# Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart

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## Abstract

First, we present a summary of the evidence for our model of the molecular mechanism of the permeability transition (MPT). Our proposal is that the MPT occurs as a result of the binding of mitochondrial cyclophilin (CyP-D) to the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane. This binding is enhanced by thiol modification of the ANT caused by oxidative stress or other thiol reagents. CyP-D binding enhances the ability of the ANT to undergo a conformational change triggered by $Ca^{2+}$. Binding of ADP or ATP to a matrix site of the ANT antagonises this effect of $Ca^{2+}$; modification of other ANT thiol groups inhibits ADP binding and sensitises the MPT to $[Ca^{2+}]$. Increased membrane potential changes the ANT conformation to enhance ATP binding and hence inhibit the MPT. Our most recent data shows that a fusion protein of CyP-D and glutathione-*S*-transferase immobilised to Sepharose specifically binds the ANT from Triton-solubilised inner mitochondrial membranes in a cyclosporin A (CsA) sensitive manner. Second we summarise the evidence for the MPT being a major factor in the transition from reversible to irreversible injury during reperfusion of a heart following a period of ischaemia. We describe how in the perfused heart [³H]deoxyglucose entrapment within mitochondria can be used to measure the opening of MPT pore in situ. During ischaemia pore opening does not occur, but significant opening does occur during reperfusion, and recovery of the heart is dependent on subsequent pore closure. Pore opening is inhibited by the presence in the perfusion medium of pyruvate and the anaesthetic propofol which both protect the heart from reperfusion injury. Third we discuss how the MPT may be involved in determining whether cell death occurs by necrosis (extensive pore opening and ATP depletion) or apoptosis (transient pore opening with maintenance of ATP). © 1998 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

When mitochondria are exposed to supraphysiological matrix concentrations of calcium, they become non-selectively permeable to small molecules ($<1500$ Da). This causes uncoupling, loss of low molecular weight components from the matrix and swelling, a phenomenon that has been recognised for several decades and is often referred to as the mitochondrial permeability transition (MPT). The sensitivity of the MPT to $[Ca^{2+}]$ is greatly increased by oxidative stress, adenine nucleotide depletion, elevated phosphate concentrations, low membrane potential and agents such as carboxyatractyloside (CAT) that stabilise the 'c' conformation of the adenine nucleotide translocase (ANT). In contrast, protection is afforded by low pH, high membrane potential, cyclosporin A (CsA), and agents that stabilise the 'm' conformation of the ANT (see [1–4]). We begin this article by providing a brief summary of our current understanding of the molecular mechanism of the MPT that may account for these observations. We then move on to consider the role of the MPT in causing the irreversible damage to a heart that occurs when it is reperfused following a period of ischaemia, a phenomenon known as reperfusion injury [2–4]. Cardiac surgeons must avoid such reperfusion injury if the outcome of open heart surgery is to be successful. We end the article by showing how this may be achieved as a result of our increasing understanding of the molecular mechanism of the MPT.

## 2. The molecular mechanism of the MPT

The size profile of solutes that can enter the mitochondrial matrix following the MPT implies the $Ca^{2+}$-dependent formation of a non-specific channel in the inner mitochondrial membrane, as does the immediate reversal of the MPT by chelation of $Ca^{2+}$ with EGTA [5,6]. However, despite strong evidence to the contrary, for many years it was argued that the MPT was induced by activation of mitochondrial phospholipase $A_2$ leading to a non-specific increase in the permeability of the inner membrane phospholipid bilayer [7]. This view became untenable when it was demonstrated that the MPT can be specifically and totally inhibited by sub-micromolar concentrations of the immunosuppressive drug cyclosporin A (CsA) without any effect on phospholipase $A_2$ activity [8–10]. Our own studies on the mechanism of the MPT were initiated by this observation. We have endeavoured to simplify the number of variables that may contribute to the regulation of the MPT and have usually used de-energised mitochondria, more recently in the presence of the calcium ionophore A23187. This ensures that mitochondrial membrane potential and matrix $[Ca^{2+}]$ are held constant, and thus factors influencing the MPT cannot be doing so indirectly through these parameters.

### 2.1. The role of mitochondrial cyclophilin in the mitochondrial permeability transition

The immunosuppressive action of CsA is mediated by a complex between cytosolic cyclophilin (CyP-A) and CsA that inhibits calcineurin, a Ca-sensitive protein phosphatase [11–13]. This led us to investigate whether a mitochondrial CyP was responsible for the effects of CsA on the MPT. Matrix CyP was assayed by its CsA-sensitive peptidyl-prolyl *cis-trans* isomerase (PPIase) activity and the $K_{0.5}$ values of CsA analogues for inhibition of this enzyme were found to be very similar to their $K_{0.5}$ values for inhibition of the MPT [10,14]. More recent work from this and other laboratories has confirmed these observations [15,16]. Furthermore, the number of binding sites required for 100% inhibition of the MPT corresponded to the concentration of PPIase within the matrix (about 50 pmol per mg protein) [10,16]. We were able to purify and N-terminal sequence the CsA-sensitive mitochondrial PPIase [17] and so confirm that it was a member of the cyclophylin (CyP) family, most probably identical to the product of the human CyP-3 gene product [18]. This is a nuclear encoded protein with a mitochondrial targeting presequence that is cleaved after translocation of the protein into the matrix. It is now more usually termed CyP-D [12,13]. Cleavage occurs at one of two points leading to mature proteins of about 17.6 kDa (minor product) and 18.6 kDa (major product). Crompton and colleagues have also purified and sequenced a mitochondrial CyP which they found associated with the inner mitochondrial membrane in a manner

suggestive of it being involved in the MPT [19,20]. This also proved to be CyP-D, but its larger size (21 kDa) suggests that it may represent the protein before cleavage of the signal sequence. We have cloned and sequenced the cDNA for rat mitochondrial CyP (accession number U68544). With the exception of the extreme N-terminal residue, the sequence corresponds exactly to the N-terminus sequence of the purified protein, thus confirming that the matrix PPIase in rat mitochondria is indeed the equivalent of human CyP-3 (CyP-D). Northern blots demonstrate that mRNA for CyP-D is present in rat muscle, heart, liver, kidney and brain and is of identical size (1.5 kb) in all tissues. This makes it unlikely that there are differently spliced tissue-specific isoforms [21].

## 2.2. CyP-D is recruited to the inner mitochondrial membrane under conditions that enhance the MPT

The PPIase activity of CyP-D suggested to us that its role in the mechanism of the MPT might be to interact with an integral membrane protein and cause a change in its conformation which, when triggered by $Ca^{2+}$, would induce pore opening. In view of the influence of the ANT conformation on this process [1–3] we suggested that the integral membrane protein might be the ANT [10,14]. A proline residue ($Pro^{61}$) on a putative matrix loop of the transporter was proposed as the binding site since an adjacent lysine shows conformation specific labeling [10,14]. One mechanism by which the MPT might be sensitised to $[Ca^{2+}]$ would be through an increased binding of CyP to its target protein. We provided evidence in support of this by demonstrating that oxidative stress induced with *t*-butyl hydroperoxide (TBH), glutathione depletion induced by diamide treatment or modification of vicinal thiols by phenylarsine oxide (PheArs) all increased CyP binding to the inner mitochondrial membrane [22,23]. These reagents all increase the sensitivity of pore opening to $[Ca^{2+}]$. In order to show this effect the mitochondrial membranes had to be prepared in iso-osmotic KSCN medium to stabilise the complex between CyP and its membrane target protein. Stabilisation could also be achieved by the addition of low concentrations of guanidinium hydrochloride, implying that it is the chaotropic properties of KSCN that are responsible for its stabilising effects [23]. This suggests that the CyP forms a complex with the target protein, inducing a conformational change that exposes more of the protein surface to the aqueous medium. Such an effect might be predicted for the formation of a channel. Another factor that enhances both CyP binding and MPT opening in response to $[Ca^{2+}]$ is an increase in matrix volume [23]. In all cases, binding of CyP was almost totally prevented by CsA. In contrast, we have been unable to show significant effects on CyP binding of several other known modulators of the MPT including matrix $[Ca^{2+}]$, [ADP], pH or membrane potential. This implies that these effectors are acting at another site that regulates the sensitivity of the MPT towards $[Ca^{2+}]$ [23,24]. However, Bernardi and colleagues have demonstrated an inhibitory effect of low pH on CyP-D binding to submitochondrial particles [15]. The effect was blocked by the histidine reagent diethylpyrocarbonate which also blocks the inhibitory effect of pH on the MPT [25]. These experiments were performed in low ionic strength media where a large number of other matrix proteins also remained bound to the membrane at low pH, and thus it is possible that the effect of pH was on non-specific binding of CyP-D to charged groups on the phospholipids or membrane proteins. Crompton and colleagues [19,20] used similar conditions when labelling the membrane bound CyP-D with photoactivatable CsA derivatives. Their data suggested that $Ca^{2+}$ might enhance and ADP diminish CyP binding under such conditions.

Despite the evidence described above, there is now a body of data to suggest that CyP binding may not be essential for pore opening, but may rather sensitise the process to $[Ca^{2+}]$. Thus Novgorodov et al. [26] and Crompton and Andreeva [27], using different techniques, have shown that at high matrix $[Ca^{2+}]$ inhibition of pore opening by CsA is overcome. We have confirmed this in both heart mitochondria [14] and liver mitochondria [22–24]. Yet under the same conditions, CsA is able to prevent almost totally the binding of CyP-D to the inner mitochondrial membrane [22,24]. In addition, studies on the megachannel of patched clamped mitochondria, which it has been suggested may represent the MPT pore, have shown that an inhibitory effect of CsA is overcome at higher $[Ca^{2+}]$ [28,29]. However,

recent data has cast doubt on whether the megachannel and the MPT pore are the same molecular entity [30].

## 2.3. The role of the adenine nucleotide translocase in the mitochondrial permeability transition

An involvement of the ANT in pore opening was first proposed by Hunter and Haworth [5] and more convincing evidence provided by LeQuoc and Le-Quoc [31] and ourselves [10,24]. The evidence was based largely around the observation that any reagent such as CAT that stabilised the 'c' conformation of the ANT, stimulated the MPT, whilst any reagent such as bongkrekic acid (BKA) that stabilised the 'm' conformation of the ANT, inhibited the MPT. Furthermore, matrix ADP is an important modulator of pore opening that acts by decreasing the sensitivity of the calcium trigger site to $[Ca^{2+}]$. There are two ADP binding sites with $K_i$ values of about 1 and 25 $\mu$M. The high affinity site is blocked by the inhibitor CAT and therefore thought to be associated with the ANT [5,6,24,26,32]. We have tested the ability of a range of other nucleotides to inhibit the MPT, and found that only ATP and deoxy-ADP inhibit with $K_{0.5}$ values 500 and 20 times greater than ADP, respectively. This correlates with their affinity for the matrix binding site of the ANT [24]. Adenine nucleotide binding is antagonised by oxidative stress induced by TBH or diamide and also by thiol reagents such as PheArs, a powerful activator of the MPT [24,33]. PheArs has the greatest effect of the reagents tested, raising the $K_{0.5}$ for ADP inhibition of the MPT to > 500 $\mu$M [24]. We have shown that this effect is accompanied by covalent modification of the ANT [24] which may explain why PheArs is a more potent stimulus of the MPT than diamide or TBH, and yet has a smaller effect on CyP binding [23].

Bernardi and colleagues have provided strong evidence that the MPT is voltage-regulated, being activated as the membrane potential becomes less negative [34–39]. We have suggested that the membrane potential is sensed by the ANT itself through an effect on adenine nucleotide binding. This is not unlikely, since the ANT is an electrogenic carrier, transporting $ATP^{4-}$ in exchange for $ADP^{3-}$ with a mechanism that may well involve a potential driven conformational change that alters the affinity of the adenine nucleotides on either side of the membrane [40–42]. In support of this hypothesis, we have demonstrated that in mitochondria depleted of adenine nucleotides by pyrophosphate treatment, not only is the MPT much more sensitive to $[Ca^{2+}]$, but it is also no longer voltage sensitive [16,24]. Oxidative stress shifts the voltage dependence of the MPT, allowing the pore to open at more negative potentials. Two distinct thiol groups have been implicated in this effect [37,43,44]. One is sensitive to oxidation of glutathione, for example by TBH or diamide, and is protected by both monobromobimane and N-ethylmaleimide. The other responds to the redox state of matrix NAD(P), and is protected by N-ethylmaleimide but not monobromobimane. It is the latter site that accounts for the well documented stimulatory effect of oxidation of matrix NADH on the MPT perhaps through the mediation of thioredoxin or lipoamide [45,46]. The ANT is known to have three cysteine residues that show differential reactivity to various thiol reagents and oxidising agents in a conformation dependent manner [47–49]. These cysteines may well represent the thiol groups that regulate both CyP-D binding and the inhibitory effects of ADP and membrane potential on the MPT [24].

Although a role for the ANT in the MPT is now generally accepted (see [1–3]), there has been debate as to whether this protein may itself form the pore or rather be a regulatory component. Recently it has been shown that the purified and reconstituted ANT can form $Ca^{2+}$-dependent channels resembling the permeability transition pore [50] which provides strong support for the proposed mechanism. What is lacking is direct evidence for an interaction of CyP-D with the ANT and we have directed a great deal of effort towards providing such a demonstration. Attempts at chemical cross-linking of the CyP-D to the ANT have so far been unsuccessful. As an alternative approach we have overexpressed CyP-D as a glutathione-S-transferase fusion protein in E. coli and have immobilised this on Sepharose-glutathione resin in order to investigate ANT binding directly (unpublished data of K. Woodfield and A.P. Halestrap). We found that when fully solubilised in 3% Triton X-100, the ANT does not bind to the GST-CyP, but this may not be surprising since the conformation of the ANT in detergent is unlikely to
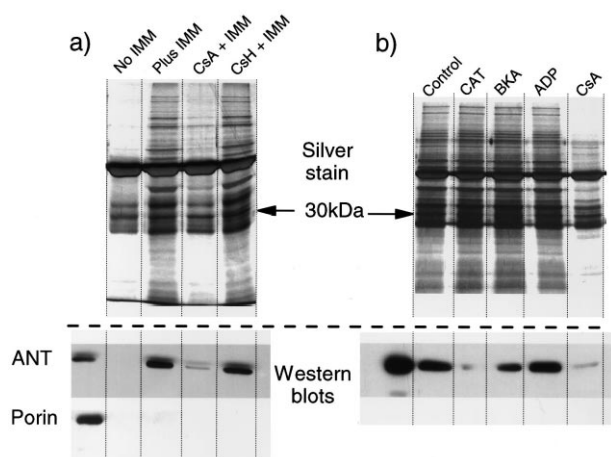
Fig. 1. Binding of the adenine nucleotide translocase to a GST-CyP-D affinity matrix. Rat liver mitochondrial inner membranes (IMM), prepared according to [164], were solubilised at 1 mg protein/ml in KPi buffer (20 mM KPi, pH 7.4, 0.1 mM EDTA, 0.5% (w/v) Triton X-100) containing where indicated 1 mM ADP, 1 μM CsA or cyclosporin H (CsH), 10 μM CAT or 20 μM BKA. Aliquots (1 ml) were incubated for 30 min at 25°C with immobilised GST-CyP-D which was synthesised by binding 0.25 mg GST-CyP-D (fusion protein between GST and CyP-D overexpressed in *E. coli*) to 0.2 ml glutathione-Sepharose resin (Pharmacia). Where the effect of CsA or CsH on binding was to be studied, the GST-CyP-D was preincubated with 25 μM CsA or CsH prior to addition of IMMs, in order to ensure saturation of CyP-D binding sites with CsA. In (a) one aliquot of immobilised GST-CyP-D was incubated in the absence of IMMs. Following incubation, the Sepharose was sedimented by centrifugation, washed 5 times with 1 ml KPi buffer and then bound protein released by incubation with 0.6 ml buffer containing 75 mM Tris, pH 8.0, 300 mM NaCl, 0.5% Triton X-100 and 10 mM GSH. The eluted proteins were separated by SDS-PAGE (12% acrylamide) and either protein bands stained with silver (top) or subjected to Western blotting (bottom) with either anti-ANT (a and b) or anti-porin antibodies (a only). The far left track of each Western blot (labelled IMM only) shows IMMs not subjected to binding on the GST-CyP-D resin but separated by SDS-PAGE to confirm the presence of ANT and porin in the IMM fraction. The antibodies were kind gifts from Dr Gerard Brandolin and Dr Vito de Pinto, respectively. Data shown are unpublished results of K.-Y. Woodfield and A.P. Halestrap.

reflect that in the membrane. However, when inner mitochondrial membranes (IMMs) were resuspended in 0.5% Triton X-100 (partial solubilisation) and then incubated with the immobilised GST-CyP-D, a range of proteins bound to the fusion protein in a CsA sensitive manner. This was demonstrated by SDS-PAGE of the GSH-eluted proteins and visual-

isation using silver staining as shown in Fig. 1a. One of the proteins that bound in a CsA-sensitive manner was the ANT as revealed by parallel Western blotting with anti-ANT antibodies. In contrast, Western blotting with anti-porin antibodies demonstrated no porin binding under the same conditions (Fig. 1a). These data alone do not allow us to conclude that specific binding of the ANT to GST-CyP-D is occurring since, in addition to the ANT, other proteins may remain in the partially-solubilised IMM fraction and be responsible for the binding of membranes containing ANT. However, the data of Fig. 1b provide very strong evidence that the interaction is through the ANT rather than through another associated protein. Thus pre-treatment of the IMMs with CAT before their partial solubilisation in 0.5% Triton X-100 almost totally abolished ANT binding to the GST-CyP-D, whilst having no appreciable effect on total protein binding. BKA had a similar but less pronounced effect. These data demonstrate that binding of the ANT to the column was conformation-dependent, suggesting that a direct interaction between the ANT and the CyP-D was responsible for removing the ANT from the partially solubilised membranes.

## 2.4. The locus of action of different modulators of the MPT

In Table 1 we summarise our current thinking on how different modulators of the MPT exert their effects. We suggest that the ANT provides a common locus for these effects. Binding of matrix adenine nucleotides to the ANT inhibits pore formation by decreasing the affinity of the trigger site for [$Ca^{2+}$]. Adenine nucleotide binding is antagonised by thiol reagents, the 'c' conformation of the carrier and membrane depolarisation which therefore sensitise the MPT to [$Ca^{2+}$]. We have suggested that the thiol group $Cys^{159}$ may be involved in these effects and may represent one of the vicinal thiols to which PheArs binds [24]. The 'm' conformation and increased membrane potential enhance nucleotide binding and inhibit the pore. These effectors are all without effect on CyP-D recruitment to the membrane. In contrast, chaotropic agents and increased matrix volume sensitise the MPT to [$Ca^{2+}$] by increasing CyP-D binding, an effect that thiol reagents

and oxidative stress also induce, perhaps through modification of Cys[56] [24]. This facilitates the transition of the ANT into its open channel state. Low pH and $Mg^{2+}$ are suggested to compete directly with $Ca^{2+}$ at the trigger site. Trifluoperazine is a potent inhibitor of the MPT under energised but not de-energised conditions [24]. It was originally thought to act indirectly through inhibition of phospholipase $A_2$, preventing the accumulation of free fatty acids which stimulate the MPT, probably through interaction with the ANT. However, inhibition occurs even without changes in free fatty acid accumulation and is now thought to be mediated by an effect on surface membrane charge that changes the voltage sensitivity of the MPT [51].

## 2.5. Other possible components of the MPT pore

The question arises as to whether there are other components of the pore, and it seems probable that the answer is yes. We have found that sub-mitochondrial particles (SMPs) maintain a succinate driven membrane potential that is not dissipated in the presence of 1 mM $[Ca^{2+}]$ whether or not 10 μM purified CyP-D is added (unpublished data of A.P. Halestrap). This demonstrates that pore opening is not occurring under such conditions and implies that a matrix component, lost on preparation of the SMPs, is required for operation of the MPT. One possibility for this protein would be a calcium binding protein that provides the calcium trigger site for the MPT. Others have suggested an involvement of the outer

membrane porin and benzodiazepine receptor [3,52] since these components have been reported to copurify with the ANT as a complex under some conditions [53]. Attractive though this proposal is, especially in the light of the recent suggestion that the MPT is involved in the release of cytochrome c during apoptosis (see below), the evidence for it is lacking. First, ligands of the mitochondrial benzodiazepine receptor are without effect on the MPT [24]. Second, the MPT can be observed in mitoplasts from which the outer membrane has been largely removed by digitonin treatment and exhibits identical properties to the MPT in normal mitochondria (A.P. Halestrap, unpublished data). Third, the data of Fig. 1a show that unlike the ANT, porin does not bind to the GST-CyP-D affinity matrix.

## 3. The role of the mitochondrial permeability transition in the ischaemic/reperfused heart

The impermeability of the mitochondrial inner membrane to all but a few selected metabolites and ions is essential for the maintenance of the membrane potential and pH gradient that drive ATP synthesis during oxidative phosphorylation. When this permeability barrier is disrupted by opening of the MPT pore, mitochondria become uncoupled and the proton-translocating ATPase actively hydrolyses rather than synthesises ATP. If this were to occur in a cell, ATP concentrations could not be maintained even by glycolysis. Unless the pore can close

Table 1
Proposed sites of action of known modulators of the mitochondrial permeability transition

| Effect via change in cyclophilin-D binding | Effect via change in nucleotide binding | Direct effect on $Ca^{2+}$ binding |
|---|---|---|
| *Activatory* | | |
| Thiol reagents (e.g. diamide PheArs) | Thiol reagents (e.g. Diamide PheArs) | High pH |
| Oxidative stress (e.g. TBH) | Oxidative stress (e.g. TBH) | |
| Increased matrix volume | 'C' conformation of ANT | |
| Chaotropic agents | | |
| | | |
| *Inhibitory* | | |
| CsA | Membrane potential | Low pH |
| | Membrane surface charge (e.g. trifluoperazine) | $Mg^{2+}$ |
| | 'M' conformation of ANT | |

The site of action of the different modulators is discussed in the text. Note that both CyP binding and ADP binding exert their effects through changes in the sensitivity of the MPT to $[Ca^{2+}]$.
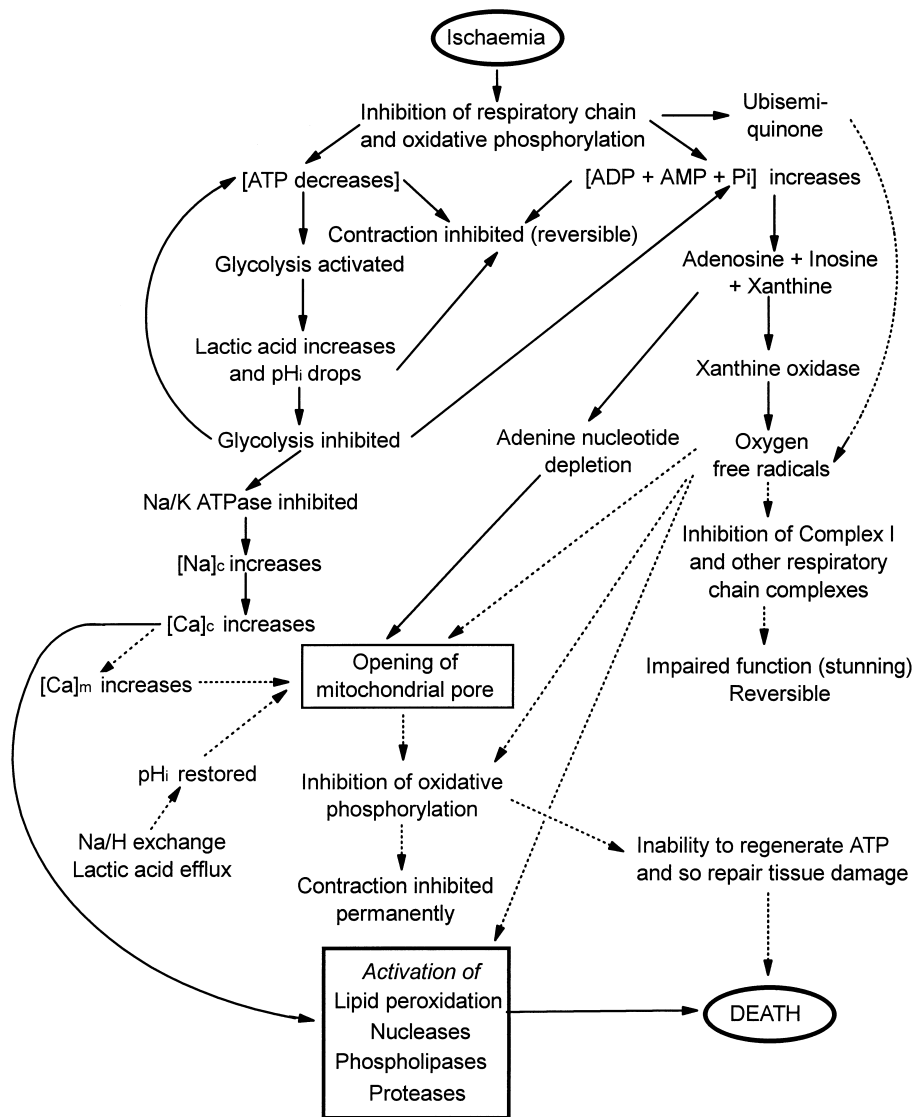
Fig. 2. Scheme summarising factors involved in reperfusion injury of the heart. The solid lines represent events that occur during the ischaemic phase and the dashed lines the subsequent effects of reperfusion. Details are given in the text.

again, the cell would be destined to die, since damage to cellular components caused by hypoxia and oxidative stress can only be repaired if ATP is available, but will continue unabated in its absence [2,54]. Eventually, the permeability barrier of the plasma membrane will be compromised through phospholipase $A_2$ action, and leakage of cell contents and disruption of ion gradients will then ensure cell death. Thus the MPT provides a mechanism by which necrotic cell death can be initiated, and there is increasing evidence that this is indeed the case as will be described below.

In the ischaemic heart, cells endeavour to maintain their ATP levels through glycolysis. This leads to an accumulation of lactic acid, a decrease in intracellular pH and a consequent inhibition of contraction and glycolysis [55,56]. The $Na^+/H^+$ antiporter endeavours to correct the decrease in $pH_i$, loading the cell with $Na^+$ which requires the $Na^+/K^+$ ATPase to drive it out [57–59]. If there is insufficient ATP to allow this, the $Na^+$ accumulates and also prevents $Ca^{2+}$ from being pumped out of the cell on the $Na^+/Ca^{2+}$ antiporter; the process may actually be reversed and allow additional $Ca^{2+}$ entry [59,60].

During ischaemia, calcium may also enter the mitochondria by reversal of the $Na^+/Ca^{2+}$ antiporter [61], but upon reperfusion $Ca^{2+}$ is rapidly taken up into the mitochondria by means of the uniporter [62,63]. The sudden influx of oxygen into a hypoxic cell induces the formation of oxygen free radicals through an interaction of oxygen with ubisemiquinone which is formed during hypoxia as a result of respiratory chain inhibition [54,64,65]. Additional oxygen free radicals are produced through the operation of xanthine oxidase [66]. This enzyme is activated during hypoxia and is presented with high concentrations of xanthine produced by the purine degradation that occurs during hypoxia [67]. The combination of oxidative stress and high $[Ca^{2+}]$ provides the ideal conditions for the MPT, especially in the presence of elevated cellular phosphate concentrations and depleted adenine nucleotide levels, both of which occur during ischaemia [2,4,54,68]. Furthermore, during the reperfusion phase the intracellular pH rapidly returns to pre-ischaemic values through the operation of the $Na^+/H^+$ antiporter, lactic acid efflux on the monocarboxylate transporter (MCT) and bicarbonate dependent mechanisms [58]. Thus the inhibitory effect of low pH on the MPT [69,70] is removed, allowing the other stimulators of the process to exert their full effect. The sequence of events described above is summarised in Fig. 2. It is well established that the reperfusion phase following a prolonged period of ischaemia is more damaging than the ischaemia itself, and activation of the MPT provides an explanation for this. Indeed morphological studies confirm mitochondria become swollen and amorphous under such conditions [2,68].

## 4. Direct measurement of pore opening during reperfusion of the ischaemic heart

In order to establish directly that the MPT occurs in intact tissues under pathological conditions it is necessary to measure mitochondrial pore opening in situ. In isolated hepatocytes subjected to oxidative stress, this has been achieved using laser scanning confocal microscopy with the intracellular green fluorescent dye, calcein, in combination with the mitochondrial membrane potential sensitive red fluorescent dye tetramethylrhodamine (TMRM) [71,72].

The calcein only enters the mitochondria when the MPT pore opens, whilst TMRM leaves the mitochondria under these conditions. This technique has been used to show that mitochondria undergo the MPT during necrotic cell death induced by oxidative stress [71,72]. CsA, especially in the presence of trifluoperazine, inhibits the MPT and protects cells from death. In isolated heart cells measurement of hyper-contracture and mitochondrial membrane potential with fluorescent dyes have been used as an indirect indicator of pore opening during re-oxygenation following anoxia and once again protection by CsA has been observed [73,74]. There was also a correlation between mitochondrial $[Ca^{2+}]$ content and subsequent cell death [62,63].

These techniques for measuring the MPT cannot easily be applied to the perfused heart and we have devised an alternative procedure that relies on the impermeability of the inner mitochondrial membrane to 2-deoxyglucose-6-phosphate [16]. The principle behind this technique is summarised in Fig. 3. Hearts are perfused in Langendorff recirculating mode with [³H]-2-deoxyglucose [DOG] which enters the heart on the glucose carrier and is then phosphorylated to DOG-6-phosphate. This is not further metabolised and is trapped within the cell. It would not be ex-
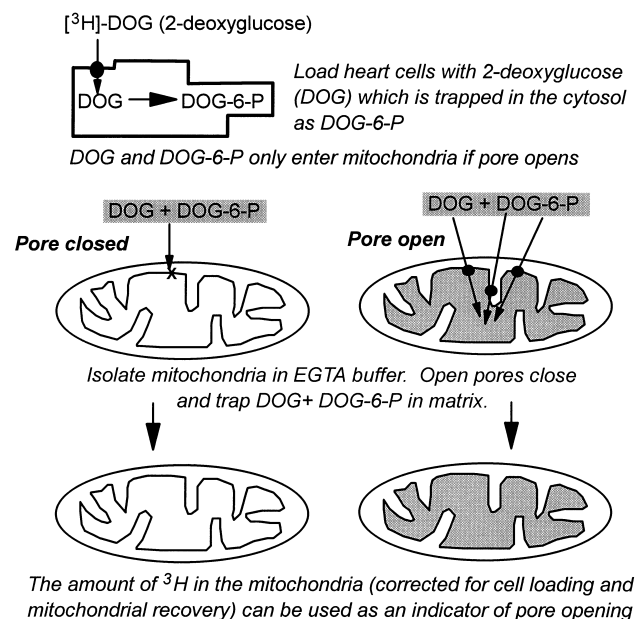


Fig. 3. The principle behind using 2-DOG to measure the opening of MPT pores in the perfused heart.
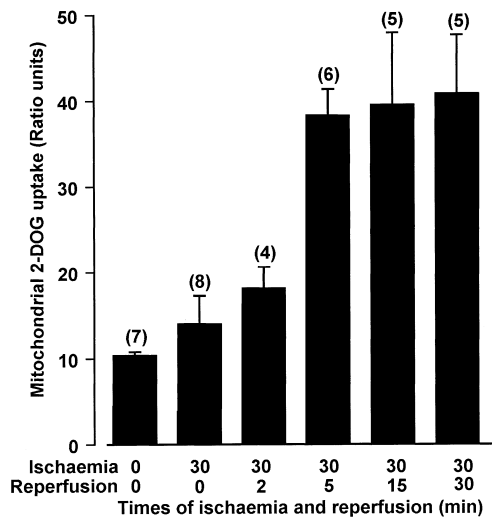
Fig. 4. The time dependence of MPT pore opening in hearts reperfused following 30 min global ischaemia. Hearts were preloaded with [³H]-DOG before 30 min global ischaemia followed by reperfusion for the times shown. Data are taken from [75].

pected to enter mitochondria unless the pore opens. After washing out extracellular [³H]-DOG, hearts are subjected to various periods of ischaemia and reperfusion as outlined above, and then mitochondria prepared rapidly and assayed for [³H]-DOG and citrate synthase (an indicator of mitochondrial recovery). From the [³H]-DOG content of the mitochondria and a small sample of total heart homogenate an estimation of pore opening is possible. As shown in Fig. 4, mitochondria prepared immediately after the ischaemic period showed no increase in DOG content, but those prepared following a period of reperfusion showed a significant increase [16]. Pore opening is maximal after 5 min of reperfusion [75] and this corresponds to the period of time over which the intracellular pH returns to pre-ischaemic values [58]. Thus our data suggest that a profound opening of the mitochondrial pore occurs only during the reperfusion phase, consistent with the predictions made above. It has been argued that pore opening is not a primary cause of cell injury but rather a secondary phenomenon that occurs following other critical damage to the myocyte, such as breakdown of the plasma membrane permeability barrier [76]. However, if the latter were to occur, DOG would be lost from the cell before it could enter the mitochondria and thus no increase in mitochondrial DOG would be measured.

## 5. Measurement of MPT reversal in hearts that recover during reperfusion

Recovery of heart function during reperfusion can be measured using pressure transducers to monitor beat, left ventricular developed pressure (LVDP), end diastolic pressure (EDP) and aortic pressure. In addition adenine nucleotide concentrations can be determined in hearts freeze-clamped after the ischaemic period or after reperfusion [77]. Reperfusion after short periods of ischaemia leads to total recovery of LVDP and ATP/ADP ratio, yet using the DOG technique, we have demonstrated that even under these conditions mitochondria undergo the MPT [16,75,77]. This suggests that the opening of the mitochondrial pores must be transient, rapid resealing allowing total recovery of mitochondrial function and heart performance. Unfortunately, when the MPT reverses, closure of the mitochondrial pores means that DOG remains trapped inside the mitochondria and the reversal is not detected. Detection requires a modification of the DOG technique, in which hearts are loaded with [³H]-DOG during re-
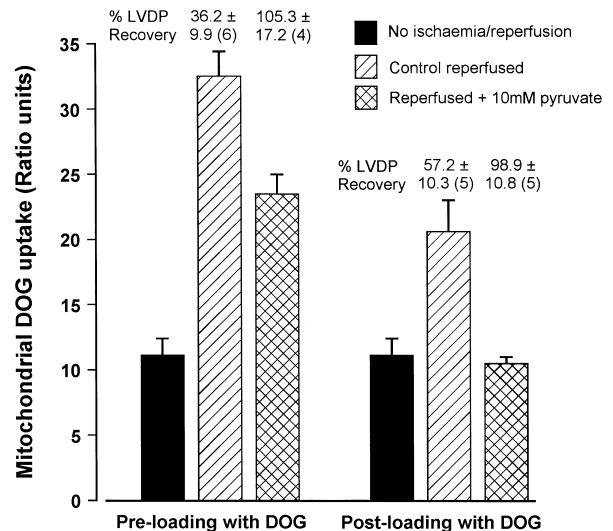


Fig. 5. The effects of 10 mM pyruvate on the opening and subsequent closure of MPT pores during reperfusion of hearts following global ischaemia. Mitochondrial DOG entrapment was measured in either (a) hearts pre-loaded with [³H]-DOG before 40 min global ischaemia and then reperfusion for 20 min or (b) hearts subjected to 40 min global ischaemia and 20 min reperfusion before post-loading with [³H]-DOG. Where indicated 10 mM pyruvate was present 10 min before ischaemia and during reperfusion. Data are taken from [113].

perfusion, once maximum recovery of the heart had been established (post-loading). If reversal of the MPT occurs during reperfusion, mitochondrial DOG entrapment following post-loading should be less than when DOG is present at the start of reperfusion (pre-loading). The data of Fig. 5 show that this is the case. After 40 min ischaemia post-loading gives about 50% less mitochondrial DOG entrapment than observed with pre-loading. Thus it would seem that if the insult caused by ischaemia/reperfusion is not too great, mitochondria can undergo a transient permeability transition, followed by closure of the pores and entrapment of the DOG. The closure is presumably brought about by the decrease in matrix $[Ca^{2+}]$ that occurs as calcium is lost from the mitochondria during the MPT. However, this will only occur if enough 'healthy' mitochondria are remaining in the cell to accumulate the released calcium and provide sufficient ATP to maintain the ionic homeostasis of the cell. The balance between the number of 'closed' and 'open' mitochondria within any cell will be critical in determining whether a cell lives or dies. If there are too many 'open' mitochondria, they will release more calcium and hydrolyse more ATP than the 'closed' mitochondria can accommodate. In contrast, if there are sufficient 'closed' mitochondria to meet the cell's ATP requirements and to accumulate released calcium without undergoing the MPT themselves, the 'open' mitochondria will close again and the cell will recover.

## 6. Protection of the heart from reperfusion injury by inhibitors of the MPT

### 6.1. Cyclosporin

If the MPT is a critical factor in the development of reperfusion injury, CsA would be expected to provide some protection from damage. This has been observed in isolated cardiac myocytes subjected to reoxygenation following a period of hypoxia [73] and in hepatocytes during chemical anoxia and oxidative stress [71,72,78,79]. To test whether such protection from reperfusion injury by CsA can be observed, hearts were perfused with control medium for 15 min before exposure to different concentrations of CsA for 3 min. Isothermic global ischaemia was then

induced for the required period before reperfusion with medium with or without CsA. Hearts treated with 0.2 μM CsA recovered significantly better than controls after periods of ischaemia of 30 min or greater, as reflected in a greater recovery of LVDP and ATP/ADP ratios, lower AMP levels and EDP (an indicator of contracture due to low ATP/ADP and elevated $[Ca^{2+}]$), and a greater recovery of functional mitochondria [16,75,77]. No protective effect of CsA was observed on the loss of total adenine nucleotides that occurs as a result of purine degradation during hypoxia. Nor was protection from inhibition of respiratory chain function (state 3 substrate oxidation) observed [16,75]. The latter effect is probably caused by oxygen free radicals, formed during ischaemia and reperfusion, directly modifying components of the respiratory chain [16,54,75,77]. Our observations are consistent with CsA exerting its effects by preventing the MPT, which is downstream of changes in total adenine nucleotides and free radicals. Furthermore, we have demonstrated that only analogues of CsA that block the MPT in isolated mitochondria are able to offer protection to the reperfused heart [16,75]. The protective effect of CsA was highly concentration dependent, showing an optimal response at 0.2 μM and declining at higher concentrations [77]. A similar concentration dependence has been observed for CsA-protection of isolated cardiac myocytes subjected to reoxygenation following a period of hypoxia [73]. Massoudy et al. [80] have also demonstrated a protective effect of CsA on the working guinea pig heart which was accompanied by an increase in NO production and blocked by co-administration of endothelin receptor antagonists. These authors concluded that CsA protected the hearts by stimulating endothelin receptor-mediated NO production which led to a reduction in oxidative stress, rather than through an effect on the MPT. However, since oxidative stress strongly activates the MPT, these two suggestions are not mutually exclusive.

Direct measurement of the MPT using the DOG preloading technique failed to detect any decrease in pore opening in the presence of CsA [16]. If anything, DOG entrapment was enhanced by the presence of CsA. We attributed this to the nature of CsA inhibition of the MPT. CsA is known to be ineffective as an inhibitor if $[Ca^{2+}]$ is very high, especially

under conditions of adenine nucleotide depletion and oxidative stress [22–24,26,27]. Such conditions may be present during the early phase of reperfusion and might therefore allow the pore to open even in the presence of CsA. The functional protection offered by CsA may rather reflect its ability to accelerate subsequent pore closure as glycolysis and remaining 'closed' mitochondria provide sufficient ATP to restore ion balances and repair other cellular damage. Paradoxically this may be reflected in an increase in DOG entrapment. In order to test this possibility it will be necessary to determine the extent of pore opening after post-ischaemic recovery of hearts using DOG entrapment (post-loading technique).

### 6.2. Antioxidants and calcium antagonists

There is an extensive literature to show that antioxidants and free radical scavengers can protect the ischaemic/reperfused heart from irreversible damage [54,81]. Whilst there may be many processes within the cardiac myocyte that are possible targets for the action of these reagents, prevention of the MPT is clearly one of them. There is also strong evidence that mitochondrial calcium overload accompanies reperfusion injury [63,82,83], and preventing this with calcium antagonists [84,85] or ruthenium red, an inhibitor of mitochondrial calcium uptake [63,86–90], also provides protection. Once again, these observations are consistent with a critical role for the MPT in reperfusion injury, but they cannot be taken as proof of such a role [76].

### 6.3. Low intracellular pH

There is an extensive literature to demonstrate that low pH ($< 7.0$) can protect a variety of cells, including cardiac myocytes [91–95] and hepatocytes [96–99] from chemically-induced oxidative stress, re-oxygenation following anoxia or reperfusion following ischaemia. These effects can be brought about by using low extracellular pH or by addition of specific inhibitors of the $Na^+/H^+$ antiporter such as amiloride [100–104]. A low intracellular pH ($pH_i$) may have several means of exerting a protective effect on the cell, but the profound inhibition of the MPT at pH $< 7.0$ [69,70] suggests that prevention of the MPT

pore may be an important one. Support for this view comes from the observation that during reperfusion the MPT pore opens over the same period of time as the $pH_i$ is restored from less than 6.5 to pre-ischaemic values ($> 7.0$) [58].

### 6.4. Pyruvate

It is well documented that pyruvate can protect hearts against ischaemia/reperfusion and anoxia/re-oxygenation injury [105–108]. The protective effects of pyruvate have been attributed to beneficial metabolic alterations [105,106,108] and to protection from free radical production [107,109] since pyruvate acts as a free radical scavenger [110]. However, an additional effect of pyruvate might be through inhibition of the MPT. The free radical scavenging effects of pyruvate would contribute to this. In addition, as a good respiratory substrate pyruvate would generate a high mitochondrial $NADH/NAD^+$ ratio, preventing oxidation of protein thiol groups critical for modulation of the MPT voltage sensor, and also a high membrane potential which would act in concert to inhibit the MPT [45,46,111]. Furthermore, pyruvate enters heart cells with a proton by means of the monocarboxylate transporter (MCT) [55,112]. This will lead to a decrease in $pH_i$ directly, but in addition pyruvate competes with lactate for transport by the MCT [55,112] which may lead to a greater intracellular accumulation of lactic acid and further lowering of $pH_i$. Such an effect would inhibit the MPT still further. We have demonstrated that the drop in perfusate pH of pyruvate-treated hearts on reperfusion is considerably greater than for control hearts, suggesting that $pH_i$ is significantly lower at the end of ischaemia and during the reperfusion phase [113]. There is also direct evidence from NMR studies that pyruvate causes a decrease in $pH_i$ in a low-flow model of ischaemia [114].

We have used the DOG technique to investigate whether the protection offered by pyruvate is associated by inhibition of the MPT [113]. Data summarised in Fig. 5 confirm that significantly less pore opening does occur on reperfusion when 10 mM pyruvate is present both before ischaemia and during reperfusion. Under these conditions hearts recover 100% of their LVDP after 40 min ischaemia, compared to only about 50% in the absence of pyruvate.

Furthermore, when the post-loading technique was used to determine the extent of pore closure after hearts had recovered their maximum LVDP, DOG entrapment had returned to pre-ischaemic values. Thus total functional recovery of the heart was accompanied by total reversal of the MPT, whereas 50% recovery was associated with about 50% decrease in DOG entrapment and thus 50% reversal of the MPT. These data are the first direct evidence that mitochondrial pore opening can reverse when hearts damaged by reperfusion recover. We cannot exclude the possibility that mitochondrial pore opening is secondary to irreversible cell damage, rather than a primary cause of it. However, as outlined above, breakdown of the plasma membrane permeability barrier cannot be the cause since if this were to occur, DOG would be lost from the cells and not trapped by the mitochondria.

## 6.5. Propofol

Propofol is an anaesthetic that is frequently used during cardiac surgery and in post-operative sedation [115]. There are reports that propofol can act as a free radical scavenger [116,117] and also that at concentrations higher than used clinically, it may inhibit the permeability transition of isolated mitochondria [118,119]. One study has suggested that propofol can attenuate the effects of hydrogen peroxide induced oxidative stress in the perfused heart [120]. Thus we have studied the effects of propofol on recovery of hearts from ischaemia. When added 10 min prior to ischaemia and during reperfusion at 2 µg/ml, a concentration similar to that employed clinically, significant protection of hearts was observed. Thus recovery of LVDP after 30 min ischaemia (means ± S.E.M.) increased from $36 \pm 8\%$ ($n = 10$) in control hearts to $70 \pm 11\%$ ($n = 8$; $P < 0.05$) in propofol-treated hearts. This was accompanied by a significant decrease in mitochondrial DOG entrapment from $22 \pm 2$ to $17 \pm 1$ DOG units ($P < 0.05$). When added to isolated heart mitochondria at the same concentration, no inhibition of the MPT was observed. Thus the protective effect of propofol may not be through a direct effect on the MPT, but through other mechanisms such as its free radical scavenging properties causing a decrease in oxidative stress or reported inhibitory effects on calcium channels [121,

122] leading to a reduction in calcium overload. Nevertheless, propofol provides another example of a reagent whose protection of the heart from reperfusion injury is accompanied by a decrease in mitochondrial pore opening in vivo.

## 6.6. Preconditioning

One of the most effective means of protecting ischaemic hearts from reperfusion injury is by subjecting them to two or three brief (3–5 min) ischaemic periods with intervening recovery periods before the prolonged period of ischaemia is initiated. This process is called preconditioning and is associated with an immediate protection which is reduced over a period of hours, but is followed about 24 h later by a second window of protection [123,124]. The precise mechanism by which preconditioning exerts its protective effects is not known, but it seems likely that several processes are involved. The second window of protection probably involves stress activated protein kinase pathways [125,126], whilst in the short term protection mediators such as adenosine, bradykinin, endothelin 1, opioids, and catecholamines released during the brief ischaemic periods may cause receptor-mediated activation of protein kinase C [123,127–129]. Depletion of glycogen during the brief ischaemic periods during preconditioning has also been proposed as an important factor since this will result in less glycogen breakdown during the prolonged ischaemic period. This in turn will lead to less lactic acid production, less $Na^+/H^+$ exchange, less accumulation of intracellular $Na^+$ and hence less increase in intracellular $[Ca^{2+}]$ [130–132]. However, as outlined above, low $pH_i$ is protective and thus this mechanism will only work if the effects of lower $[Ca^{2+}]$ outweigh those of a smaller decrease in $pH_i$. There is also evidence for $K_{ATP}$ channel activation being involved in the mechanism of preconditioning, since the effects are blocked by sulphonylureas, which are potent inhibitors of the ATP-$K^+$ channel [133–135]. Another mechanism proposed for preconditioning is that the mitochondrial ATPase inhibitor protein is activated during the brief ischaemic periods [136,137]. This would ensure that those mitochondria that have undergone the MPT break down less of the ATP generated by glycolysis and by the remaining 'closed' mitochondria. Although controversial [137], such a

mechanism would enable hearts to remain protected from reperfusion injury even when a significant number of mitochondria remained in an 'open' state. Thus it is of interest that, although there are reports that mitochondrial damage is reduced in preconditioned hearts [138], we have been unable to detect any decrease in mitochondrial pore opening determined using the DOG entrapment technique (P.M. Kerr and A.P. Halestrap, unpublished data).

## 7. The mitochondrial permeability transition and apoptosis in the heart

It is now recognised that in the failing heart and hearts damaged by reperfusion injury some cells undergo apoptotic cell death as opposed to necrosis. This is particularly pronounced in areas surrounding a myocardial infarct, i.e. areas that experience a less pronounced ischaemic insult than that which leads to necrosis [139–145]. There is recent evidence that the MPT may act as the 'central executioner' of cells subjected to a range of insults, such as oxidative stress, growth factor removal or exposure to cytokines. Indeed, the mitochondria may not determine only whether cells live or die, but also whether death occurs by apoptosis or necrosis [146,147]. Thus, in some cells changes in $\Delta\psi$ occur during early stages of apoptosis and can be inhibited by CsA which also inhibits apoptosis [148,149]. Furthermore, mitochondria are required to induce apoptosis in a cell free system and appear to do this by release of an apoptosis inducing factor which is most probably cytochrome *c* [150–152]. The anti-apoptotic gene product, bcl-2, is associated with the mitochondrial outer membrane and has been reported to inhibit the MPT and prevent release of cytochrome *c* and consequent caspase activation [153–155].

Attractive though this hypothesis may be, there are cell types that demonstrate apoptosis without early changes in $\Delta\psi$ or inhibition by CsA [153,156–158]. Nor does CsA protect cell types from all apoptotic stimuli and may even induce apoptosis under some circumstances [156,159–161]. However, it does seem likely that cells experiencing only a modest insult may undergo a transient opening of the MPT pore which then closes again, enabling ion gradients and ATP production to be re-established. Under such conditions, sufficient swelling of the outer membrane may occur to release cytochrome *c* and set in motion the apoptotic cascade that causes an organised, non-inflammatory cell death by apoptosis. The controlled nature of apoptosis requires that tissue ATP content is maintained, and where this is not the case cell death becomes necrotic [162,163]. Such a situation occurs when a cell experiences a sufficient insult to cause MPT pore opening that is both extensive and prolonged. Not only is cytochrome *c* released, but mitochondria remain uncoupled and so unable to generate the ATP required for maintaining cellular ionic homeostasis and repairing tissue damage. Under these conditions damage continues unabated leading ultimately to rupture of the plasma membrane and cell death. This uncontrolled necrotic form of cell death is inflammatory and further exacerbated as neutrophil invasion leads to yet more damage. Thus the decision between apoptosis and necrosis may rest on the extent of the MPT and can account for the observation that apoptosis and necrosis both occur in the reperfused heart, with the least damaged areas showing a preponderance of apoptosis over necrosis.

## Acknowledgements

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