Cytochrome *c* release from isolated rat liver mitochondria can occur independently of outer-membrane rupture: possible role of contact sites

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Percoll-purified rat liver mitochondria were shown to contain BAX dimer and rapidly (< 2 min) release 5–10% of their cytochrome *c* when incubated in a standard KCl incubation medium under energized conditions. This release was not accompanied by release of adenylate kinase (AK), another intermembrane protein, and was not inhibited by Mg²⁺, dATP, inhibitors of the permeability transition or ligands of the peripheral benzodiazepine receptor. However, release was greatly reduced by the presence of 5% (w/v) dextran (40 kDa), which caused a decrease in the light scattering (*A*₅₂₀) of mitochondrial suspensions. Dextran also inhibited both mitochondrial oxidation of exogenous ferrocytochrome *c* in the presence of rotenone and antimycin, and respiratory-chain-driven reduction of exogenous ferricytochrome *c*. Hypo-osmotic medium or digitonin treatment of mitochondria caused a large additional

release of both cytochrome *c* and AK that was not blocked by dextran. Polyaspartate, which stabilizes the low conductance state of the voltage-dependent anion channel (VDAC), increased cytochrome *c* release. VDAC and BAX are both found at the contact sites between the inner and outer membranes and dextran is known to stabilize these contact sites in isolated mitochondria. Thus our data suggest that regulation of a specific permeability pathway for cytochrome *c* may be mediated by changes in protein–protein interactions within contact sites. The adenine nucleotide translocase is known to bind to VDAC and thus provides an additional link between the specific cytochrome *c* release pathway and the permeability transition.

Key words: adenylate kinase, apoptosis, BAX, mitochondrial permeability transition, VDAC.

INTRODUCTION

Mitochondria are now known to play a central role in both necrotic and apoptotic cell death (see [1–3]). In necrosis, the opening of the mitochondrial permeability transition (MPT) pore (MPTP) may well be the point of no return for the cell, since once mitochondria have undergone the permeability transition they become uncoupled. Not only are they unable to synthesize ATP, they actively hydrolyse it, thus preventing glycolysis from maintaining ATP supplies [3]. Since ATP is required to maintain cellular integrity and repair damage, the cell can no longer control its fate and dies by necrosis. In contrast, apoptosis is a controlled form of cell death that requires maintenance of cellular ATP and mitochondrial function, at least in the early stages [4]. The role of the mitochondria here appears to be in the release of cytochrome *c*, and probably other apoptosis-inducing factors, which initiate or amplify the apoptotic cascade [1,5]. In the presence of dATP, the released cytochrome *c* binds with APAF-1 (apoptosis-activating factor 1) and pro-caspase 9 to form a complex that leads to the proteolytic activation of pro-caspase 9 to active caspase 9. This then cleaves pro-caspase 3 to caspase 3, which directly or indirectly activates a range of enzymes critical for the rearrangement of the nucleus, cytoskeleton and plasma membrane that are characteristic of apoptosis [1,2].

The release of cytochrome *c* involves the pro-apoptotic members of the Bcl-2 family, such as BAX, BAD and BID, whose effects are antagonized by the anti-apoptotic members of the family such as Bcl-2 and Bcl-X_L [1,2]. It is unclear how these proteins interact in order to maintain the required balance between cell survival and proliferation on the one hand and apoptosis on the other. Proteolytic modifications and phos-

phorylations of the different Bcl-2 family members have been suggested to provide the link between cell-surface receptors and stimulation of cytochrome *c* release (see [2]). In particular, activation of members of the tumour-necrosis-factor receptor family leads to death-domain-dependent activation of caspase 8 and it has recently been shown that caspase 8 can proteolytically cleave a BID precursor to form active BID, which migrates to the mitochondria where it interacts with BAX to cause cytochrome *c* release. This then activates caspase 9 and hence caspase 3, which then sets in motion the apoptotic cascade [2]. What is still unresolved is the mechanism by which BID in association with BAX causes the release of cytochrome *c* from the intermembrane space. Two possibilities have been proposed. One is outer-membrane rupture caused by either the MPT or some other mechanism such as increased mitochondrial potassium uptake [6,7]. The second involves BAX, alone or in combination with other proteins, forming a specific pathway (channel) for cytochrome *c* transport [8–10]. The former mechanism would be associated with release of other intermembrane proteins such as adenylate kinase (AK), and there are data to suggest that for some toxins that induce apoptosis, such as etoposide and staurosporin, this may be the case [7,11]. However, breakage of the outer mitochondrial membrane is not a very precise mechanism for regulating a sophisticated process such as apoptosis and a more specific cytochrome *c* release mechanism might be expected. Indeed, it has been shown that cytochrome *c* release can occur without any sign of mitochondrial swelling and outer-membrane breakage; rather mitochondrial shrinkage may be observed [12–14].

In this paper we investigate the release of cytochrome *c* by a well-characterized liver mitochondrial preparation and dem-

Abbreviations used: ANT, adenine nucleotide translocase; AK, adenylate kinase; CsA, cyclosporin A; MPT, mitochondrial permeability transition; MPTP, MPT pore; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; VDAC, voltage-dependent anion channel.

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onstrate that cytochrome *c* can be released rapidly without the concomitant loss of AK. This process is reduced greatly in the presence of dextran, which maintains inner- and outer-membrane interactions at contact sites, and is enhanced by polyaspartate, which binds to the voltage-dependent anion channel (VDAC) and favours its low conductance state. Since Bcl-2 and VDAC are associated with contact sites, these data suggest that a specific permeability pathway for cytochrome *c* can open when contact sites are disrupted.

MATERIALS AND METHODS

Mitochondria

Male Wistar rats (225–250 g in weight and allowed free access to food and water) were used for the preparation of liver mitochondria as described previously [15]. The isolation medium was 300 mM sucrose/2 mM EGTA/10 mM Tris/HCl/0.5% BSA, pH 7.4, and the mitochondria were subjected to Percoll-gradient centrifugation to remove microsomal and plasma-membrane contamination. Mitochondria were washed in media without added BSA and stored on ice in this medium at about 30 mg of protein/ml. Unless otherwise stated, all subsequent experiments were performed within 2 h of isolation. Mitoplasts were prepared from mitochondria by removal of the outer membrane by digitonin treatment [16].

Chemicals

Leupeptin, antipain, diazepam, protoporphyrin IX and poly-L-aspartate were from Sigma-Aldrich (Poole, Dorset, U.K.), Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone) was from Calbiochem-Novabiochem UK (Nottingham, U.K.) and Dextran FP40 (pyrogen-free) and all enzymes and cofactors were from Boehringer Mannheim UK (Lewes, East Sussex, U.K.). Purified mouse monoclonal antibodies against cytochrome *c* and BAX were obtained from PharMingen International (Beckton Dickinson UK, Oxford, U.K.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) respectively. All other chemicals were of the highest purity commercially available.

Determination of cytochrome *c* and AK release from mitochondria

The standard incubation medium (pH 7.2) contained 25 mM KCl, 20 mM Mops, 10 mM Tris, 2 mM EGTA, 0.2 μ M rotenone, 1 mM succinate and either sucrose or additional KCl to give the desired osmolarity. For iso-osmotic media (300 mosmol/l), additional KCl (100 mM) or sucrose (200 mM) were added. Mitochondria (1 mg of protein) were incubated in 1 ml of medium for 1 min at 25 °C in the presence or absence of 5% (w/v) dextran (40 kDa) and other additions as indicated. After incubation mitochondria were placed on ice and then pelleted by centrifugation. The supernatant and pellet fractions were subjected to SDS/PAGE and analysed by Western blotting using purified mouse anti-cytochrome *c* monoclonal antibody with enhanced chemiluminescence detection. Quantification was by densitometry using a commercial scanner and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Various film exposures were employed to ensure determinations were made under conditions in which the response of the film was linear with respect to loading. AK activity in the same samples (20 μ l) was determined spectrophotometrically at 25 °C in 1 ml of medium (pH 7.5) containing 130 mM KCl, 6 mM MgSO₄, 100 mM Tris/HCl, 0.2 mM NADH, 0.5 mM ATP, 0.5 mM phosphoenolpyruvate, 0.5 μ M rotenone, 7.5 μ M oligomycin, 20 units/ml pyruvate kinase and 20 units/ml lactate dehydrogenase.

The reaction was started by adding 0.75 mM AMP and followed by the decrease in A_{340} .

Polarographic and spectrophotometric determination of cytochrome *c* permeation across the outer membrane

NADH-cytochrome *b₅*-dependent oxidation of exogenous cytochrome *c* was determined polarographically using a Clarke-type oxygen electrode at 30 °C. Mitochondria (4 mg of protein) were suspended in 1 ml of standard KCl or sucrose incubation medium without succinate but supplemented with 0.25 mM NADH, 0.5 μ M antimycin A and, when required, 5% (w/v) dextran (40 kDa). After a basal rate of oxygen consumption was obtained (1 min), 10 μ M cytochrome *c* was added and the new rate determined. For the spectrophotometric determination of exogenous cytochrome *c* reduction, mitochondria (2 mg of protein) were suspended in 7 ml of iso-osmotic KCl incubation medium without succinate but supplemented with 6 μ M cytochrome *c* and, when required, 5% (w/v) dextran (40 kDa). The suspension was divided between two cuvettes and incubated at 25 °C in a split-beam spectrophotometer with continuous monitoring of A_{550} . At the required time 1 mM succinate, 1 mM KCN and 0.12 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) were added to the sample cuvette only.

Measurement of the permeability transition and changes in mitochondrial conformation

Mitochondria (1 mg of protein) were incubated at 25 °C in 3.5 ml of standard iso-osmotic KCl medium but containing only 0.5 mM EGTA, which was absent for MPT assays, and other additions as required. Light scattering (A_{520}) was used to monitor changes in mitochondrial volume or conformation as described previously [15].

Protein assay

Protein was determined by the Bradford method using BSA as a standard.

Statistics

Results are expressed as means $\pm$ S.E.M. and statistical significance calculated using a two-tailed Student's *t* test.

RESULTS

In the absence of a colloidal osmotic support, isolated mitochondria rapidly release cytochrome *c*

Isolated rat liver mitochondria were incubated at 25 °C under energized conditions in a standard iso-osmotic medium containing physiological concentrations of K⁺ (125 mM KCl) designed to mimic conditions *in vivo*. Following the required period of incubation the release of cytochrome *c* was determined by sedimentation of the mitochondria and analysis of the cytochrome *c* content of the supernatant and pellet by SDS/PAGE and Western blotting. In Figure 1(A) we show that after 2 min in KCl medium (300 mosmol/l) about 5–6% of the total mitochondrial cytochrome *c* was detectable in the supernatant. This increased to about 20% in hypotonic medium (50 mosmol/l), which would be expected to cause mitochondrial swelling and outer-membrane rupture. In Figure 1(B) we show that a similar amount of cytochrome *c* was released by disruption of the outer membrane by digitonin treatment. In these experiments the release of AK was determined enzymically and this

Figure 1 Release of cytochrome *c* by isolated rat liver mitochondria incubated in KCl medium

Mitochondria (1 h post-isolation) were incubated at 25 °C in KCl medium for the times indicated before sedimentation of mitochondria by centrifugation, as described in the Materials and methods section. Where indicated the medium also contained 0.12 mg/ml digitonin (DIG) and 5% (w/v) dextran (40 kDa). The cytochrome *c* content of the supernatant (S) and (in **A**) mitochondrial pellet (P) were determined by Western blotting following SDS/PAGE. The percentages shown below (**A**) represent the percentage of the total mitochondrial cytochrome *c* found in the pellet and supernatant fractions. In (**D**) cytosol (post-mitochondrial supernatant clarified by centrifugation at 100 000 *g* for 15 min) and mitochondria (15 µg of protein per track) were separated by SDS/PAGE and the presence of BAX revealed by Western blotting.

also was increased substantially by both hypo-osmotic and digitonin treatments. These experiments also suggested that relatively more cytochrome *c* was released in normal KCl medium than was AK. In Figure 1(C) we show the time course of cytochrome *c* appearance in the supernatant, which was found to be maximal within 2 min and thereafter declined. This decline might have been due to proteolysis, although in three separate experiments addition of the proteinase inhibitors leupeptin and

antipain (both at 4 µg/ml) to the incubation medium gave little reduction in the loss of cytochrome *c*.

Cytochrome *c* release is not accompanied by outer-membrane rupture

The rapid release of mitochondrial cytochrome *c* could reflect a specific release mechanism involving pro-apoptotic members of the Bcl-2 family known to stimulate cytochrome *c* release such as BAX. Indeed, using anti-BAX antibodies we were able to demonstrate the presence of the dimeric (active) form of the protein (46 kDa) in mitochondria, as illustrated in Figure 1(D). Relatively little of the monomeric form of BAX (23 kDa) was detected in mitochondria, although this form of BAX was present in the cytosol. The stability of the dimeric form of BAX during SDS/PAGE despite the denaturing conditions has been observed by others [17,18] and is not uncommon for proteins with hydrophobic membrane-spanning domains [19]. However, it might appear more likely that the cytochrome *c* release we observed was due merely to the instability of the outer membrane in our incubation medium. This possibility was investigated by comparing the release of cytochrome *c* and AK from mitochondria incubated in both KCl and sucrose media of varying osmolarity. Since cytochrome *c* binding to the inner membrane is known to decrease at higher ionic strengths, the use of both media was important to discriminate between the potential effects of decreasing ionic strength and osmolarity. In addition, we also investigated the effects of 5% (w/v) 40 kDa dextran on cytochrome *c* release in order to mimic the colloidal osmotic pressure by cytosolic proteins. The media routinely employed for incubating mitochondria *in vitro* usually lack such colloidal osmotic support, which may be important to prevent osmotic swelling of the intermembrane space.

The data of Figure 1(C) demonstrate that 5% (w/v) dextran (40 kDa) does indeed cause a substantial inhibition of cytochrome *c* release when mitochondria were incubated in KCl (125 mM) medium. However, the data of Figure 2(A) and Table 1 suggest that this effect does not merely reflect a reduction in the swelling of the intermembrane space and outer-membrane rupture since the release of AK remains unaffected by the presence of dextran. In contrast, when mitochondria were swollen by progressively decreasing the osmolarity of the incubation medium, AK was released, whether or not dextran was present, and was accompanied by additional release of cytochrome *c* (Figure 2). As noted in Figure 1(B), similar data were obtained with digitonin treatment, which specifically permeabilizes the outer membrane. Very much less cytochrome *c* release was observed when mitochondria were incubated in iso-osmotic medium containing only 25 mM KCl (with sucrose providing the remainder of the osmotic support), although AK release was similar to that observed in KCl medium (see Figure 2B). However, extensive release of both cytochrome *c* and AK was still obtained in this medium when swelling was induced by reducing the sucrose concentration. These data imply that the greater release of cytochrome *c* observed in iso-osmotic KCl medium than sucrose medium does not merely reflect less tight binding of cytochrome *c* to the inner membrane. The release of cytochrome *c* from mitochondria was slightly greater when mitochondria were stored on ice for longer periods. Thus after 2 h on ice, subsequent incubation of mitochondria for 2 min in KCl medium caused a $58 \pm 12\%$ (mean $\pm$ S.E.M. of nine different mitochondrial preparations) greater increase in cytochrome *c* release than after 1 h on ice. However, AK release was also increased by $31 \pm 7\%$ under the same conditions, suggesting that some damage to the outer membrane may also be occurring with time. The ability of

Figure 2 The effect of osmolarity on cytochrome *c* and AK release from isolated mitochondria in the presence or absence of dextran

Isolated mitochondria (1 mg of protein) were incubated for 1 min at 25 °C in standard medium containing 25mM KCl (see the Materials and methods section) in the presence or absence of 5% (w/v) dextran and increasing amounts of KCl (**A**) or sucrose (**B**) to vary the osmolarity as indicated. After incubation mitochondria were sedimented by centrifugation and cytochrome *c* content and AK activity of supernatants were assayed by Western blotting and enzymic assay respectively. The blots were quantified by scanning and band intensities expressed as a percentage of the value determined for 50 (**A**) or 100 mosM (**B**) medium without added dextran. Data for the AK activity determined enzymically are expressed similarly. The experiment shown is representative of two experiments showing similar results.

Table 1 Effect of dextran and other factors on the release of cytochrome *c* and AK from isolated rat liver mitochondria

Mitochondria were stored on ice for 1 or 2 h after isolation before incubation for 2 min at 25 °C in KCl medium with additions as indicated. After sedimentation of mitochondria by centrifugation the cytochrome *c* and AK contents of the supernatant were measured as described in the Materials and methods section. Data are expressed as a percentage of the corresponding control and as means $\pm$ S.E.M. for three separate mitochondrial preparations for dextran, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and cyclosporin A, and for four preparations for polyaspartate (37 kDa). The release of cytochrome *c* and AK 2 h after isolation were $158 \pm 12\%$ and $131 \pm 7\%$ of that at 1 h post-isolation, respectively ($n = 9$). Statistically significant differences are indicated as follows: * $P < 0.05$, ** $P < 0.001$.

Time post-isolation...	Cytochrome <i>c</i>		AK	
	1 h	2 h	1 h	2 h
Control	100	100	100	100
5% Dextran	65 $\pm$ 5*	34 $\pm$ 1**	94 $\pm$ 8	80 $\pm$ 9
54 μ M Polyaspartate	318 $\pm$ 134	172 $\pm$ 17*	124 $\pm$ 13	110 $\pm$ 8
3 μ M FCCP	174 $\pm$ 23	108 $\pm$ 23	117 $\pm$ 4	117 $\pm$ 11
0.2 μ M Cyclosporin A	131 $\pm$ 15	102 $\pm$ 12	100 $\pm$ 1	105 $\pm$ 3

Figure 3 Dextran inhibits oxidation of exogenous cytochrome *c* by mitochondria in KCl medium

NADH-cytochrome *b₅*-dependent oxidation of exogenous cytochrome *c* was determined polarographically using a Clarke-type oxygen electrode at 30 °C as described in the Materials and methods section. Mitochondria (4 mg of protein) were suspended in 1 ml of KCl or sucrose medium containing 0.25 mM NADH, 0.5 μ M antimycin A and, where indicated, 5% (w/v) dextran (40 kDa). Cytochrome *c* (10 μ M) was added where indicated. Rates of oxygen consumption in nmol of O/min per mg of protein are given alongside each trace. Data are representative of several similar experiments whose results are summarized in the text.

dextran to inhibit cytochrome *c* release was unaffected by storage on ice (see Table 1).

Cytochrome *c* can move in both directions across the mitochondrial outer membrane

In order to establish whether the release of cytochrome *c* from mitochondria was a bidirectional process involving a pore, rather than a unidirectional transport process, we studied the respiratory-chain-dependent oxidation of externally added ferrocytochrome *c*, as shown in Figure 3. In these experiments endogenous substrate oxidation is prevented by the presence of rotenone and antimycin and added oxidized cytochrome *c* is reduced to ferrocytochrome *c* through the action of NADH-cytochrome *b₅* reductase on the outer-mitochondrial membrane [20]. In sucrose medium, cytochrome *c*-dependent oxidation of NADH consumed oxygen at the rate of about 12 nmol of O atoms/min per mg of protein whether or not dextran was present. However, in KCl medium without added dextran, the rate of oxygen uptake was about 50% greater but was decreased significantly by the presence of dextran. In the experiment shown involving mitochondria isolated from two rats, four separate determinations of oxygen uptake in the presence and absence of dextran were made over a 4 h period. No significant changes in the inhibition caused by dextran were observed over this time period, during which the mitochondria were stored at 0 °C. The mean inhibition ($\pm$ S.E.M.) caused by dextran was $22 \pm 4.5\%$. With a second mitochondrial preparation the inhibition caused by dextran was 26% after 1 h at 0 °C and 32% after 5 h. These data suggest that the NADH/cytochrome *c*-dependent oxygen uptake observed in sucrose medium represents the contribution by mitochondria with ruptured outer membranes that allow cytochrome *c* free access to cytochrome oxidase. The additional oxidation observed in KCl medium that is largely prevented by dextran correlates with the additional release of cytochrome *c*

Figure 4 The effects of dextran and outer-membrane removal on the succinate-driven reduction of extramitochondrial cytochrome *c*

Mitochondria or mitoplasts (1 mg of protein/ml) were incubated in a split-beam spectrophotometer at 25 °C in standard KCl buffer (see the Materials and methods section) containing 6 μ M cytochrome *c* and, when required, 5% (w/v) dextran, but no succinate. A_{550} was monitored continuously, and additions of 1 mM succinate, 1 mM KCN and 0.15 mM TMPD were made as indicated. The data shown are typical of three separate mitochondrial preparations for which 5% dextran inhibited the rate of cyanide-induced cytochrome *c* reduction by $23 \pm 1.7\%$ (mean $\pm$ S.E.M., $P < 0.01$).

(but not AK) observed in this medium (Figure 2) and appears to reflect a specific dextran-sensitive cytochrome *c*-transport mechanism across the outer membrane.

This conclusion was further strengthened by studying the reduction of exogenous ferricytochrome *c* by electrons donated from the cyanide-inhibited respiratory chain. Under these conditions, electrons from succinate oxidation are passed to complex III, which then donates electrons to exogenous cytochrome *c*. This is only possible if the outer membrane is broken or if there is a bidirectional cytochrome *c* transport mechanism. It may also be mediated by addition of TMPD, which is lipid soluble and hence can shuttle electrons across the outer membrane. The data of Figure 4 show that reduction of external cytochrome *c* can occur under such conditions, but is greatly stimulated by the addition of 0.15 mM TMPD or when the outer membrane is removed by digitonin treatment. The rate of cytochrome *c* reduction seen in the absence of TMPD could reflect only the small fraction of mitochondria with damaged outer membranes. However, addition of 5% dextran caused a $23 \pm 1.7\%$ inhibition in the rate of cytochrome *c* reduction (mean $\pm$ S.E.M. of three separate mitochondrial preparations; $P < 0.01$), implying that some of the cytochrome *c* was being reduced through the operation of the same mechanism that caused the cytochrome *c* release. The similarity of this dextran-mediated inhibition to that observed on NADH/cytochrome *c* oxidation described above increases confidence that this is the correct interpretation. Using these two methods we were able to screen a variety of potential effectors of the cytochrome *c* permeability pathway. No effects of ATP, ADP (+ oligomycin), dATP, Mg^{2+} , bongkreikic acid or cyclosporin A (CsA) were observed.

VDAC but not the peripheral benzodiazepine receptor or MPTP may be involved in cytochrome *c* release

It has been suggested that the MPT may be involved in cytochrome *c* release, but this should be calcium-dependent, whereas we observe cytochrome *c* release in the presence of EGTA. Furthermore, we have been unable to detect any effect of CsA on the release of cytochrome *c* (Table 1). Nor was dextran able to inhibit the rate of the light-scattering change induced by

Figure 5 The effects of 5% dextran on the MPT of isolated liver mitochondria

Mitochondria were incubated under energized conditions in a split-beam spectrophotometer before addition of $50 \mu\text{M}$ Ca^{2+} at the time shown. Further details are given in the Materials and methods section. The experiment shown is typical of three similar experiments on separate mitochondrial preparations.

Figure 6 Effect of polyaspartate on mitochondrial cytochrome *c* release

After isolation (1 or 2 h), mitochondria were incubated in the presence or absence of $54 \mu\text{M}$ polyaspartate (PAA; 37 kDa) and pelleted by centrifugation as described in the Materials and methods section. The cytochrome *c* content of the supernatant was determined by Western blotting following SDS/PAGE. The data shown are typical of three independent experiments for which quantitative analysis is given in Table 1.

calcium overload under energized conditions (Figure 5), which suggests that the MPT is not inhibited by dextran. This is not surprising since the MPT involves a change in the inner- rather than outer-membrane permeability, most probably mediated by a calcium-triggered, cyclophilin-dependent conformational change of the adenine nucleotide translocase (ANT) [21,22]. However, it has been proposed that the ANT may interact with VDAC and possibly the peripheral benzodiazepine receptor in the outer membrane [23,24]. We have been unable to detect any effect of a range of concentrations of either protoporphyrin IX or diazepam (both ligands of the benzodiazepine receptor) on cytochrome *c* release (results not shown). This suggests that the benzodiazepine receptor is not involved in the release mechanism. Nor did de-energization of mitochondria with uncoupler ($3 \mu\text{M}$ carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, FCCP) have a significant effect on cytochrome *c* release (Table 1). However, the addition of $54 \mu\text{M}$ polyaspartate (37 kDa), which causes VDAC to take up its low conductance state for anions [25], caused a significant increase in cytochrome *c* release (Figure 6 and Table 1).

Figure 7 The effects of 5% dextran on the conformational changes induced in isolated energized liver mitochondria by sub-micromolar $[\text{Ca}^{2+}]$

The experimental protocol was as described in the Materials and methods section. After 1 min of incubation in the spectrophotometer, CaCl_2 was added to the sample cuvette alone to give the calculated free $[\text{Ca}^{2+}]$ indicated [27]. The experiment shown is typical of three similar experiments on separate mitochondrial preparations.

The potential role of contact sites in cytochrome *c* release

The inner and outer mitochondrial membranes are closely apposed at various points known as contact sites, which are believed to be associated with an interaction between the ANT and VDAC. Dextran has been shown to increase the number of such contact sites in isolated mitochondria [26], and thus the ability of dextran to inhibit cytochrome *c* release is consistent with their disruption being associated with the unmasking of a cytochrome *c*-release pathway. One potential indicator of the effect of dextran on the contact sites may be the light scattering of the mitochondrial suspension. Indeed, we observed that the initial light scattering of liver mitochondria suspended in energized KCl medium was significantly less in the presence of 5% dextran, the A_{520} values at 1 and 2 h post-isolation (as means $\pm$ S.E.M. of four separate preparations) being $74.3 \pm 7.6\%$ ($P < 0.05$) and $64.6 \pm 9.0\%$ ($P < 0.02$) of control values respectively. Furthermore, although dextran had no effect on the rate of the light-scattering change occurring during the MPT induced by calcium overload, the maximum extent of the light-scattering change observed was diminished (Figure 5). These data are consistent with the formation of contact sites changing the conformation of mitochondria and leading to a decrease in their light scattering.

During the course of the latter experiments we observed that, in the presence of dextran, the exposure of energized mitochondria to sub-micromolar concentrations of Ca^{2+} (buffered with EGTA) caused a rapid decrease in light scattering, which was barely visible in the absence of dextran (Figure 7). The effect, which we have observed previously in heart mitochondria [15,27], was not inhibited by CsA and was quite distinct from the MPT, which required much greater loading of mitochondria with Ca^{2+} . Calcium has been demonstrated to increase contact-site formation in the perfused heart [28] and thus these data are

also consistent with decreased light scattering detecting a conformational change that reflects greater contact-site formation.

DISCUSSION

The data we present in this paper demonstrate that Percoll-purified isolated rat liver mitochondria contain BAX dimer and can release cytochrome *c* independently of AK, and thus by implication without outer-membrane rupture. The ability of dextran to inhibit this process may reflect its ability to maintain the structure of contact sites between the inner and outer membrane [26]. Such contact sites are thought to involve an interaction between the ANT and VDAC that allows the communication between the inner and outer membranes [29–31] and our data suggest that their disruption may unmask a latent pathway for cytochrome *c* release. In this context it may be significant that both BAX and Bcl-2 have been shown to be concentrated at the contact sites [32,33]. Evidence for a role of VDAC in the cytochrome *c*-release mechanism comes from the ability of polyaspartate to enhance cytochrome *c* release. Polyaspartate binds to VDAC and induces the low anion-conductance state [25], and it is known that when VDAC changes from the high- to the low-conductance conformation there is a large-scale rearrangement of protein domains [34]. Thus it is possible that the low-conductance conformation of VDAC may activate a cytochrome *c*-release pathway by some protein–protein interaction within the contact sites. A role for the low-conductance conformation of VDAC in cytochrome *c* release is supported by the observation that apoptosis induced by growth-factor withdrawal is associated with impaired ATP/ADP exchange into mitochondria [35]. The low anion-conductance state of VDAC is known to greatly inhibit ATP/ADP transport into mitochondria [36]. In contrast, Bcl-X_L over-expression facilitates mitochondrial ATP/ADP exchange and inhibits cytochrome *c* release [35], suggesting that it stabilizes the high-conductance state of VDAC associated with low cytochrome *c* permeability. In contrast, it would be envisaged that BAX might interact with VDAC to favour the low-conductance conformation and so enhance cytochrome *c* release. It may also be significant that VDAC possesses a tight binding site for cytochrome *c* on its intermembrane surface [34].

Recently Shimizu et al. [10] have reported that when VDAC is reconstituted into proteoliposomes, the addition of either BAX or BAK enables permeation by cytochrome *c*, which was blocked by Bcl-X_L. VDAC alone did not allow cytochrome *c* permeation, suggesting that BAX and BAK cause a conformational change in VDAC that allows it to transport cytochrome *c*, while Bcl-X_L antagonizes this effect. At first glance, our data might appear compatible with this hypothesis, if it is assumed that a VDAC interaction with the ANT at contact sites might prevent the cytochrome *c* translocation. However, closer examination of the data of Shimizu et al. [10] reveals discrepancies between their data and those of others, including ourselves. First, Shimizu et al. suggest that it is the high-conductance conformation of VDAC that allows cytochrome *c* permeation, yet our own data and those of Vander Heiden et al. [35] suggest that cytochrome *c* release and apoptosis are associated with the low-conductance conformation of VDAC. Second, Priault et al. [37] have demonstrated that BAX can induce cytochrome *c* release in VDAC-deficient yeast. Shimizu et al. used proteoliposomes containing VDAC reconstituted from Triton-solubilized protein to demonstrate cytochrome *c* release. Since even very low concentrations of Triton X-100 can exert profound effects on both the inhibitor sensitivity of VDAC [38] and on the general permeability of

liposomes [39], results obtained in this way must be treated with caution.

If VDAC present at the contact sites does participate directly or indirectly in the cytochrome *c*-release mechanism, there is the possibility that the conformation of the MPTP in the inner membrane may communicate directly with the cytochrome *c*-release mechanism independently of outer-membrane rupture. The MPTP is thought to be associated with contact sites and to be formed by a conformational change in the ANT, possibly in association with VDAC [1,2,15,40,41]. This may provide an explanation of how Bcl-2 may inhibit both the specific (MPTP-independent) cytochrome *c*-release mechanism and the MPTP-dependent mechanism. The latter is most likely to operate under conditions of oxidative stress and calcium overload, which are known to stimulate the opening of the MPTP. Examples include reperfusion of tissues following a period of ischaemia or the addition of chemical toxins where MPTP opening has been shown directly and treatment with CsA or over-expression of Bcl-2 may offer protection (see [2,41–43]). However, cell death is often necrotic under such circumstances unless MPTP opening is transient (see for example [44,45]), since mitochondria will be uncoupled and not maintain sufficient ATP concentrations to support apoptosis [2,4,41].

Our results also suggest the presence of a mechanism for the removal of released cytochrome *c* (Figure 1C). The mechanism of this is unknown but it was insensitive to standard protease inhibitors. If such a mechanism also exists within the cell, it provides a means by which apoptosis could be prevented or reversed if the apoptotic signal is weak or transient. This would ensure that cells do not enter apoptosis too readily. It might also explain how cells that have released cytochrome *c* following growth-factor withdrawal can be rescued from apoptosis by re-administration of growth factors and re-establish their normal compartmentation of cytochrome *c* within the mitochondria [13].

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