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EVALUATING CYTOCHROME C DIFFUSION IN THE INTERMEMBRANE SPACES OF MITOCHONDRIA DURING CYTOCHROME C RELEASE

Kieran Gillick and **Martin Crompton**
Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.

Summary

Truncated Bid (tBid) releases cytochrome c from mitochondria by inducing Bak (and Bax) pore formation in the outer membrane. An important issue is whether a second tBid action, independent of Bak and Bax, is also required to enhance cytochrome c mobility in the intermembrane spaces. To investigate this, we have developed a kinetic analysis enabling changes in the diffusibility of cytochrome c in the intermembrane spaces of isolated mitochondria to be differentiated from changes resulting from Bak activation. Cytochrome c diffusibility in the intermembrane spaces was unaffected by changes in [tBid] over the range 0.5 – 19 pmol / mg mitochondrial protein, when tBid-dependent Bak activation was increased several thousand fold. However, high [tBid] (100 pmol / mg) did increase diffusibility about two-fold. This was attributable to the Permeability Transition. The basal cytochrome c diffusibility in the absence of tBid was determined to be about 0.2 min^{-1}, sufficient to support cytochrome c release with a half time of 3.4 min. It is concluded that tBid has a monofunctional action at low concentrations and, more generally, that the basal cytochrome c diffusibility in the intermembrane spaces is adequate for rapid and complete cytochrome c release irrespective of the mode of outer membrane permeabilisation.

Keywords

Bid; Bak; cytochrome c; diffusion; mitochondria; apoptosis

Introduction

Mitochondria play a key role in apoptosis by releasing cytochrome c and other apoptogenic proteins to the cytosol (including Apoptosis Inducing Factor, endonuclease G, Omi and Smac/Diablo). These proteins are normally retained in the mitochondrial intermembrane spaces, but pass into the cytosol on outer membrane permeabilization (reviewed in Martinou and Green, 2001). In the cytosol, cytochrome c forms a complex (apoptosome) with Apaf-1 and procaspase-9 leading to caspase-9 activation and subsequent processing of other caspases. The release of cytochrome c and other intermembrane space proteins is controlled by members of the Bcl-2 family (reviewed in Danial and Korsmeyer, 2004). Some of these are anti-apoptotic, inhibiting permeabilization of the mitochondrial outer membrane. These contain three or four Bcl-2 homology (BH) domains (eg Bcl-2, Bcl-X_L, Mcl-l). Other Bcl-2 relatives bring about outer membrane permeabilization and are pro-apoptotic. The proapoptotics comprise proteins with multiple BH domains (ie Bax, Bak) and proteins with

Correspondence to: Prof. M Crompton, Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT Tel +44 20 7679 2207 Fax +44 20 7679 7193 m.crompton@biochemistry.ucl.ac.uk.

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only a single (BH3) domain (eg Bid, Bad, Bik, Bim). The functional interactions between single and multi BH domain proteins are complex, but becoming clearer. Bax and Bak oligomerize to form pores in the mitochondrial outer membrane large enough for the egress of cytochrome c and other intermembrane space proteins (Dejean et al., 2005; Korsmeyer et al., 2000; Kuwana et al., 2002). In order to oligomerize, Bax and Bak undergo conformational change brought about by BH3-only proteins (Desagher et al., 1999; Ruffolo and Shore, 2003). For Bak, an integral outer membrane protein, this first involves displacement of Bak from inhibitory interactions with Bcl-X_L and Mcl-l (Willis et al., 2005). The function of BH3-only Bid in this respect is greatly enhanced by caspase-8 cleavage of Bid to form truncated Bid (tBid), in which the BH3 domain is fully exposed and free to interact with Bax, Bak and antiapoptotic proteins. Thus death-receptor-activation of caspase-8 can lead via tBid and Bax or Bak to cytochrome c release from mitochondria, and both Bid (Yin et al., 1999) and Bax or Bak (Zong et al., 2001) are necessary.

In addition tBid was reported to alter the organisation of the mitochondrial inner membrane independently of its BH3 domain and of Bax and Bak, (Kim et al., 2004; Scorrano et al., 2002). The restructuring is believed to enable cytochrome c release by opening the junctions between the intracristal and intermembrane spaces, and allowing cytochrome c to diffuse from within the intracristal spaces to outer membrane Bak and Bax pores. These changes have been attributed to opening of the inner membrane Permeability Transition (PT) pore (Alirol et al., 2006; Scorrano et al, 2002). Consistent with this, release of cytochrome c from isolated mitochondria was attenuated by cyclosporin A (CSA) (Karpinich et al., 2006; Scorrano et al., 2002; Zhao et al., 2003) which inhibits cyclophilin-D, a component of the PT pore. In other studies, however, cytochrome c release was unaffected by cyclophilin-D inhibition (Brustovetsky et al., 2005; Eskes et al., 1998) or by cyclophilin-D knockout (Baines et al., 2005; Nakagawa et al., 2005). It is unclear therefore whether tBid has a secondary action in enabling cytochrome c diffusion within mitochondria and whether any such action is PT mediated.

To address this question, we need a means of measuring changes in the diffusibility of cytochrome c in the intracristal / intermembrane spaces during its release from mitochondria. Current techniques do not allow this (Discussion). Here, we describe a kinetic analysis of tBid-induced cytochrome c release from isolated mitochondria that differentiates basic steps in the process, including cytochrome c mobility in the intermembrane spaces. The analyses reveal that, although cytochrome c mobility is facilitated via the PT at high tBid concentrations, a substantial mobility exists even in the absence of tBid. This basal mobility is sufficient for rapid and complete cytochrome c release at low concentrations of tBid, when it has no effect on internal cytochrome c mobility.

Results

tBid-induced release of cytochrome c in isolated B50 cell mitochondria

Cytochrome c release was measured routinely by quantification of secondary antibody fluorescence in Western blots. Preliminary experiments established that the fluorescence signal was linear with cytochrome c over the range used (0 – 60 μg mitochondrial protein) and that incubation for up to 120 min without tBid, or Bid, caused no detectable cytochrome c release. We also confirmed that cytochrome c released from the mitochondria was quantitatively recovered in the incubation medium. Figure 1 compares the ability of tBid to release cytochrome c with respect to full length Bid. After 30 min half-maximal release was obtained with 10 nM tBid and 140 nM Bid. Thus tBid was about 14-fold more potent than Bid under these conditions, which is similar to the degree of activation on Bid cleavage reported for other cell types (generally 10- to 20-fold; Yin, 2006). The [tBid] giving half maximal release (EC_{50} value) depended on the amount of mitochondria. For example, the

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EC_{50} value increased from 3.7 nM to 22 nM as mitochondrial protein in the reaction volume (20 μl) was increased from 4 μg to 31 μg (Figure 2A), yielding a direct proportionality between the EC_{50} for tBid and mitochondrial protein (Figure 2B). This indicates that essentially all of the added tBid was bound to mitochondria (otherwise, increasing the amount of mitochondria would result in a less-than-proportional increase in EC_{50}).

tBid triggers the formation of outer membrane pores from both Bak and Bax, and tBid-induced cytochrome c efflux is believed to occur exclusively via these pores (Desagher et al., 1999; Korsmeyer et al., 2000; Kuwana et al., 2002; Ruffolo and Shore, 2003; Zong et al, 2001). Whereas Bax is cytosolic and migrates to mitochondria only during apoptosis (Capano and Crompton, 2002; Wolter et al., 1997) Bak is constitutively mitochondrial (Griffiths et al., 1999). In agreement, isolated B50 cell mitochondria contained Bak, but not Bax (Figure 3A). Moreover, cytochrome c release was completely prevented by anti-Bak antibody G-23 (Figure 3B), which blocks Bak interaction with tBid and prevents Bak-mediated cytochrome c efflux (Scorrano et al, 2002; Wei et al., 2000). Accordingly, kinetic analyses (below) of tBid-induced cytochrome c release were formulated on the basis of tBid-induced formation of Bak pores.

tBid-induced change in Bak conformation

Bak conformational change from the “closed” to the “open” form (Figure 4) was monitored by tryptic cleavage (Ruffolo and Shore, 2003). Whereas the “closed” conformation resists tryptic cleavage, a trypsin cleavage site is exposed by the conformation change, so that the “open” Bak is digested to fragments that are unreactive with the antibody used. The technique is demonstrated in Figure 5, where mitochondria were incubated with or without tBid for 30 min and then treated with varying [trypsin] on ice for a further 10 min. Only tBid-incubated samples were digested. About 50-100 μg trypsin/ml was needed for maximal digestion; under standard conditions, 80 μg/ml was used. Since cytochrome c within mitochondria is shielded from added trypsin by the outer membrane it would be predicted that mitochondrial cytochrome c would resist trypsin, and this was confirmed in both the presence and absence of tBid (Figure 5C). It was also important that no further Bak conformational change and cytochrome c release occurred during the period of Bak digestion on ice. In fact, 10 min incubation on ice of tBid-treated mitochondria, immediately prior to the 10 min trypsin treatment, had no detectable effect on the changes measured in Bak conformation or cytochrome c release (not shown). Thus both Bak conformation change and cytochrome c release were effectively stopped during the period of Bak digestion. This enabled Bak conformation change and cytochrome c release to be measured on the same mitochondrial pellets

Kinetic analyses of Bak conformation change and cytochrome c release are consistent with tBid-catalysed and autocatalysed Bak activation and allow cytochrome c diffusibility to be determined

Figure 6 shows the measured time courses of Bak conformation change (solid symbols) and of cytochrome c release (open symbols) elicited at two concentrations of tBid. At the lower [tBid], the onset of both processes was delayed by about 20 min. Once begun, however, the rate of change in Bak conformation was largely independent of [tBid]. This behaviour is consistent with a pronounced autocatalytic action of Bak (Ruffolo and Shore, 2003). tBid initiated the process of Bak conformation change but, once underway, it proceeded autocatalytically. According to this model (Fig 4), the fractional Bak activation with time after tBid ($[Bak^*]_t$) is given by

$$[Bak^*]_t = 1 - \frac{c+f}{c \cdot e^{(c+f)\cdot t} + f} \quad \text{(Equation 2, Methods)}$$

where c and f are the rate constants for the tBid-catalysed and autocatalysed Bak activation, respectively (Fig 4). The curves fitting the Bak data are the best fits given by equation 2 and yield the given values of the rate constants c and f.

The fractional mitochondrial cytochrome c content with time after tBid ($[Cytc]_t$) according to Fig 4 is given by

$$[Cytc]_t = \frac{\exp\left[a.\left(-t + (c+f)\left[\frac{t}{f} - \frac{1}{f\cdot(c+f)} \cdot \ln\left(c \cdot e^{(c+f)\cdot t} + f\right)\right]\right)\right]}{\exp\left[\frac{-a \cdot \ln(c+f)}{f}\right]} \quad \text{(Equation 3, Methods)}$$

where a is the rate constant for cytochrome c diffusion and reflects the maximal number of Bak pores, their intrinsic permeability to cytochrome c, and any limitation due to restricted cytochrome c diffusion in the intermembrane/intracristal spaces. The curves fitting the cytochrome c data are the best fits according to equation 3 when constrained to have the c and f values indicated by the corresponding Bak data. Both equations give good fits to the experimental data. Thus, Bak activation could be accurately modelled in terms of two rate constants, c and f. The same values of c and f also allowed accurate modelling of cytochrome c release enabling the values of the diffusibility constant a to be determined (Fig 6)

Changes in the rate constant for cytochrome c diffusion indicate changes in the diffusibility of cytochrome c in the intermembrane spaces

In the two-step procedure (Fig 6), the values of c and f were first obtained from Bak data and equation 2. Using these values, equation 3 was then best-fitted to cytochrome c data to give the cytochrome c diffusibility constant, a. It is important to note that since a is a rate constant given by (Methods, penultimate equation):

$$\text{Rate of cytc efflux} = a\,[\text{fractional Bak activation}][\text{fractional cytc content}]$$

the value of a (the rate of cytochrome c release per unit fractional Bak activation and per unit fractional cytochrome c) should be independent of the number of formed Bak pores in the outer membrane. In order to check this, we needed a means of changing Bak activation at constant [tBid] (since diffusibility may be tBid-dependent). It was found that the rate of Bak activation was markedly reduced by washing the mitochondria in saline. In Figure 7, saline-washing produced a 17-fold decrease in the rate of autocatalysis (f). The resultant decrease in the rate of Bak activation decreased the rate of cytochrome c release. However, the value of the diffusibility constant a was essentially unchanged. In four such experiments, saline-washing decreased the autocatalytic constant f by 91 +/− 6% (mean +/− S.E.M.), but had no significant effect on the diffusibility constant a (+12 +/−16%). Thus changes in the value of the rate constant a reflect changes in the ease of diffusion (diffusibility) of cytochrome c within the intracristal and intermembrane spaces, and not across the outer membrane.

The effect of saline-washing may reflect the loss of superficially-associated proteins that facilitate autocatalysis. This aspect was not pursued, since it was not the object of the study. Nevertheless, it was evident that saline-washing did remove some proteins, most obviously

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at around 280 kDa and 23 kDa (Figure 7B, arrows). However, saline-washing caused no loss of Bak (Figure 7B).

Cytochrome c diffusibility in the intermembrane spaces is independent of tBid at low concentrations; determination of the "basal" cytochrome c diffusibility in the absence of tBid

In Figure 6, the value of the cytochrome c diffusibility constant *a* was little changed when the concentration of tBid was increased 30-fold i.e. from 0.10 (0.5 nM tBid) to 0.15 (15 nM tBid). In contrast, the same change in [tBid] produced a 7000-fold increase in tBid-dependent Bak activation (*c*). These data were obtained in the absence of Ca^{2+}. Since the complete absence of Ca^{2+} may preclude the PT (which can affect diffusibility, below), experiments were also conducted in the presence of [Ca^{2+}] in the physiological range (1μM free Ca^{2+}; Nicholls and Budd, 1998). As shown in Figure 8A, the diffusibility constant for cytochrome c under these conditions was also essentially independent of [tBid] over the range 0.5 – 19 nM. In the same experiment, Bak activation (*c*) was acutely dependent on [tBid] (Figure 8C). In a number of such experiments the constant *c* was proportional to $[tBid]^n$, where the value of n varied between 2 and 3. The value of n cannot be meaningfully estimated, however, since this would require knowing the free [tBid], whereas nearly all added tBid was bound (Figure 2). The power dependence of *c* on [tBid] is considered in the Discussion.

In summary, irrespective of the presence or absence of physiological [Ca^{2+}], intermembrane space diffusibility of cytochrome c was essentially unaffected by tBid over the range 0.5 – 19 pmol mg mitochondrial protein. The cytochrome c diffusibility in the absence of tBid could be obtained by extrapolation to zero [tBid]. In Figure 8A this yielded a "basal" diffusibility value for cytochrome c in the absence of tBid of 0.13 min^{-1}. In five such experiments, a mean "basal" *a* value for cytochrome c of 0.19 min^{-1} was obtained (Figure 8B) . This value is considered further in the Discussion. It is clear however that the "basal" diffusibility was sufficient for rapid and complete cytochrome c release (eg Figures 6 & 7).

The influence of the permeability transition on cytochrome c diffusibility

Isolated B50 cell mitochondria are relatively resistant to the Ca^{2+}-induced PT, but succumb readily when cyclophilin-D is overexpressed (Li et al, 2004). To investigate the influence of the PT, therefore, cyclophilin-D-overexpressed {CyP-D(+)} mitochondria were used, which contain about 10-fold more cyclophilin-D than wild type. The PT was induced by addition of sufficient Ca^{2+} in the presence of inorganic phosphate. PT pore opening was monitored from the release of matrix-entrapped calcein (M_r 622), which permeates freely through the PT pore in the inner membrane (M_r cut-off, about 1500; Crompton, 1999) and through the voltage dependent anion channel (porin) in the outer membrane (M_r cut-off >3000, Bathori et al, 2006). Addition of high [Ca^{2+}] induced rapid loss of calcein, and the release was largely blocked by the cyclophilin-D inhibitor, cyclosporin A (CSA), indicative of PT-mediated calcein efflux (Figure 9A). The release of calcein was accompanied by complete dissipation of the mitochondrial inner membrane potential (data not shown; equivalent data for CyP-D(+) mitochondria were published previously: Li et al., 2004). However, the Ca^{2+}-induced PT did not lead to cytochrome c release (Figure 9B), even though tBid did induce rapid cytochrome c release irrespective of [Ca^{2+}]. This indicates that the outer membrane remained intact during the Ca^{2+}-induced PT. Conversely, the slow, basal release of calcein in the presence of low, buffered [Ca^{2+}] was not detectably increased by 20 nM tBid (Figure 9A), indicating that the PT did not contribute to the tBid-induced cytochrome c release. In agreement, the cytochrome c diffusibility constant during tBid action at low [Ca^{2+}] was unchanged by CSA (Figure 9C). Thus, 20 nM tBid did not induce PT pore opening nor did the PT release cytochrome c in the absence of tBid.

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However, high [Ca^{2+}], sufficient to induce the PT, did increase the diffusibility rate constant for cytochrome c during tBid action, and this increase was quantitatively prevented by CSA, indicating that the Ca^{2+}-induced PT facilitated cytochrome c diffusion (Figure 9C). High [Ca^{2+}] also inhibited tBid-induced Bak activation to a small extent (not shown). To establish whether the PT-induced change was general, we also applied the two-step procedure to measurements of smac release. This yielded similar diffusibilities to cytochrome c under most conditions. Since the PT did not release cytochrome c in the absence of tBid (Figure 9B) ie did not rupture the outer membrane, the PT-induced increase in cytochrome c and smac diffusibility must have occurred internally to the outer membrane ie in the intermembrane and intracristal spaces. The rather lower sensitivity of smac diffusibility to the PT than cytochrome c diffusibility may reflect different distributions of the two proteins in the intermembrane spaces. In conclusion, when the PT occurred, it increased the internal diffusibility of cytochrome c and smac in the intermembrane spaces.

Although low [tBid] did not induce the PT (Fig 9), high [tBid] did. Thus, in contrast to 20 nM tBid, 100 nM tBid (100 pmol / mg mitochondrial protein) did induce the release of entrapped calcein and this was largely blocked by CSA (Figure 10), indicating that the higher [tBid] promoted the PT. Since release of calcein was unaffected by saline-washing (not shown), these experiments were conducted with saline-washed mitochondria to allow determination of the effects of high [tBid] on cytochrome c diffusibility (in unwashed mitochondria, Bak activation occurred too quickly at high [tBid] for satisfactory kinetic measurements). Figure 10B shows that the cytochrome c diffusibility constant was increased approximately two-fold by 100 – 200 nM tBid, and that this was prevented by CSA. Thus high [tBid] promote the PT with consequent increase in cytochrome c diffusibility in the intermembrane spaces. However, this involvement of the PT appears to be confined to high [tBid]; in particular, the PT was not part of tBid action at concentrations up to 20 nM (corresponding to 20 pmol tBid bound / mg mitochondrial protein; Figure 9).

Discussion

Evidence that cytochrome c is normally sequestered in the intracristal spaces (Bernardi and Azzone, 1981), away from the outer membrane, has raised the question of whether this results in a restricted mobility to the outer membrane which needs to be facilitated for efficient cytochrome c release, and whether Bid and Bik perform such a function (Germain et al, 2005; Kim et al, 2004; Scorrano et al, 2002). To date, the mobility of cytochrome c between the intracristal and intermembrane spaces has been assessed from its capacity to transfer electrons from externally-added ascorbate and from outer membrane cytochrome b_5 to cytochrome oxidase in the (presumably) cristal membranes (Frezza et al, 2006; Germain et al., 2005; Scorrano et al., 2002). Although informative, these techniques are limited in important respects. Since the oxidation / reduction rates depend on the local concentration of cytochrome c they are not applicable when cytochrome c is actually released from mitochondria, and are restricted to protocols under which release does not occur eg Bak-deficient mitochondria. Also chemical reactivity is measured, rather than flux. Thus it is not easy to evaluate the impact that any observed changes would have on the actual rate at which cytochrome c is released. Moreover, they cannot be applied to intermembrane space proteins that do not undergo oxidation / reduction. Here, we apply a novel procedure that overcomes these limitations in allowing the mobilities of cytochrome c and other intermembrane space proteins to be obtained during the actual process of Bak-mediated release. The model (Figure 4) was set up specifically to test whether a second action of tBid mobilizes cytochrome c in the intermembrane spaces independently of Bak (Introduction). A different analytical model would be needed to test whether Bak activation itself influences mobility in the intermembrane spaces.

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The rate of cytochrome c release will be determined by the number of Bak pores and their intrinsic permeability and by the ease with which cytochrome c diffuses internally to these pores from its location in the intracristal / intermembrane spaces. In order to differentiate changes in the ease of internal diffusion from changes due to Bak pores, it was essential to know the degree of Bak activation at any point in time. The purpose of modelling Bak activation was simply to enable this. The model of Bak activation used was the minimal model required to enable Bak activation to be estimated. It necessarily incorporated both tBid-induced and autocatalytic components but, beyond this, probably only approximated the complex events taking place. But it was adequate for purpose, since the values obtained for constants *f* and *c* (eg Figures 6 & 7) yielded an accurate estimate of total Bak activation with time (ie what was required) when inserted into equations 2 and 3. Since the diffusibility is obtained as a rate constant (ie rate per unit of Bak activation) it is independent of the degree of Bak activation and pore formation. Experimentally, this is evident from the fact that diffusibility did not change in parallel with Bak activation either when [tBid] was changed (Figures 6 & 8) or after saline washing (Figure 7). Thus, although the value of the diffusibility constant *a* is determined by diffusion across both the intermembrane spaces and the outer membrane, changes in the value of *a* specifically indicate changes in diffusibility in the intermembrane / intracristal spaces (as they affect the overall diffusibility between intracristal space and external medium) eg an n-fold increase in the value of *a* means that an increase in internal diffusibility has occurred sufficient to produce an n-fold increase in the rate of cytochrome c release at maximal Bak activation.

The technique revealed that relatively low [tBid] (<20 pmol / mg mitochondrial protein) did not increase cytochrome c diffusibility in the intermembrane spaces (or, at least, not sufficiently to produce a significant change in the rate of cytochrome c release from the mitochondria; Figures 6 & 8). Yet, once begun, cytochrome c release occurred quickly and completely. Evidently, the basal diffusibility was sufficient to support this. Quantitatively, the measured “basal” cytochrome c diffusibility constant (about 0.2 min^{-1}; Figure 8) corresponds to a half time for cytochrome c release of 3.4 min. Since the basal diffusibility refers to zero [tBid], it should be applicable to cytochrome c release under all apoptotic stimuli, not merely those involving tBid. It is useful to compare this half time value with kinetic measurements of cytochrome c release *in vivo.* Imaging of GFP-tagged cytochrome c release in HeLa cells showed that, following a lag period, the release from mitochondria in any cell, once underway, was largely complete within 4-7 min under a range of apoptotic stimuli (Goldstein et al., 2000). Our rates are broadly comparable to these. Although between different cell types, the comparison suggests that the basal cytochrome c diffusibility may suffice for physiological rates of cytochrome c release.

A recurrent question concerns PT involvement in cytochrome c release (Garrido et al., 2006). The PT is typically induced by high [Ca^{2+}], and results in non-selective permeabilization of the mitochondrial inner membrane to low M_r solutes and expansion of the matrix space (Crompton, 1999). It is blocked by CSA. Previous findings that tBid can promote the PT and that tBid-induced cytochrome c release is attenuated by CSA (in some studies) has led to the PT being widely considered as an essential part of tBid action. According to this, tBid not only initiates Bak and Bax activation but also triggers the PT in a Bak/Bax-independent manner, thereby providing a mechanism for opening cristal junctions and allowing otherwise sequestered cytochrome c to diffuse from the cristal spaces into the intermembrane space and to the outer membrane Bak and Bax pores (Alirol et al., 2006; Frezza et al., 2006; Gazaryan and Brown, 2007; Scorrano et al 2002;Zhang and Armstrong, 2007). Yet we found no indication for such a bimodal action of tBid at low concentrations when neither the PT (Figure 9) nor internal cytochrome c diffusibility (Figure 8) was promoted by tBid. Nevertheless, high [tBid] did increase cytochrome c diffusibility in a PT-dependent manner (Figure 10), and the increased diffusibility was comparable to that

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associated with the Ca^{2+}-induced PT (Figure 9C). The importance of the PT-induced change may be evaluated from its effect on the overall rate of cytochrome c release. From the present measurements, the PT produced an approximate 2-fold increase in the rate of cytochrome c release. On this basis, the PT seems unlikely to be an essential feature of tBid action, a conclusion reinforced by the high basal cytochrome c diffusibility (above) and the requirement of high [tBid] for PT induction.

The reason why tBid can promote the PT is not known. But, as a possible scenario (Crompton, 2003), tBid binds cardiolipin tightly (Kim et al., 2004), and has been reported to cause cardiolipin transfer from the inner membrane where it is abundant to the outer membrane where it is scarce (Fernandez et al., 2002). The function of this may be to facilitate outer membrane permeabilisation, since cardiolipin markedly promotes tBid-induced Bax (and Bak?) pore formation (Kuwana et al., 2002). Cardiolipin is also believed to stabilize the native structure of the adenine nucleotide translocase in the inner membrane (Brustovetsky et al., 2002), so that excessive cardiolipin transfer may destabilize the translocase and increase its tendency to deform into the PT pore. This would be more likely to occur at high [tBid]. Evidence that tBid binding to cardiolipin in isolated mitochondria can destabilize the translocase has been reported (Gonzalvez et al., 2005).

Although tBid (0-20 nM) had no effect on cytochrome c diffusibility, it had a profound effect on Bak activation. The apparent power dependence of Bak conformation change on [tBid] (Figure 8) suggests, at first sight, an involvement of tBid oligomers in Bak activation, or that tBid monomers operate cooperatively. Grinberg et al (2002) detected tBid/tBid interactions in mitochondria by FRET (using tagged tBids) and that a fraction of tBid was crosslinked into trimers on binding to mitochondria. However, in that study, homo-oligomerisation did not correlate with Bak activation. It is more likely, therefore, that tBid binds first to proteins that have a higher affinity for it than Bak, so that very little of total tBid is available for Bak at low [tBid], but progressively more becomes available as the higher affinity proteins become saturated with tBid. In particular, there is gathering evidence that Bak is maintained in an inactive state bound to Bcl-X_L and Mcl-1, and that tBid must first displace Bak by binding to these proteins before Bak is activated (Kim et al., 2006; Uren et al., 2007; Willis et al., 2005). With excess Bcl-X_L and Mcl-1, the observed relation (Figure 8C) between Bak activation and total tBid would be expected. The data derived from the analysis, therefore, are broadly consistent with current models of Bak activation incorporating both Bak displacement from antiapoptotics and autocatalysis.

In conclusion, a procedure has been developed allowing basic parameters of tBid action on isolated mitochondria to be extracted from kinetic data. It enables Bak activation to be resolved into tBid-dependent and autocatalytic components and shows that, whereas tBid initiates Bak activation, Bak activation proceeds largely autocatalytically once begun. This initial trigger for Bak activation appears to be the only function of tBid required for the release of cytochrome c. In particular, the analysis reveals that the “basal” (tBid-independent) diffusibility of cytochrome c within intracristal and intermembrane spaces is sufficient to support rapid and complete loss of cytochrome c from mitochondria. A secondary effect of tBid of inducing the PT and increasing cytochrome c mobility only occurs at high [tBid] and is not essential for cytochrome c release. We conclude, therefore, that tBid probably acts in a monofunctional manner in inducing cytochrome c release.

Materials and methods

Preparation of rat tBid

Total RNA from rat liver was used for the synthesis of Bid first strand cDNA. This was PCR-amplified, ligated into pET-15b (Novagen), and transformed into E. coli DH5α.

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Following induction, cells were extracted and the His-tagged Bid was purified using Ni-NTA-agarose beads. The tag was removed with thrombin and the Bid protein was purified to a single band on SDS-PAGE by FPLC (Pharmacia) using cation exchange (mono-S), gel filtration (superdex 75) and hydrophobic interaction (phenyl sepharose) columns. tBid was generated by incubation with caspase 8 (Sigma) until cleavage was complete as judged by SDS-PAGE.

B50 cell culture and isolation of mitochondria

B50 cells from a rat neuronal cell line (European collection of Cell Culture, Salisbury, U.K.) and a B50 clone overexpressing cyclophilin-D {CyP-D(+) cells}were cultured as previously (Li et al., 2004). For mitochondrial isolation, cells were extracted in 395 mM sucrose / 10 mM Hepes (pH 7.4) / 0.5 mM EGTA (SHE) containing 45 μg/ml phenylmethylsulphonyl fluoride, and pepstatin, leupeptin, antipain and chymostatin (all at 5 μg/ml) as protease inhibitors. The cells were ruptured by passage five times through a 19 gauge needle using a lab-made, air-pressure-driven device producing a flow rate through the needle of 30 m/s. This gave reproducible cell rupture with minimally damaged mitochondria (as judged by the retention of cytochrome c, otherwise lost on breakage of the outer membrane). The extract was centrifuged at 1000 g for 5 mins, and then at 10000g for 8 min to sediment the mitochondria. The mitochondria were washed once in SHE by suspension / sedimentation. For saline washed mitochondria, mitochondria were washed initially in 150 mM KCl / 10 mM Hepes (pH 8.0) / 0.5 mM EGTA, followed by SHE, and suspended finally in SHE.

Cytochrome c release, Bak conformation change and calcein release

For cytochrome c release alone, mitochondria (20 μg protein) were incubated in 125 mM KCl / 50 mM sucrose / 0.2 mM EGTA / 5 mM succinate / 5 mM Na_2HPO_4 (pH 7.0) / 2 mM $MgCl_2$ / 1 μM rotenone (standard reaction medium, SRM) containing tBid in a final reaction volume of 20 μl, unless otherwise indicated. Reactions were stopped by centrifugation at 13000g for 3 min, and the mitochondrial pellets extracted in SDS-PAGE sample-loading buffer (10 mM Tris-HCl, pH 6.8 / 4% (w/v) SDS / 10% glycerol / 1 mM mercaptoethanol) at 90°C for 4 min. For Bak conformation change, and for simultaneous Bak and cytochrome c measurements, mitochondrial reactions (20 μl) were stopped by transfer to precooled tubes containing 5 μl trypsin (50 μg / ml) and incubated at 0°C for 10 min. Trypsin inhibitor was added and the mitochondria were then sedimented and extracted as above. Following standard SDS-PAGE and blotting, blots were developed with antibodies for cytochrome c (Pharmingen), Bax (Santa Cruz Biotech.), smac / Diablo (Calbiochem) and Bak (NT antibody, Upstate, the epitope for which contains a trypsin cleavage site. G23 antibody, Santa Cruz Biotech). As fluorescent secondary antibodies, IRDye800-conjugated anti-mouse IgG (Molecular probes) and IRDye680-conjugated anti-rabbit IgG (LiCor) were used. Bands were quantified using the Odyssey imaging system (LiCor). Results are expressed as fractional change. If S is the fluorescence signal from any band of cytochrome c or Bak, and S_o is the signal at zero time or zero tBid, then the fractional change = $(S_o - S) / S_o$. Both tBid-induced cytochrome c release and Bak conformation change proceeded to completion, or almost to completion, with time i.e. maximal measured fractional change was 0.92 – 1.00 in all cases (corresponding to 92–100% completion). To correct for less than 100% completion, values were scaled using the following: corrected fractional change = measured fractional change / maximal measured fractional change. This allowed best-fitting of Regression curves over the range 0 – 1.00 (below).

Calcein was loaded into mitochondria as the lipophilic acetoxymethyl ester; this is hydrolysed to the free acid in the mitochondrial matrix where it is entrapped. Mitochondria (3 mg protein / ml SHE) were incubated with 10 μM calcein ester for 30 min at 25°C and

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then washed twice in SHE. For release, calcein–loaded mitochondria in SRM were centrifuged at intervals and calcein in the supernatant determined fluorimetrically.

A kinetic model for analysing tBid-induced cytochrome c release from isolated mitochondria

Expressions were formulated for the rate of cytochrome c release from mitochondria in which Bak pore formation is complete (Equation 1, below) and for the time dependence of Bak pore formation (Equation 2, below). These two expressions were then combined to give the time dependence of cytochrome c release (Equation 3, below). To simplify the equations, all changes are represented as fractional changes (where a change from 0 to 1 indicates a change from 0 to 100%).

When Bak pore formation is complete, the rate of cytochrome c efflux will be given by

$$-\frac{d[Cytc]}{dt}=a\cdot[Cytc]_t \quad \text{Equation 1}$$

where $[Cytc]_t$ is the fractional internal concentration of cytochrome c at time t, and the rate constant a for cytochrome c diffusion reflects the maximum number of Bak pores, their intrinsic permeability to cytochrome c and any limitation due to restricted intramitochondrial diffusion of cytochrome c.

When Bak pore formation is incomplete, then the fractional completion at any time must be considered. According to the autocatalytic model (Ruffolo and Shore, 2003; Results), tBid triggers a conformational change in Bak from a “closed” to an “open” conformation. The “open” conformation then catalyses the conversion of further “closed” conformers to the “open” form. “Open” conformers self assemble into oligomeric pores for cytochrome c efflux. The process is depicted in Figure 4, where the “open” conformation is designated Bak* and where c and f are the rate constants for the tBid-induced and the Bak*-induced conformation changes in Bak. For simplicity, reversible self- assembly into pores is assumed to be fast, so that the number of Bak pores with time is in direct proportion to the number of “open” conformers The composite rate constant for pore formation will be

$$c+f\cdot[Open]_t$$

where $[Open]_t$ has a value from 0 to 1, and the rate of “open” conformer formation will be

$$\frac{d[Open]}{dt}=(c+f\cdot[Open]_t)\cdot[Closed]_t$$

Then, by integration

$$[Open]_t=1-\frac{c+f}{c\cdot e^{(c+f)\cdot t}+f} \quad \text{Equation 2}$$

where $[Open]_t$ is the fractional Bak* at any time after addition of tBid as a function of the two rate constants c and f (Fig 4). Combining equations 1 and 2

$$-\frac{d[Cytc]}{dt}=\left(1-\frac{c+f}{c\cdot e^{(c+f)\cdot t}+f}\right)\cdot a\cdot[Cytc]_t$$

and, by integration

$$[Cytc]_t = \frac{\exp\left[a.\left(-t + (c+f)\left[\frac{t}{f} - \frac{1}{f\cdot(c+f)}\cdot\ln\left(c\cdot e^{(c+f)\cdot t} + f\right)\right]\right)\right]}{\exp\left[\frac{-a\cdot\ln(c+f)}{f}\right]} \quad \text{Equation 3}$$

where $[Cytc]_t$ is the fractional mitochondrial cytochrome c at any time (t) after addition of tBid as a function of the three rate constants *c*, *f* and *a* (Fig 4). In practice a two step procedure was used: The rate constants for Bak* formation, *c* and *f*, were first obtained from measurements of Bak conformation change and application of equation 2. These derived values of *c* and *f* were then inserted into equation 3, which was then best-fitted to measured cytochrome c release data ($[Cytc]_t$), to give the rate constant *a* for cytochrome c diffusion. Curves according to equations 2 and 3 were best-fitted to the experimental points by Regression analysis using SigmaPlot 10 (Systat software) and R^2, the coefficient of determination, as an index of fit ($0 \leq R^2 \leq 1$).

Acknowledgments

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Abbreviations

Cytc	cytochrome c
PT	permeability transition

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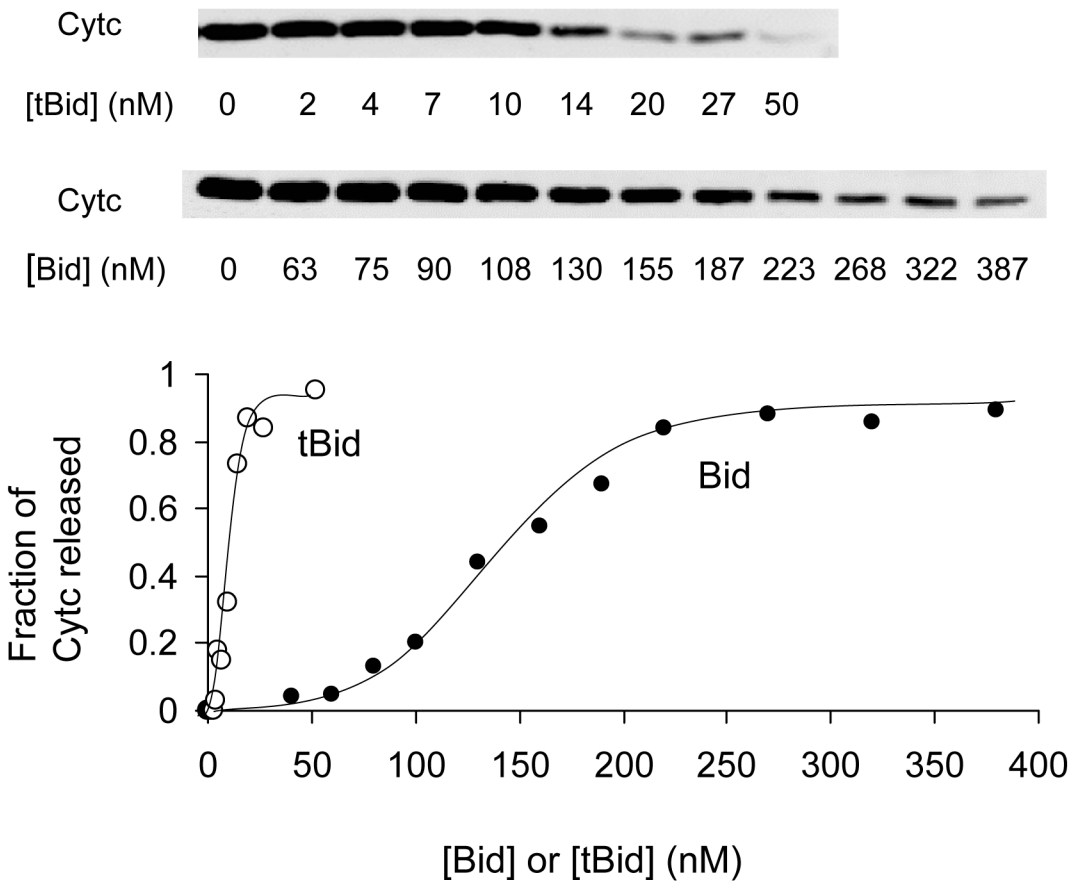

Fig. 1.

Bid- and tBid-induced cytochrome c release from B50 cell mitochondria. Mitochondria were incubated for 30 min with either Bid or tBid, as indicated, and then sedimented. The mitochondrial pellets were analysed for cytochrome c in Western blots using fluorescent secondary antibodies as shown. Bands were quantified by fluorescence imaging and fractional cytochrome c release calculated (where 0-1 indicates 0-100% release). Cytc = cytochrome c.

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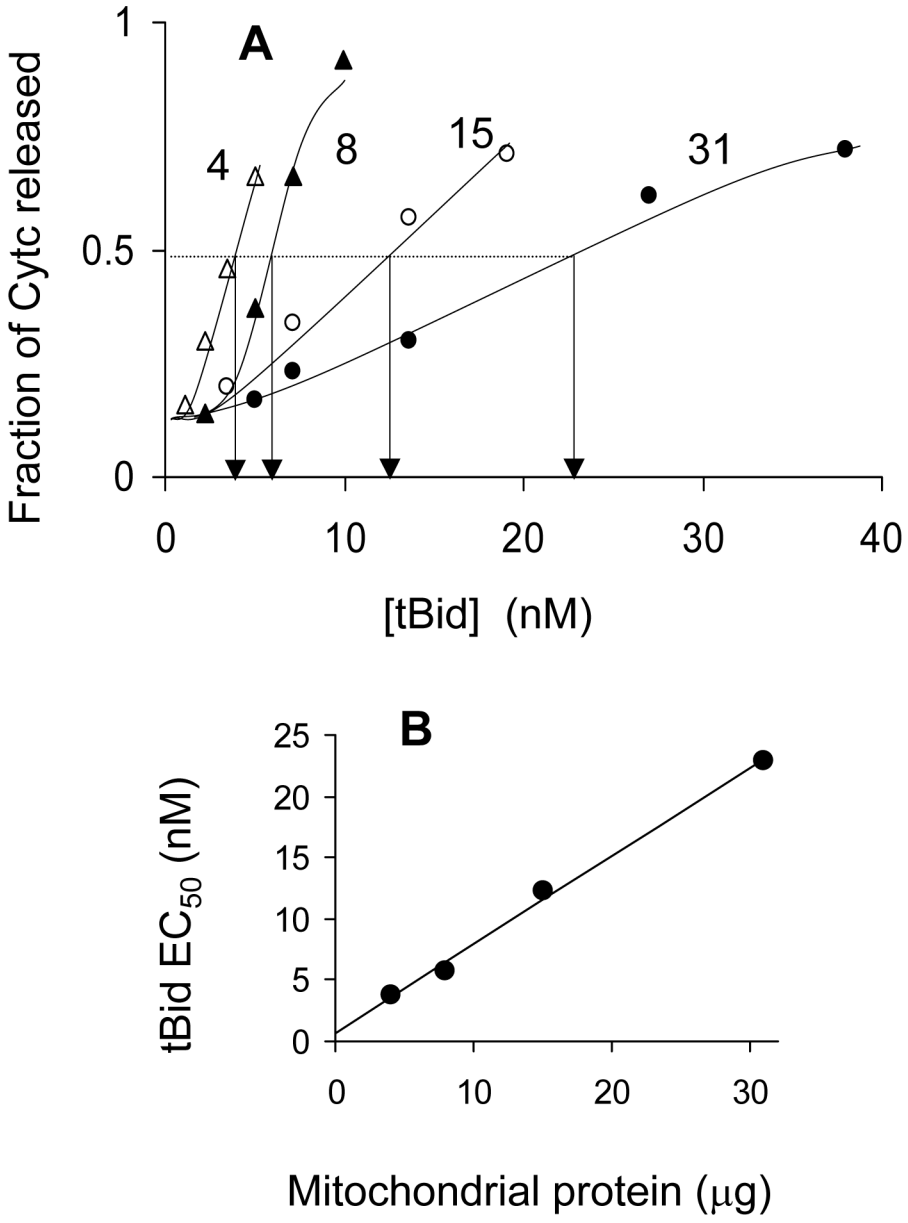

Fig. 2.

The dependence of tBid-induced cytochrome c release on mitochondrial concentration (A) tBid (15 nM)-induced cytochrome c release was measured with either 4, 8, 15, or 31μg of mitochondrial protein per reaction volume (20 μl) as indicated. Arrows show the [tBid] giving half-maximal cytochrome c release (EC_{50} values). (B) The EC_{50} values are plotted against mitochondrial protein per reaction.

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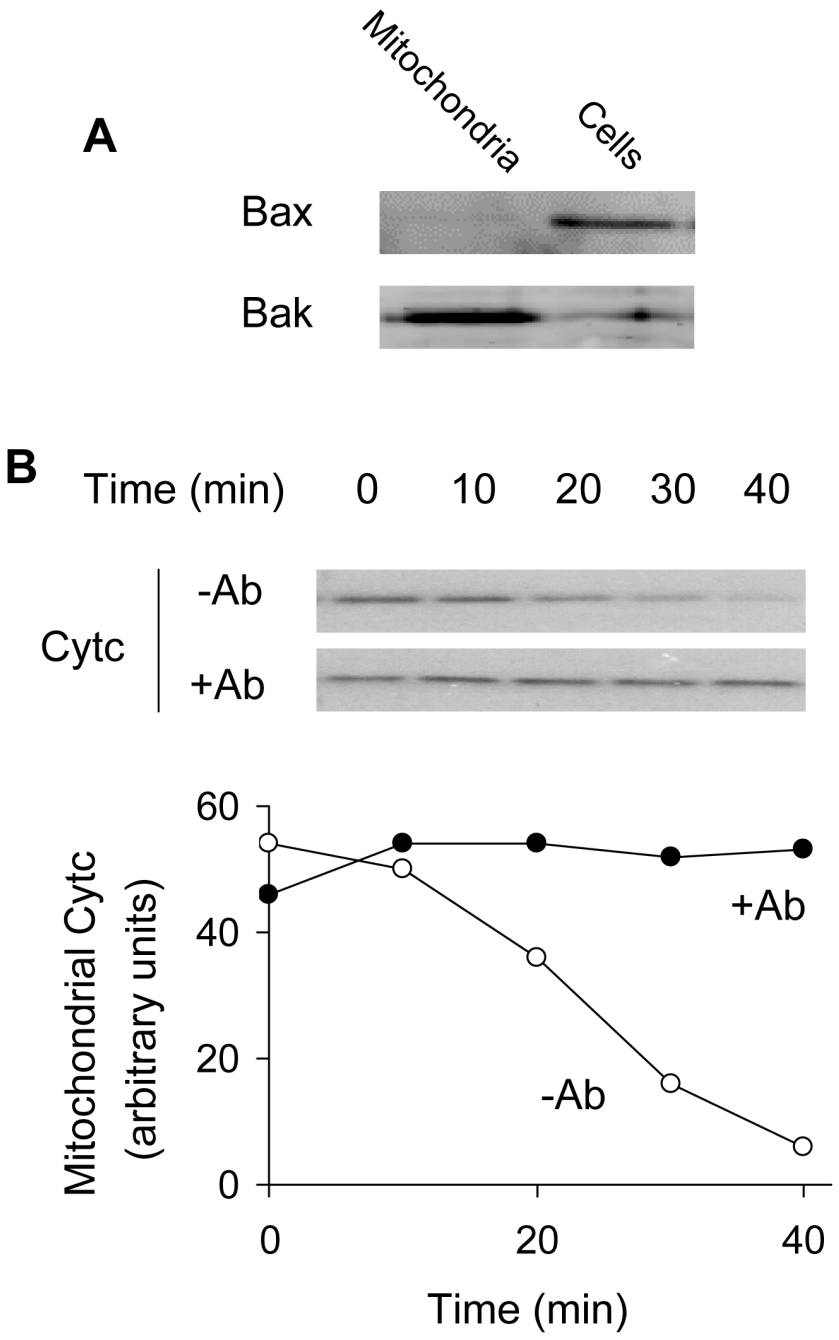

Fig. 3.

Isolated B50 cell mitochondria contain Bak, but not Bax; inhibition of tBid-induced cytochrome c release by anti-Bak antibodies. (A) Mitochondria and whole B50 cells, each containing 20 μg protein, were analysed in Western blots developed with anti-Bak and anti-Bax antibodies. (B) Mitochondria (150 μg protein) were preincubated with or without anti-Bak G-23 antibodies (1 μg) for 30 min. tBid was added and mitochondrial cytochrome c determined at the times indicated.

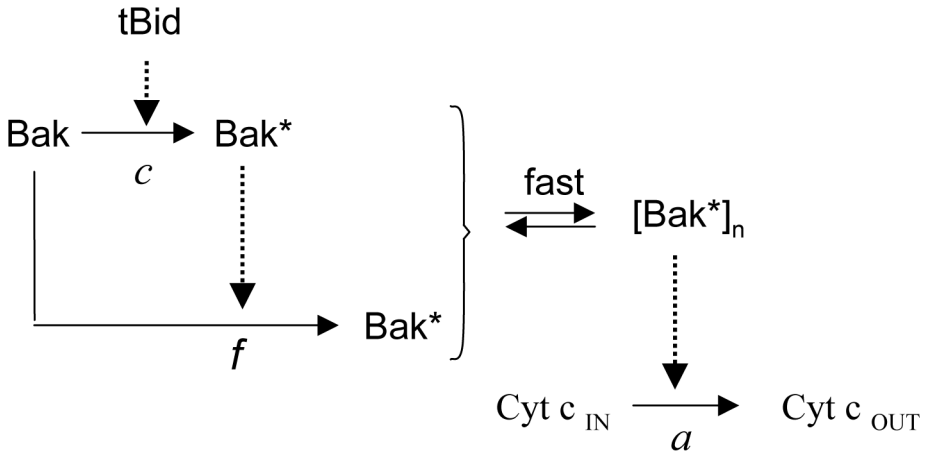

Fig. 4.

Scheme of tBid-induced, Bak-mediated cytochrome c release. In the inactive state, Bak assumes a “closed” conformation designated Bak. tBid induces a change in conformation to an “open” conformer, Bak*. “Open” conformers self assemble into an outer membrane pore $[Bak^*]_n$ through which cytochrome c permeates. Bak is also activated autocatalytically by Bak*. The rate constants for tBid-induced and autocatalytic Bak* formation are c (min^{-1}) and f (min^{-1}) respectively. The rate constant for cytochrome c permeation from the intracristal / intermembrane spaces to the external medium is a (min^{-1}).

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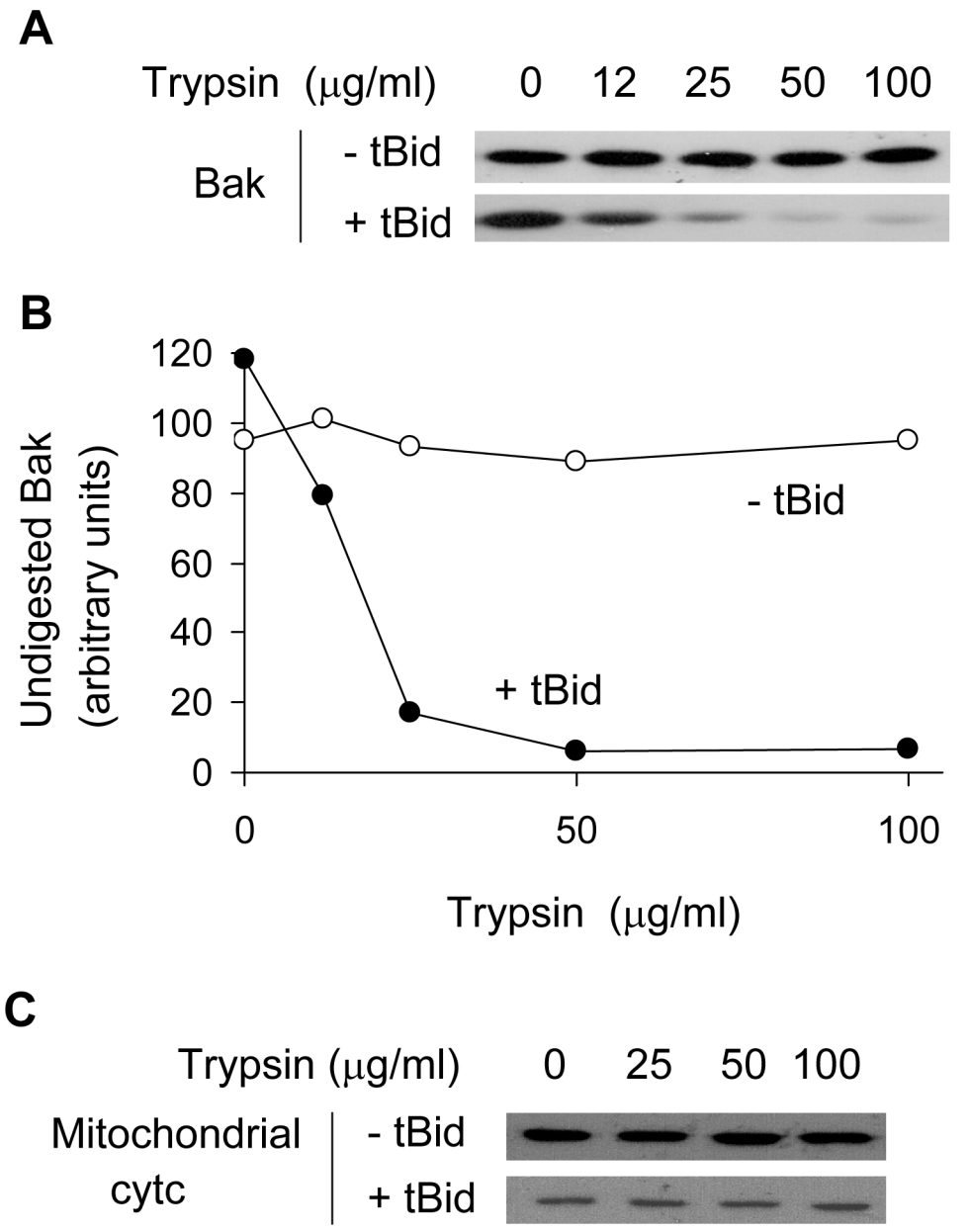

Fig. 5.

Rat mitochondrial Bak is digested by trypsin after tBid treatment, but mitochondrial cytochrome c is resistant. (A) Mitochondria were incubated with 5 nM tBid for 30 min and then with the indicated concentration of trypsin at 0°C for 10 min. Western blots were probed with anti-Bak antibody which only recognizes uncleaved Bak. (B) The Bak signal was used to calculate undigested Bak. (C) Mitochondria were incubated with or without 5 nM tBid for 9 min (yielding partial release of cytochrome c) and then with trypsin as indicated for 10 min at 0°C. Mitochondria were sedimented and their cytochrome c analysed in immunoblots.

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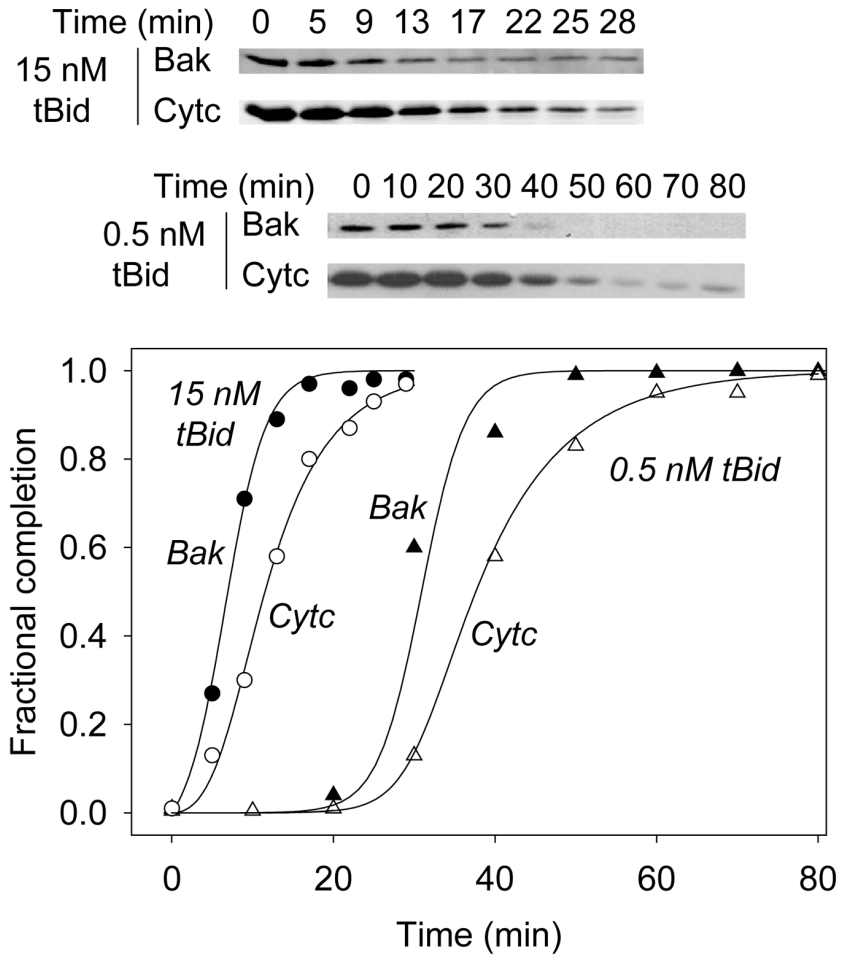

[tBid]:	15 nM		0.5 nM	
Data:	Bak	Cytc	Bak	Cytc
c	0.03	0.03	4×10^{-6}	4×10^{-6}
f	0.37	0.37	0.37	0.37
a	-	0.150	-	0.100
R^2	0.996	0.996	0.976	0.993

Fig. 6.

Kinetic analyses of Bak activation and cytochrome c efflux at two [tBid]. The immunoblots show mitochondrial cytochrome c and trypsin-resistant Bak following addition of either 15 nM tBid or 0.5 nM tBid to mitochondria at zero time. The blots were used to determine the time courses of Bak conformation change (closed symbols) and cytochrome c release (open symbols). 15 nM tBid, circles. 0.5 nM tBid, triangles. The Bak data were best-fitted according to equation 2 (Methods) by the curves shown, yielding the *c* and *f* values given (*f* values constrained equal). These values were then used to derive the best fits for the cytochrome c data according to equation 3 (Methods) to yield the curves shown and the *a* values given.

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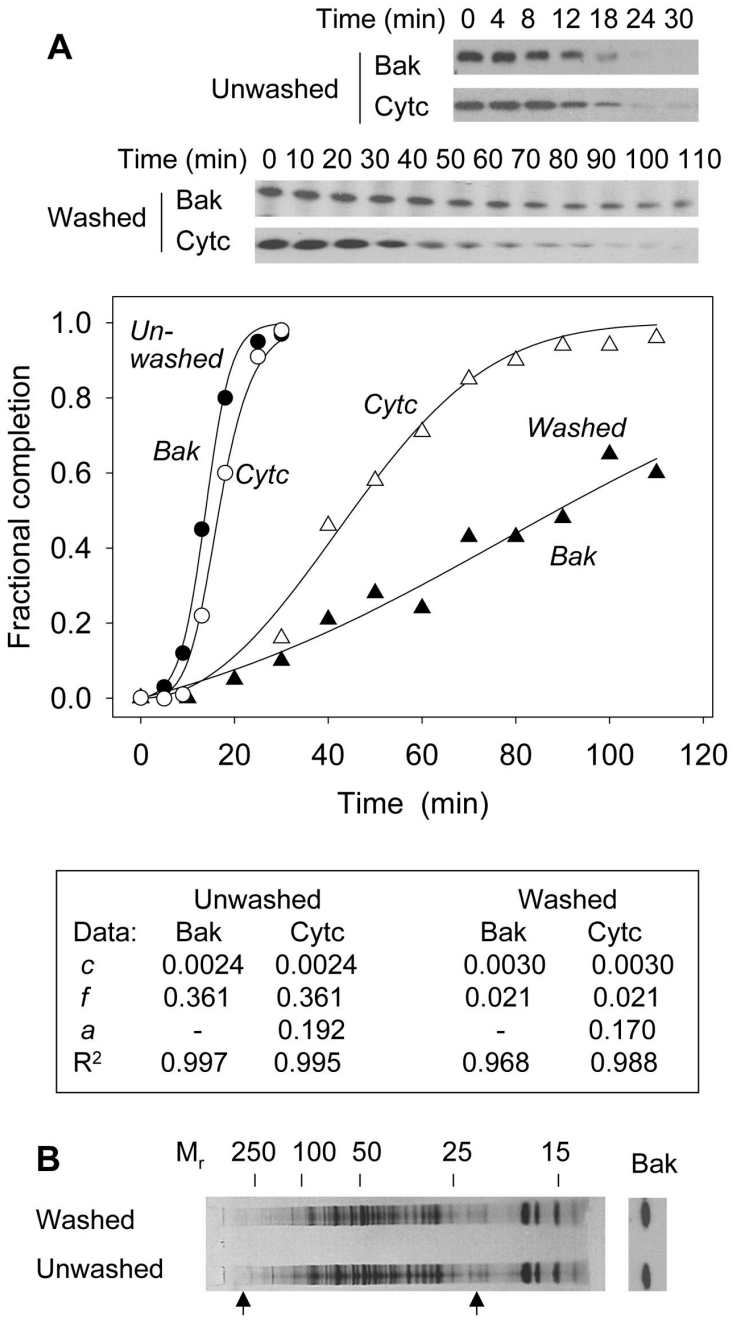

	Unwashed		Washed	
Data:	Bak	Cytc	Bak	Cytc
c	0.0024	0.0024	0.0030	0.0030
f	0.361	0.361	0.021	0.021
a	-	0.192	-	0.170
R^2	0.997	0.995	0.968	0.988

Fig. 7.

Saline washing of mitochondria selectively suppresses autocatalysis of Bak conformation change. (A) Conventional (designated “unwashed”) and saline-washed (“washed”) mitochondria were prepared from the same cell homogenate. tBid-induced Bak conformation change (closed symbols) and cytochrome c release (open symbols) were measured as in Figure 6. The Bak data were best-fitted according to equation 2 (Methods) to yield the *f* and *c* values given. These values were then used to obtain the best fits for the cytochrome c data according to equation 3 (Methods) to give the curves shown and the *a* values given. (B) SDS-PAGE gels (Coomassie stained) and Bak immunoblots of the

washed and unwashed mitochondrial preparations. Arrows show bands reduced in the washed preparation

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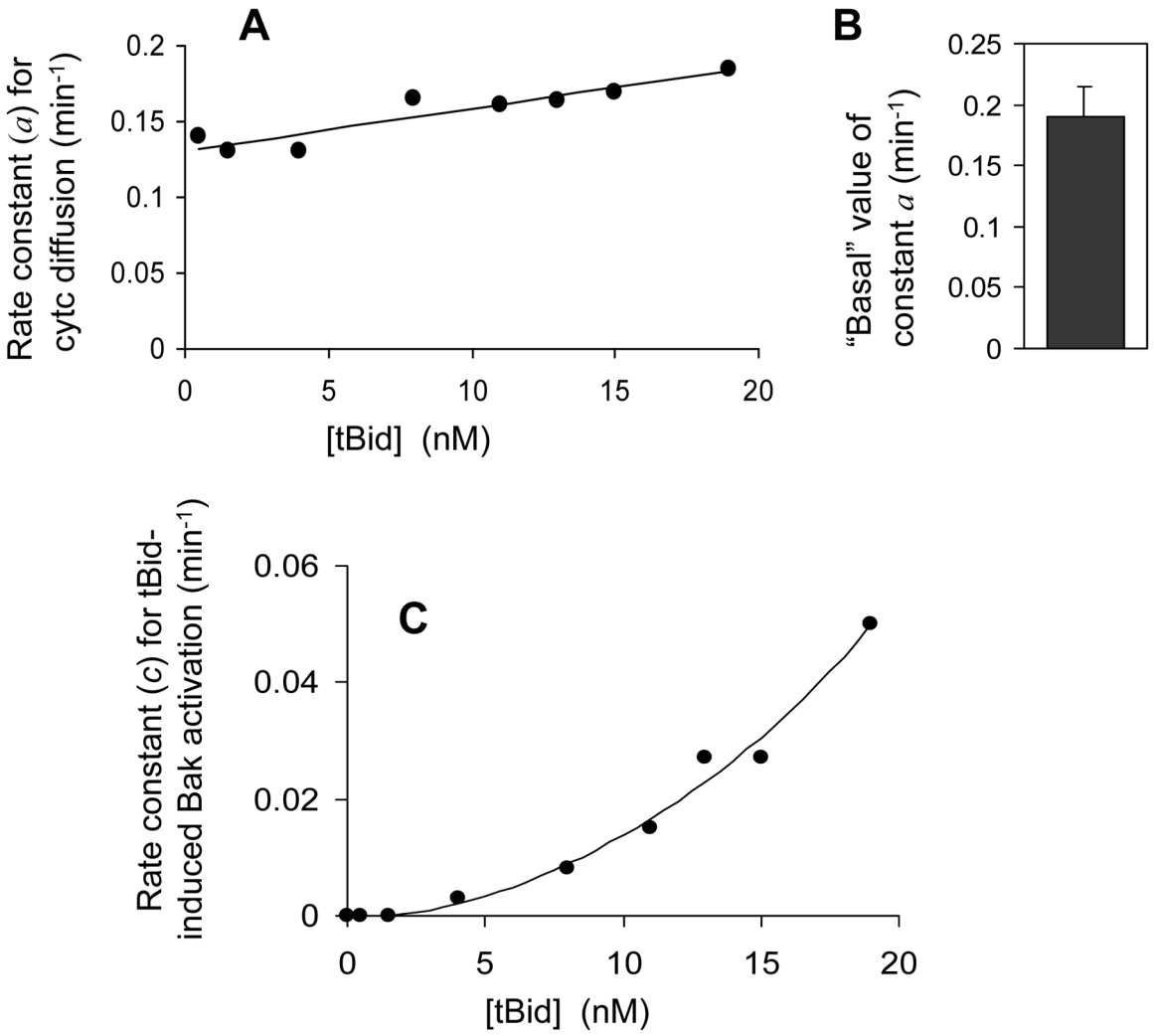

Fig. 8.

The dependence of cytochrome c diffusibility and Bak conformation change on [tBid]. (A, C) The rate constants *a* and *c* at varying [tBid] were evaluated in a single mitochondrial preparation by the two-step procedure used in Figure 6, but with 1 μM Ca^{2+} buffer (5 mM EGTA / 4 mM Ca^{2+}). In (B) the "basal" values of the cytochrome c diffusibility constant *a* were determined in 5 mitochondrial preparations by extrapolation of lines (as in A) to zero [tBid]; mean +/− S.E.M.

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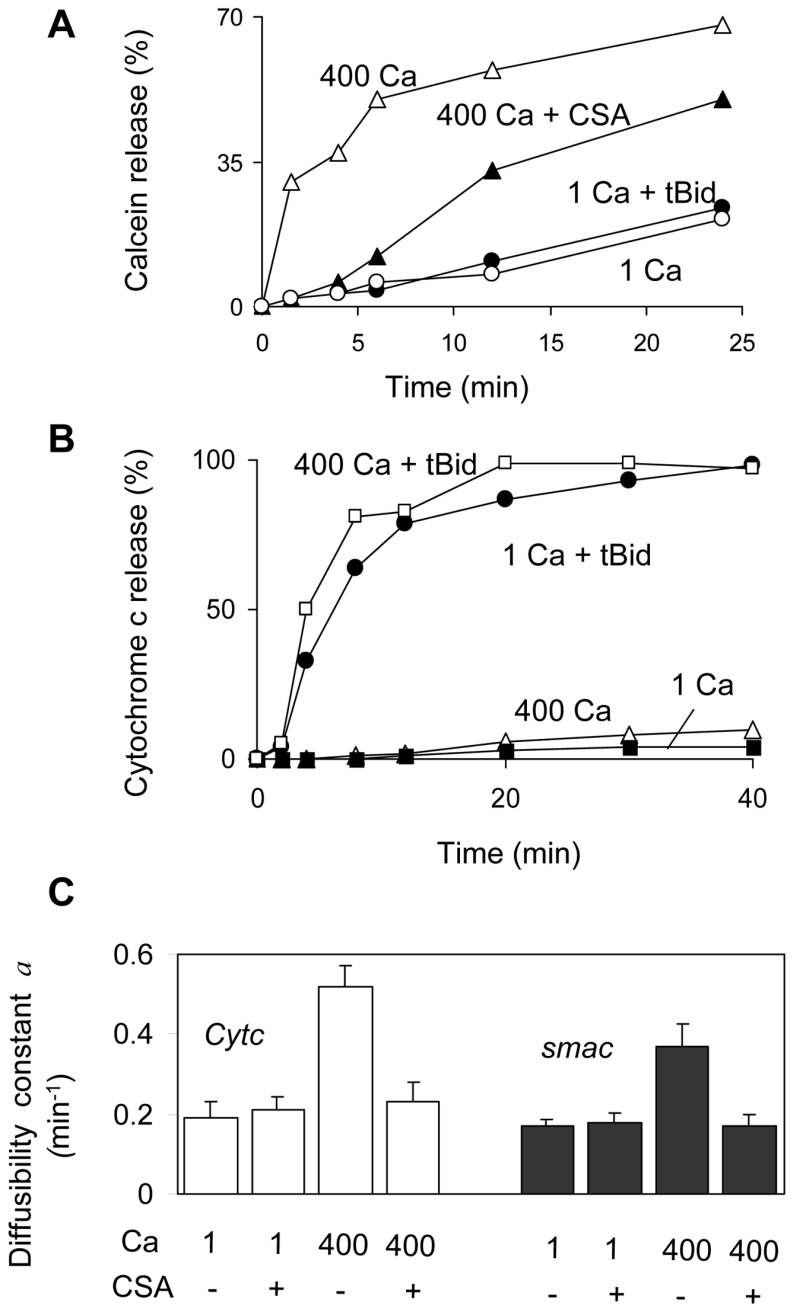

Fig. 9.

The PT pore increases diffusibility of intermembrane space proteins, but is not involved in the action of tBid at low concentrations. CyP-D(+) mitochondria were used throughout. (A) Calcein-loaded mitochondria were incubated in SRM containing either 400 μM Ca^{2+} (400 Ca) with/without 1 μM CSA or 1 μM buffered free Ca^{2+} (1 Ca) with/without 20 nM tBid. The Ca^{2+} buffer comprised 5 mM EGTA and 4 mM $CaCl_2$. After incubation for the time specified mitochondria were sedimented and calcein in the supernatant determined fluorimetrically. (B) Calcein-loaded mitochondria were incubated in SRM containing 400 μM Ca^{2+}, 1 μM free Ca^{2+} and 20 nM tBid, as indicated. Cytochrome c release was measured at the times specified. (C) The two-step procedure was used to determine the

diffusibility rate constants for cytochrome c (open columns) and smac (closed columns) in the presence of 20 nM tBid. Other additions were 400 μM Ca^{2+}, 1 μM buffered free Ca^{2+}, and 1 μM CSA as indicated. Means +/− S.E.M., 4 experiments. The data of (A) and (B) are representative of 3 such experiments.

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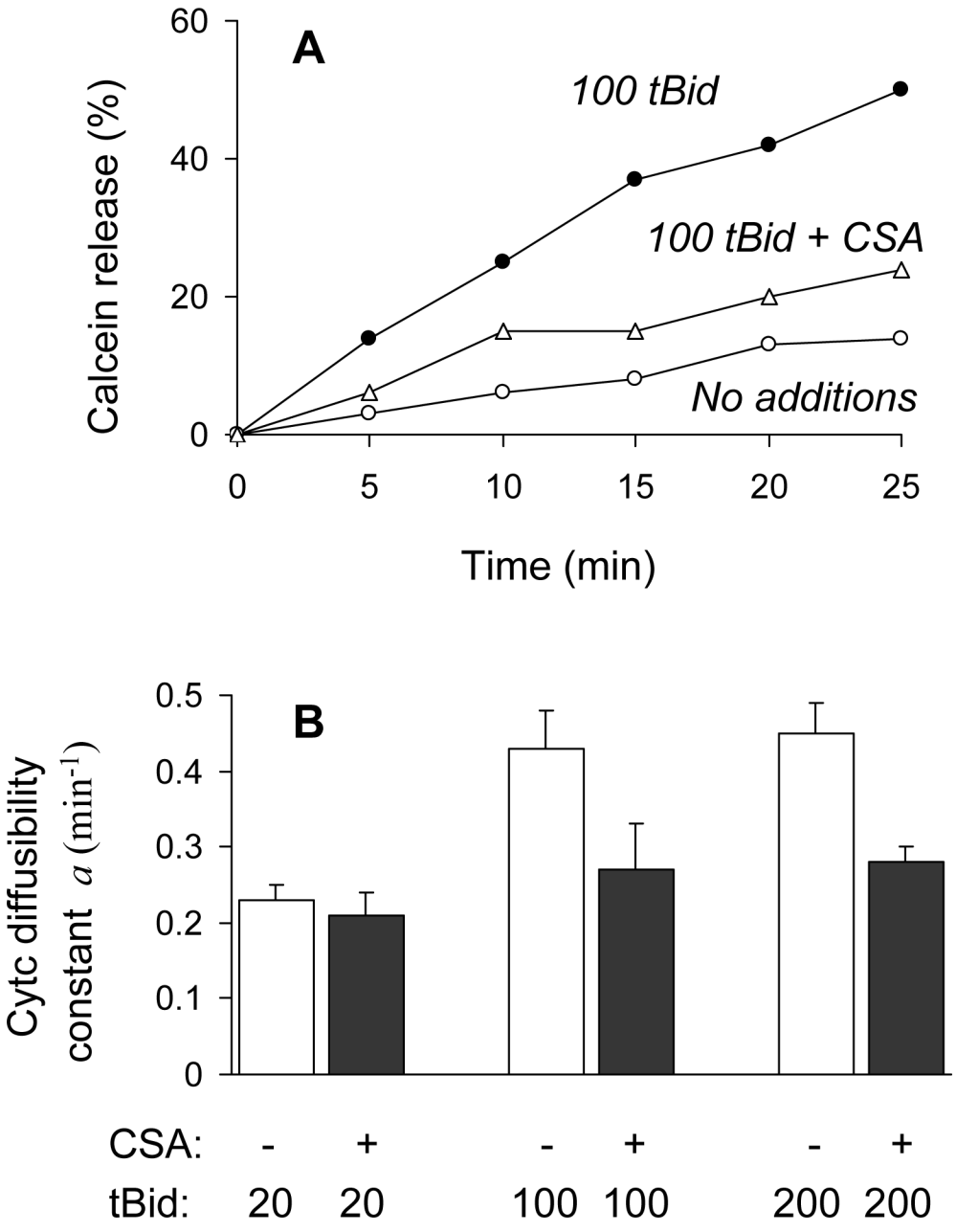

Fig. 10.

High concentrations of tBid induce the PT and increase cytochrome c diffusibility in the intermembrane spaces. CyP-D(+) mitochondria were used and were saline-washed. All experiments were conducted with 1 μM buffered free Ca^{2+}. Additions: 20 nM tBid, 100 nM tBid, 200 nM tBid and 1 μM CSA, as indicated. (A) Calcein-loaded mitochondria were incubated in SRM; other details as in Figure 9A. Data are representative of 3 such experiments. (B) The two-step procedure was used to determine the diffusibility constant for cytochrome c. Means +/− S.E.M. (4 experiments)