.02 0.68 ± 0.07</td>
</tr>
</tbody>
</table>

Hearts were perfused with or without ischaemic preconditioning (IPC) according to the protocols shown in Fig. 1. Loading with 2-deoxy$[^{3}H]$glucose ($[^{3}H]$DOG) was performed either before 30 min global ischaemia (pre-loading) or after ischaemia and 25 min reperfusion (post-loading) as indicated. Hearts that were subjected to the same procedures but not subjected to ischaemia and reperfusion were used as non-ischaemic controls. At the end of the perfusion hearts were rapidly homogenised and mitochondria prepared for determination of $[^{3}H]$-DOG entrapment and citrate synthase (CS) activity as described in Methods where explanations of the units used and how they were calculated are given. Data are presented as mean values ± S.E.M. of the number of hearts shown. Statistical significance of IPC versus control hearts was determined by Student’s $t$ test (*$P<0.05$; **$P<0.01$). MPTP, mitochondrial permeability transition pore; IC, ischaemic contracture; EDP, end diastolic pressure, LVDP, left ventricular developed pressure.

Opening of the MPTP in mitochondria isolated from preconditioned hearts is more sensitive to $[Ca^{2+}]$ than in control mitochondria

The decrease in MPTP opening in the preconditioned hearts could be due to a direct effect of IPC on the MPTP itself, or be exerted via an indirect mechanism. In order to establish whether the MPTP itself was directly inhibited, mitochondria were rapidly prepared from control or IPC hearts at one of three points during the perfusion protocol: prior to ischaemia, at the end of ischaemia or after reperfusion. The $[Ca^{2+}]$ sensitivity of MPTP opening in these mitochondria was then measured using a swelling
assay under de-energised conditions in the presence of a calcium ionophore (A23187). These conditions eliminate factors that might have indirect effects on MPTP opening such as changes in membrane potential or calcium transport into, and accumulation within, the mitochondrial matrix. As such this technique should detect direct effects of IPC on the MPTP mechanism. Typical traces for mitochondria from control and IPC hearts are shown in Fig. 2A and demonstrate that mitochondria from IPC hearts are actually more sensitive to MPTP opening than those from control hearts. This contrasts with mitochondria from hearts that had been treated with the novel MPTP inhibitor, SfA (Clarke et al. 2002) at 0.2 μM for 10 min, followed by a 30 s washout prior to mitochondrial isolation. These mitochondria were almost totally insensitive to MPTP opening as shown in Fig. 2B. Similar results were obtained with 0.2 μM CsA (data not shown). The extent of MPTP opening was expressed in terms of the initial rate of swelling as described previously (Halestrap & Davidson, 1990; Halestrap et al. 1997b). Mean data for mitochondria from eight control and IPC hearts are shown in Fig. 2C and confirm that the increased sensitivity of MPTP-opening to [Ca^{2+}] caused by IPC is reproducible and statistically significant. Mean data are also presented for mitochondria isolated from control and IPC hearts subjected to 30 min global ischaemia with or without reperfusion. In both cases, opening of the MPTP in these mitochondria was more sensitive to [Ca^{2+}] than for mitochondria isolated prior to ischaemia. There was some indication (not statistically significant) that MPTP opening remained more sensitive in mitochondria from IPC hearts when they were isolated at the end of ischaemia, but the difference between control and IPC hearts was totally lost following reperfusion.

These data demonstrate that there is not a direct effect of IPC on the MPTP mechanism, but do not exclude the possibility that the MPTP is inhibited indirectly through other effects on the mitochondria relevant to the in vivo situation, such as calcium handling or free radical
production. In order to reveal any such effects we have measured MPTP opening under energised conditions upon progressive additions of calcium. In contrast to de-energised conditions, in the presence of ionophore, where the matrix \([\text{Ca}^{2+}]\) is the same as the extra-mitochondrial \([\text{Ca}^{2+}]\), under energised conditions almost all the added calcium is accumulated within the matrix and pore opening is initiated after a lag phase. Typical traces for mitochondria from control and IPC hearts, prepared on the same day, are shown in Fig. 3. We found that the absolute amount of calcium required to initiate pore opening under energised conditions exhibited more day-to-day variation than under de-energised conditions. The reasons for this are unknown. Nevertheless, in a total of six experiments we found no evidence for any inhibitory effect of IPC on MPTP opening in energised mitochondria.

SfA and CsA are less effective than IPC at inhibiting MPTP opening during reperfusion

SfA and CsA are both very potent inhibitors of the MPTP that provide substantial protection of hearts from reperfusion injury whether measured in terms of functional recovery or LDH release (Griffiths & Halestrap, 1993, 1995; Clarke et al. 2002). However, previous experiments failed to demonstrate a significant decrease in mitochondrial entrapment of pre-loaded \([\text{H}]\)DOG by

### Table 2. The effects of SfA and CsA on MPTP opening and closure in situ measured by \([\text{H}]\)DOG entrapment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-ischaemic control</th>
<th>DOG pre-loading experiments</th>
<th>DOG post-loading experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control n=12</td>
<td>SfA n=10</td>
<td>CsA n=10</td>
</tr>
<tr>
<td></td>
<td>Control n=10</td>
<td>SfA n=11</td>
<td>CsA n=3</td>
</tr>
<tr>
<td>Pre-ischaemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>2.4 ± 0.2</td>
<td>3.1 ± 0.4</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>61.9 ± 3.4</td>
<td>60.1 ± 1.9</td>
<td>61.4 ± 2.9</td>
</tr>
<tr>
<td>Ischaemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to IC (min)</td>
<td>—</td>
<td>13.4 ± 0.5</td>
<td>13.8 ± 0.9</td>
</tr>
<tr>
<td>Max IC (mmHg)</td>
<td>—</td>
<td>25.2 ± 1.6</td>
<td>25.4 ± 1.8</td>
</tr>
<tr>
<td>Post-ischaemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>—</td>
<td>42.3 ± 5.9*</td>
<td>35.8 ± 5.9**</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>—</td>
<td>25.4 ± 5.7*</td>
<td>38.4 ± 7.4**</td>
</tr>
<tr>
<td>LVDP recovery (%)</td>
<td>—</td>
<td>40.3 ± 10.8*</td>
<td>58.7 ± 10.8**</td>
</tr>
<tr>
<td>Total DOG uptake</td>
<td>66.1 ± 3.3</td>
<td>28.3 ± 2.5</td>
<td>31.2 ± 1.9</td>
</tr>
<tr>
<td>(d.p.m. per gram heart × 10^{-3})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery of mitochondria</td>
<td>15.5 ± 0.6</td>
<td>12.6 ± 0.7*</td>
<td>12.0 ± 0.8</td>
</tr>
<tr>
<td>Mitochondrial DOG entrapment</td>
<td>15.6 ± 0.80</td>
<td>69.3 ± 7.7*</td>
<td>72.5 ± 4.9*</td>
</tr>
<tr>
<td>DOG units per gram heart</td>
<td>88.2 ± 5.3</td>
<td>6.91 ± 0.92*</td>
<td>7.61 ± 0.87*</td>
</tr>
<tr>
<td>DOG units per unit recovered CS</td>
<td>10.94 ± 1.11</td>
<td>10.0 ± 2.3**</td>
<td>14.3 ± 4.2**</td>
</tr>
<tr>
<td>LDH release (mUnits ml^{-1} perf)</td>
<td>0.6 ± 0.1</td>
<td>36.8 ± 6.2</td>
<td>43.5 ± 10.2</td>
</tr>
</tbody>
</table>

Hearts were perfused with or without 0.2 \(\mu\)M sanglifehrin A (SfA) or cyclosporin A (CsA) according to the protocols shown in Fig. 1. Lactate dehydrogenase (LDH) was measured in the perfusate for the first 10 min of reperfusion. All other procedures were performed as in Table 1. Data are presented as mean values ± S.E.M. of the number of hearts shown. Statistical significance of SfA or CsA versus control hearts was determined by Student’s t test (*P < 0.05; **P < 0.01).

### Figure 3. The effects of IPC treatment of hearts on the [Ca^{2+}] sensitivity of MPTP opening in isolated energised mitochondria

Mitochondria were isolated from control hearts and those subjected to IPC and pore opening determined under energised conditions as described under Methods. Additions of [Ca^{2+}] were made as indicated.
CsA treatment, despite a significant increase in the recovery of LVDP (Griffiths & Halestrap, 1995). We have suggested that this apparent anomaly may reflect the inability of CsA and SfA to inhibit the MPTP when matrix 
\([Ca^{2+}]\) is high and accompanied by conditions of oxidative stress and low adenine nucleotide concentrations, conditions which occur during reperfusion (Griffiths & Halestrap, 1995; Halestrap et al. 1997b; Clarke et al. 2002). In contrast, we have shown previously that a significant decrease in mitochondrial \([^{3}H]DOG\) entrapment is associated with protection of hearts from reperfusion injury induced by pyruvate and propofol. Both of these agents, like IPC, reduce the production of reactive oxygen species and may also reduce calcium overload (Kerr et al. 1999). Nevertheless, the fact that two potent but distinct inhibitors of the MPTP, CsA and SfA, do provide substantial protection of hearts from reperfusion injury implies that pore opening is playing a role in their protective mechanism even if this is not detected by differences in the entrapment of pre-loaded \([^{3}H]DOG\). As discussed previously (Griffiths & Halestrap, 1995) this may be because MPTP inhibitors such as CsA and SfA can enhance MPTP closure later in reperfusion, despite having no effect on the initial pore opening early in reperfusion.

Resealing of the MPTP can be demonstrated by comparing mitochondrial entrapment of pre-loaded and post-loaded \([^{3}H]DOG\) (Kerr et al. 1999). In Table 2 we present such data for hearts reperfused in the presence or absence of CsA or SfA. In contrast to our earlier data (Griffiths & Halestrap, 1995), these experiments did demonstrate a small but significant \((P < 0.05)\) decrease in mitochondrial entrapment of pre-loaded \([^{3}H]DOG\), although the effect was considerably less than was observed with IPC treatment (Table 1). SfA produced a similar effect to CsA. Values (DOG units g\(^{-1}\) ± S.E.M.) were 88.2 ± 5.3 for control reperfused hearts, and decreased to 69.3 ± 7.7 \((P < 0.05)\) and 72.5 ± 4.9 \((P < 0.05)\) for SfA- and CsA-treated hearts, respectively. Both agents produced a significant improvement in recovery of the heart, whether this was measured using haemodynamic parameters (LVDP and EDP) or lactate dehydrogenase (LDH) release. As might be expected, there was a strong inverse correlation \((P < 0.001)\) between the recovery of the LVDP and the EDP upon reperfusion for control, drug-treated and IPC-treated hearts (data not shown).

The relationship between the recovery of haemodynamic function and LDH release is analysed in more detail in Fig. 4, and this reveals a biphasic relationship irrespective of the perfusion protocol. In the first phase, a major decline in haemodynamic function occurs before LDH release is greatly stimulated, whilst in the second phase, more extensive damage is reflected in increased LDH release with little further change in EDP (Fig. 4A) or LVDP (Fig. 4B), which indicates that heart function is already severely compromised. This is important because it suggests that stunning of heart function occurs with relatively little necrosis (phase 1), yet is still associated with MPTP opening. This relationship held true irrespective of the experimental group, SfA and CsA increasing the number of hearts in the first phase in which little necrosis occurs. One explanation of these data is that as more mitochondria undergo the permeability transition ATP production falls progressively leading to impaired haemodynamic function of the heart (stunning). However, with

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**Figure 4. Correlation between the release of LDH and the EDP (A) or percentage recovery of LVDP (B) during reperfusion of hearts subject to 30 min global ischaemia**

Data are shown for control hearts and those treated with 0.2 \( \mu \)M CsA or SfA prior to ischaemia using both the pre-loading (open symbols) and post-loading (filled symbols) protocols shown in Fig. 1. Mean data for all relevant parameters are shown in Table 2.
yet further and prolonged MPTP opening, ATP levels fall further causing progressive necrotic damage and greater LDH release. This conclusion is supported by the inverse correlation between the percentage recovery of the LVDP and the mitochondrial entrapment of preloaded \[^{3}\text{H}\]DOG \((P < 0.01)\) shown in Fig. 5A. A similar positive correlation \((P < 0.01)\) between EDP and mitochondrial \[^{3}\text{H}\]DOG entrapment was also observed (data not shown). In both cases, these correlations held true for all experimental groups, despite the control recoveries in the drug-treated experiments being less than in the IPC experiments. The reason for this difference is unclear but we have found that the time of year can affect the absolute degree of recovery observed.

Limitations in the use of mitochondrial DOG entrapment to measure MPTP opening \textit{in situ}

Table 2 also contains data for mitochondrial entrapment of post-loaded \[^{3}\text{H}\]DOG in control, SfA-treated and CsA-treated hearts. Once it became clear that CsA behaved similarly to SfA no further experiments were performed and thus data from only three hearts are presented. Despite protecting hearts from reperfusion injury, neither SfA nor CsA treatment gave any significant decrease in entrapment of post-loaded \[^{3}\text{H}\]DOG, values being 43.2 ± 2.0 and 43.8 ± 2.0 for control and SfA-treated hearts, respectively. This is in marked contrast to IPC where values were 40.9 ± 3.8 and 27.0 ± 1.6 \((P < 0.01)\) for control and IPC hearts, respectively. Thus when all the data are taken together, the inverse correlation between the recovery of LVDP and the entrapment of post-loaded \[^{3}\text{H}\]DOG is relatively weak \((r = -0.442; P < 0.02)\) as shown in Fig. 5B. These data suggest that, at least with SfA and CsA, there may be some limitation in the use of post-loading to determine the extent to which the MPTP has closed upon reperfusion. Consideration of the theory behind the technique reveals one aspect of this. The mitochondrial \[^{3}\text{H}\]DOG entrapment is calculated from the ratio of mitochondrial \[^{3}\text{H}\]DOG per unit recovered citrate synthase to total heart \[^{3}\text{H}\]DOG per gram of heart. Following reperfusion, those heart cells that have undergone necrosis will have increased plasma membrane permeability, as reflected in the loss of lactate dehydrogenase, and thus will not load with any \[^{3}\text{H}\]DOG. Nor will their mitochondria remain intact and be recovered in the mitochondrial pellet because the breakdown in plasma membrane permeability will overwhelm the myocyte with calcium causing total mitochondrial disruption. Hence totally necrotic cells will not be revealed in measurements of DOG-entrapment. In contrast, myocytes that are energetically compromised during reperfusion because of some degree of pore opening, will metabolise less \[^{3}\text{H}\]DOG to \[^{3}\text{H}\]DOG-6P, but the latter will remain within the cell and still yield a meaningful measure of MPTP opening.

In order to explore this possibility we have measured the total \[^{3}\text{H}\]DOG loading of the heart in the different experimental groups. Data are presented in Tables 1 and 2 and Fig. 6. It should be noted that the control data for total heart \[^{3}\text{H}\]DOG loading in Table 2 was about 20% of the control data of Table 1. The two sets of experiments were performed 10 months apart, with the same batch of \[^{3}\text{H}\]DOG. We have shown that the lower loading in the later experiments reflects the progressive degradation of the radiolabelled material upon storage. In subsequent experiments we have used a new batch of \[^{3}\text{H}\]DOG and found that loading was restored to the earlier values. Reassuringly, values for mitochondrial \[^{3}\text{H}\]DOG entrapment were almost identical between the two sets of

![Figure 5. Correlation between the percentage recovery of LVDP during reperfusion of hearts subject to 30 min global ischaemia and the mitochondrial DOG entrapment](image)

Data are presented for control hearts and those subjected to IPC or treated with 0.2 µM CsA or SfA prior to ischaemia. Separate control experiments were performed for the IPC experiments (filled symbols) and the SfA/CsA experiments (open symbols). Mean data for all relevant parameters are shown in Tables 1 and 2.
experiments performed at the different levels of $[^{3}H]$DOG loading. Thus, whatever breakdown products are formed, they do not affect the measurement of pore opening. Indeed, our data confirm that the DOG technique provides a valid estimate of the extent of MPTP opening that is independent of the amount of $[^{3}H]$DOG loading, as would be predicted for a ratiometric technique.

For all experimental groups in Tables 1 and 2, using the pre-loading protocol there was no significant correlation between the total $[^{3}H]$DOG content of the heart following ischaemia–reperfusion and the recovery of LVDP (Fig. 6A) or LDH release (data not shown). This would be predicted since all hearts were preloaded with $[^{3}H]$DOG prior to any insult and the only cause of a decrease in loading should be loss by necrotic cells. However, from the LDH released during 30 min of reperfusion and the total heart enzyme activity it can be estimated that the number of myocytes undergoing sufficient damage to disrupt their plasma membrane is < 5% of the total. Thus the loss of pre-loaded $[^{3}H]$DOG through this route will be too small to be detected and explains why no effect of SfA, CsA or IPC on this parameter was observed. In contrast, in the post-loading experiments, there was a significant correlation between total heart $[^{3}H]$DOG loading and the recovery of LVDP (Fig. 6B). These data are consistent with the well-established decline in mitochondrial function and ATP production in myocytes following ischaemia–reperfusion. This would decrease the conversion of $[^{3}H]$DOG to $[^{3}H]$DOG-6P. Protection of the mitochondria from pore opening with IPC and agents such as CsA or SfA can account for the observed improvement in ATP production (Griffiths & Halestrap, 1993; Vuorinen et al. 1995; Pucar et al. 2001), which in turn would be expected to enhance $[^{3}H]$DOG conversion to $[^{3}H]$DOG-6P and thus increase the total heart $[^{3}H]$DOG loading.

**DISCUSSION**

**IPC inhibits opening of the permeability transition pore through an indirect mechanism**

In the present paper we provide the first direct evidence that the protection of hearts from ischaemia–reperfusion injury by IPC is associated with an inhibition of MPTP opening in situ. However, it is unlikely that this inhibition reflects a direct effect of IPC on the MPTP mechanism, since mitochondria isolated from hearts subjected to IPC are actually more sensitive to pore opening than are...
control mitochondria (Fig. 2). This is in direct contrast to hearts pre-treated with SfA or CsA where protection of hearts from reperfusion injury is associated with inhibition of the MPTP that is maintained upon mitochondrial isolation (Fig. 2 and Clarke et al. 2002). Nor could we find evidence for inhibition of the MPTP in energised mitochondria isolated from IPC hearts (Fig. 3). Under such conditions any differences in membrane potential and calcium transport induced by IPC should be reflected in changes in MPTP opening. The increased sensitivity of the MPTP to [Ca$^{2+}$] in mitochondria isolated from IPC hearts would be consistent with some loss of adenine nucleotides and an increase in reactive oxygen species that are both known to occur during IPC (VandenHoek et al. 1998; Schulz et al. 2001) and are both powerful activators of the MPTP (Crompton, 1999; Kowaltowski et al. 2001a; Halestrap et al. 2002). Thus our data lead us to conclude that IPC-mediated inhibition of MPTP opening during reperfusion occurs through indirect mechanisms such a decrease in calcium loading of the cardiac myocyte (Schulz et al. 2001; Murata et al. 2001), and a decreased production of reactive species upon reperfusion (Narayan et al. 2001; Ozcan et al. 2002). Both of these phenomena have been observed in response to ischaemic and diazoxide-mediated preconditioning (VandenHoek et al. 2000; Ylitalo et al. 2001).

**The relative merits of inhibiting the MPTP directly or indirectly for protecting hearts from reperfusion injury**

The ability of IPC to inhibit MPTP opening upon reperfusion is similar to that observed previously for hearts protected by perfusion with either pyruvate or propofol (Kerr et al. 1999; Javadov et al. 2000). In each case, not only is the protection associated with fewer mitochondria undergoing the permeability transition upon initial reperfusion, but also, as reperfusion progresses, more resealing of the MPTP. This is revealed by a comparison of mitochondrial DOG entrapment determined using the pre-loading and post-loading protocols. In all these cases, protection of the heart is associated with an inhibition of pore opening through indirect mechanisms, probably involving decreased calcium loading and/or oxidative stress (Kerr et al. 1999). In contrast, when SfA or CsA was used to inhibit the MPTP directly, a less profound inhibition of initial pore opening upon reperfusion was detected with the DOG pre-loading technique, whilst no increase in subsequent pore closure could be detected using the post-loading protocol. A major limitation in the use of CsA and SfA that may explain the limited effect of these agents on mitochondrial entrapment of pre-loaded DOG is that neither inhibits MPTP opening when mitochondria are exposed to a sufficiently strong stimulus (Griffiths & Halestrap, 1995; Brustovetsky & Klingenberg, 1996; Halestrap et al. 1997b; Brustovetsky & Dubinsky, 2000; Clarke et al. 2002). The elevated matrix calcium in the presence of oxidative stress and adenine nucleotide depletion that is associated with reperfusion after a period of ischaemia provides just such a powerful stimulus (Halestrap et al. 1998; Suleiman et al. 2001). In contrast, IPC, pyruvate or propofol, which probably act by decreasing calcium overload and oxidative stress, do give a more profound inhibition of MPTP opening in the early stages of reperfusion (Kerr et al. 1999; Javadov et al. 2000). As reperfusion continues and intracellular [Ca$^{2+}$] and reactive oxygen species decline again, SfA and CsA may become able to block the MPTP totally, leading to better recovery of mitochondrial function. This is reflected in significantly improved haemodynamic recovery of the hearts (LVDP, EDP) and prevention of necrotic damage (LDH release). Without such protection, the continuing ATP deprivation and calcium overload would lead to further MPTP opening and a progressive increase in the number of myocytes undergoing necrosis. This explanation would require that mitochondria with open pores detected by the post-loading technique are in intact but ‘stunned cells’. The amount of pore opening must be insufficient to cause the major ATP loss and metabolic disturbance that would lead to necrosis, but sufficient to depress haemodynamic function. This scenario is consistent with data from other laboratories demonstrating that in the coronary occlusion–reperfusion model of reperfusion injury, in which necrosis rather than stunning is the major determinant of injury, CsA causes a similar decrease in infarct size to IPC- and diazoxide-induced preconditioning (Weinbrenner et al. 1998; Minners et al. 2000; Hausenloy et al. 2002). If protocols such as IPC reduced the stimulus for MPTP opening whilst SfA and CsA inhibit the MPTP directly, it might be predicted that SfA and IPC should have additive effects. However, using IPC hearts we have extended the ischaemic period to 41 min to induce similar damage (LDH release and haemodynamic function) to that experienced by control hearts subject to 30 min ischaemia. Under these conditions SfA failed to provide any additional protection above that offered by IPC, whether measured in terms of LDH release, haemodynamic function or DOG entrapment (data presented as Supplementary Material).

Unfortunately, because totally necrotic cells will have lost both cytosolic and mitochondrial [$^{3}$H]DOG, the presence of open mitochondria in such cells will not be detected. This may partially explain why the post-loading technique failed to detect a decrease in pore opening with SfA despite the improvement in heart function and decrease in LDH release. One means by which it might be possible to make some correction for the problems caused by necrosis would be to take account of the mitochondrial yield, since it would be predicted that mitochondria from necrotic cells would be totally disrupted and not recovered in the mitochondrial pellet. Thus in Tables 1 and 2 we have included data for the mitochondrial [$^{3}$H]DOG entrapment
corrected by the recovery of mitochondria expressed as units of citrate synthase per gram heart. This correction does offer some improvement but still fails to show an effect of CsA and SfA on entrapment of post-loaded \([^{3}H]\)DOG. Although it is important to recognise these shortcomings of the DOG-entrapment technique, they are confined to those situations in which mitochondrial pores have opened sufficiently to cause full-blown necrosis and hence loss of plasma membrane integrity. Since the extent of this can be estimated from LDH release, it is not a major problem in the global ischaemia–reperfusion model used here where the plasma membrane of the majority of cells remains intact. Furthermore, the mitochondrial DOG-entrapment technique is the only method currently available for the direct measurement of MPTP opening and subsequent closure in the perfused heart. However, in situations associated with extensive necrotic damage, such as in the coronary vessel occlusion model where the extent of injury is determined by measurement of infarct size, the mitochondrial DOG-entrapment technique would not be useful.

In conclusion, protection of hearts from ischaemia–reperfusion injury by IPC is associated with indirect inhibition of the MPTP, as is the case with protection by pyruvate and propofol. Indeed, the amount of pore opening is a critical factor in determining the extent of both stunning and necrotic injury. This conclusion is further strengthened by the observation that direct inhibition of the MPTP with SfA and CsA can also protect heart from reperfusion injury, both in terms of haemodynamic function (stunning) and LDH release (necrosis). However, it seems that in the global ischaemia–reperfusion model used here, targeting the MPTP directly with SfA or CsA is less effective at protecting the heart than ameliorating those conditions, such as oxidative stress and calcium overload, that are responsible for initiating pore opening in the first place.

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**Supplementary material**

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and contains supplementary material in the form of a table entitled:

**The effects of SfA on heart function, MPTP opening and LDH release in preconditioned hearts**

The table presents data on the effects of combining IPC with SfA treatment on the recovery of hearts from ischaemia–reperfusion.