tcBid promotes Ca^{2+} signal propagation to the mitochondria: control of Ca^{2+} permeation through the outer mitochondrial membrane

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Calcium spikes established by IP_3 receptor-mediated Ca^{2+} release from the endoplasmic reticulum (ER) are transmitted effectively to the mitochondria, utilizing local Ca^{2+} interactions between closely associated subdomains of the ER and mitochondria. Since the outer mitochondrial membrane (OMM) has been thought to be freely permeable to Ca^{2+} , investigations have focused on IP_3 -driven Ca^{2+} transport through the inner mitochondrial membrane (IMM). Here we demonstrate that selective permeabilization of the OMM by tcBid, a proapoptotic protein, results in an increase in the magnitude of the IP_3 -induced mitochondrial $[\text{Ca}^{2+}]$ signal. This effect of tcBid was due to promotion of activation of Ca^{2+} uptake sites in the IMM and, in turn, to facilitation of mitochondrial Ca^{2+} uptake. In contrast, tcBid failed to control the delivery of sustained and global Ca^{2+} signals to the mitochondria. Thus, our data support a novel model that Ca^{2+} permeability of the OMM at the ER-mitochondrial interface is an important determinant of local Ca^{2+} signalling. Facilitation of Ca^{2+} delivery to the mitochondria by tcBid may also support recruitment of mitochondria to the cell death machinery.

Keywords: apoptosis/Bid/calcium/mitochondria/outer mitochondrial membrane

Introduction

Early studies with isolated mitochondria established the dogma that the outer mitochondrial membrane (OMM) allows free passage of Ca^{2+} (for references see Mannella, 1992). Thus Ca^{2+} transport through the inner mitochondrial membrane (IMM) mediated by an electrogenic uniporter emerged as the sole determinant of mitochondrial Ca^{2+} uptake. Although the Ca^{2+} uptake sites in the IMM exhibit relatively low affinity for Ca^{2+} , inositol trisphosphate (IP_3) receptor- and ryanodine receptor-driven cytosolic $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_c$) spikes that peak at $<1\ \mu\text{M}$ are delivered effectively to the mitochondria (Rizzuto *et al.*, 1993). Recently, it has been demonstrated that propagation of the $[\text{Ca}^{2+}]_c$ spikes to the mitochondria is facilitated by a local Ca^{2+} control between IP_3 /ryanodine receptors and the mitochondrial uptake sites at closely apposed domains of

the endoplasmic reticulum (ER) and mitochondria (Rizzuto *et al.*, 1998; Csordás *et al.*, 1999). The ER-mitochondrial communication is likely to be supported by physical links between the two organelles (Frey and Mannella, 2000; Wang *et al.*, 2000) and displays a functional organization similar to synapses (Csordás *et al.*, 1999). However at the ER-mitochondrial interface, the OMM is inserted between what would be pre- and postsynaptic structures. If the regions of the OMM between the interfacing ER Ca^{2+} release channels and uptake sites are not freely permeable, mitochondrial Ca^{2+} uptake sites may fail to sense the transient, high $[\text{Ca}^{2+}]_c$ microdomain in the vicinity of the activated IP_3 /ryanodine receptors. Thus, the presence of structures that ensure free passage of Ca^{2+} through the OMM is of critical importance at the ER-mitochondrial interface.

Ca^{2+} may traverse the OMM through the pores formed by the voltage-dependent anion channel (VDAC) (reviewed in Mannella, 1992). In particular, the closed conformation of the VDAC shows cation selectivity (Benz and Brdiczka, 1992). Interestingly, at the contact sites, the VDAC is envisaged to interact with an IMM transporter, the adenine nucleotide translocator (ANT), to form the permeability transition pore (PTP) complex, and this complex may promote Ca^{2+} release from the mitochondria under physiological and pathophysiological conditions (Ichas and Mazat, 1998; Bernardi, 1999; Crompton, 1999; Hajnóczky *et al.*, 2000). However, it is unclear whether the VDACS also form complexes with the IMM Ca^{2+} uniporters, which may facilitate mitochondrial Ca^{2+} uptake. In addition to the OMM permeability established by the VDAC, Bcl-2 family proteins induce a large increase in the permeability of the OMM during apoptosis (Green and Reed, 1998; Vander Heiden and Thompson, 1999; Desagher and Martinou, 2000; Korsmeyer *et al.*, 2000; Kroemer and Reed, 2000). This is due to an interaction between proapoptotic (e.g. Bid, Bad, Bak and Bax) and antiapoptotic (Bcl-2 and Bcl-x_L) proteins in the mitochondrial membrane, and this interaction may also involve the VDAC (Shimizu *et al.*, 1999) or ANT (Marzo *et al.*, 1998a). The increased OMM permeability allows for the release of large proteins from the intermembrane space to the cytosol [cytochrome *c* (Liu *et al.*, 1996), apoptosis-inducing factor (Susin *et al.*, 1999a), caspases (Krajewski *et al.*, 1999; Samali *et al.*, 1999; Susin *et al.*, 1999b), Smac/DIABLO (Du *et al.*, 2000; Verhagen *et al.*, 2000), endonuclease G (Li *et al.*, 2001)]. So Ca^{2+} may certainly traverse the newly formed holes in the OMM. In many apoptotic paradigms, the PTP opening results in permeabilization of the IMM simultaneously with the OMM (Marchetti *et al.*, 1996; Lemasters *et al.*, 1998; Marzo *et al.*, 1998b; Scorrano *et al.*, 2001) but, in other models, selective permeabilization of the OMM occurs and the IMM damage is delayed (e.g. von Ahsen *et al.*, 2000;

Mootha *et al.*, 2001; Waterhouse *et al.*, 2001). We thought that investigation of the effect of selective OMM permeabilization on the IP₃-induced [Ca²⁺]_m rise may allow us to revisit the role of OMM permeability in the control of calcium signal propagation from ER to mitochondria in the cells.

In response to engagement of the death receptors, a BH3-only Bcl-2 family protein, Bid, is cleaved by caspase-8 and, subsequently, the truncated C-terminus Bid (tcBid) induces release of apoptotic factors from mitochondria (Li *et al.*, 1998; Luo *et al.*, 1998; Gross *et al.*, 1999). tcBid has been demonstrated to translocate from cytosol to the mitochondria (Li *et al.*, 1998; Luo *et al.*, 1998; Gross *et al.*, 1999), and to exhibit high affinity for the lipid domains of the OMM at the contact sites (Lutter *et al.*, 2000, 2001). Studies with different experimental systems have resulted in numerous mechanisms that may mediate tcBid-induced cytochrome *c* release. These include ion channel activity (Schendel *et al.*, 1999), destabilization of the lipid bilayer by tcBid on its own (Kudla *et al.*, 2000) and interaction of tcBid with proapoptotic Bcl-2 family proteins [Bax (Wang *et al.*, 1996; Desagher *et al.*, 1999; Eskes *et al.*, 2000) and Bak (Wei *et al.*, 2000)] or components of the PTP (Zamzami *et al.*, 2000). Although the exact mechanism underlying tcBid-induced membrane permeabilization remains elusive, several reports showed that the IMM barrier function is maintained during tcBid-induced release of apoptotic factors from the mitochondria (e.g. von Ahsen *et al.*, 2000; Mootha *et al.*, 2001; Waterhouse *et al.*, 2001). Here we first show that tcBid evokes selective permeabilization of the OMM in permeabilized RBL-2H3 cells that we have established previously as a model for local Ca²⁺ signalling between IP₃ receptors and mitochondria (Csordás *et al.*, 1999; Pacher *et al.*, 2000). We show that treatment with tcBid promotes propagation of the IP₃-induced [Ca²⁺]_c signal to the mitochondria. In contrast, we find that tcBid does not affect the [Ca²⁺]_m rise during sustained and global [Ca²⁺]_c elevations. From our data, a novel model emerges that the permeability of the OMM limits the delivery of IP₃-linked [Ca²⁺]_c spikes and oscillations to the mitochondria. This is because the short-lasting high [Ca²⁺]_c microdomains in the vicinity of IP₃ receptors require free passage of Ca²⁺ through the OMM to establish optimal activation of the mitochondrial Ca²⁺ uptake sites. Interestingly, an increase in the OMM permeability also delays the deactivation of the Ca²⁺ uptake sites during IP₃-induced Ca²⁺ release. Thus our study confirms that the OMM permits Ca²⁺ delivery to the IMM during sustained [Ca²⁺]_c elevations, but challenges the freely Ca²⁺-permeable model of the OMM and reveals an important role for the OMM in the local control of Ca²⁺ signalling between ER and mitochondria.

Results and discussion

Permeabilization of the OMM by tcBid enhances the IP₃-induced [Ca²⁺]_m signal

Recent evidence suggests that tcBid associates with the OMM at the contact sites (Lutter *et al.*, 2000, 2001), evokes selective permeabilization of the OMM and, in turn, cytochrome *c* release (Li *et al.*, 1998; Luo *et al.*, 1998; Gross *et al.*, 1999). We speculated that tcBid may

Fig. 1. tcBid induces selective permeabilization of the OMM and promotes propagation of the IP₃-induced [Ca²⁺]_c signal to the mitochondria in suspensions of permeabilized RBL-2H3 cells. (A) The permeabilized cells were treated with tcBid (200 nM for 5 min) or solvent, then centrifuged, and the supernatants (cytosol) and pellets (membrane) were resolved by 15% SDS-PAGE followed by immunoblotting for cytochrome *c* (left panel). To evaluate the respective roles of cell permeabilization and tcBid in cytochrome *c* release, cells were exposed to varying concentrations of digitonin for 5 min prior to treatment with solvent or tcBid (100 nM for 5 min) (right panel). (B) ΔΨ_m was monitored in suspensions of permeabilized cells incubated in the presence of tcBid (red) or solvent (black). Additions were: tcBid (200 nM), CaCl₂ (2 μM, 2Ca), IP₃ (10 μM) and uncoupler (Unc; FCCP/oligomycin 5 μg/ml of each). Inset: tcBid (20 nM)-induced mitochondrial depolarization in the presence of oligomycin. Incubations were carried out in the presence of cytochrome *c* (10 μM; purple trace), cyclosporin A (5 μM; blue trace) or solvent (red trace). (C) Cytosolic [Ca²⁺]_c was followed with rhod2/FA added to the medium (lower panel) and [Ca²⁺]_m was measured using compartmentalized fura2FF (upper panel). tcBid- (200 nM), CaCl₂- (2 μM, 2Ca) and IP₃- (10 μM) induced [Ca²⁺]_c and [Ca²⁺]_m responses were recorded in the absence or presence of uncoupler (Unc; FCCP/oligomycin 5 μg/ml of each). The data are representative of 3–7 different experiments.

allow us to determine whether propagation of [Ca²⁺]_c signals into the mitochondria is limited by the Ca²⁺ permeability of the OMM in the cells. Treatment of digitonin-permeabilized RBL-2H3 cells with tcBid (200 nM for 5 min) resulted in redistribution of cytochrome *c* from mitochondria to the cytosol (Figure 1A, left), indicating permeabilization of the OMM. Notably, incubation of the

cells with digitonin *per se* caused permeabilization of the plasma membrane (<5% Trypan Blue exclusion), but did not evoke cytochrome *c* release (Figure 1A, right). The OMM barrier resisted even a 3-fold higher concentration of digitonin (Figure 1A, right). Furthermore, in the absence of digitonin, treatment with tcBid did not result in cytochrome *c* redistribution from mitochondria to the cytosol (Figure 1A, right), suggesting that tcBid did not permeabilize the plasma membrane and OMM in intact cells.

Although depletion of mitochondrial cytochrome *c* inhibits the respiratory chain activity, if the IMM is intact, generation of $\Delta\Psi_m$ may be maintained at the expense of added ATP. Measurements of $\Delta\Psi_m$ confirmed that tcBid elicited only a small depolarization when mitochondria were energized with 2 mM succinate (complex II substrate) in the presence of 2 mM MgATP (Figure 1B, compare with the complete depolarization induced by uncoupler). In contrast, the $\Delta\Psi_m$ dissipated rapidly if tcBid was added to cells pre-treated with an inhibitor of the F_1F_0 -ATPase, oligomycin (5 μ g/ml) (Figure 1B, inset). The oligomycin-dependent depolarization was inhibited effectively by exogenous cytochrome *c* (20 μ M), illustrating that depletion of cytochrome *c* accounted for the depolarization (Figure 1B, inset). Notably, tcBid-induced mitochondrial depolarization was not inhibited by cyclosporin A (5 μ M), an agent that abolished the loss of $\Delta\Psi_m$ mediated by opening of the PTP complex ($n = 2$, not shown). These results show that exposure to tcBid evoked permeabilization of the OMM, whereas the integrity of the IMM was preserved in RBL-2H3 cells. After cytochrome *c* depletion, reverse operation of the mitochondrial F_1F_0 -ATPase allowed for generation of $\Delta\Psi_m$.

Simultaneous measurements of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ (Figure 1C, left) showed that IP_3 -induced Ca^{2+} release evoked a $[Ca^{2+}]_c$ rise (lower part) closely followed by a $[Ca^{2+}]_m$ increase (upper traces). The global $[Ca^{2+}]_c$ increase caused by IP_3 was similar to the effect of 2 μ M $CaCl_2$, whereas only IP_3 evoked a large $[Ca^{2+}]_m$ response, illustrating the effective delivery of IP_3 -induced Ca^{2+} release to the mitochondria, that has been shown to be due to a local Ca^{2+} transfer between IP_3 receptors and mitochondrial Ca^{2+} uptake sites (Rizzuto *et al.*, 1998; Csordás *et al.*, 1999). Addition of tcBid (200 nM) did not change the basal $[Ca^{2+}]_c$ or $[Ca^{2+}]_m$ much, and did not affect the initial $[Ca^{2+}]_c$ elevation evoked by IP_3 , but caused a marked increase in the $[Ca^{2+}]_m$ rise (Figure 1C, left, upper red versus black traces). On average, the $[Ca^{2+}]_m$ signal was increased by $19.5 \pm 4.2\%$ ($n = 7$, $P < 0.01$). Notably, tcBid also accelerated the decay of the IP_3 -induced $[Ca^{2+}]_c$ rise (Figure 1C, left, lower red versus black traces) and this effect could not result from a rapid reuptake of released Ca^{2+} to the ER, since IP_3 was added together with the ER Ca^{2+} pump inhibitor, thapsigargin (Tg). However, this experiment could not exclude the possibility that tcBid affected IP_3 -induced Ca^{2+} mobilization from the ER. To further discriminate between potential effects of tcBid on ER and mitochondrial Ca^{2+} handling, we repeated the above experiment in the presence of uncoupler (FCCP + oligomycin) that eliminated the driving force of mitochondrial Ca^{2+} uptake. The IP_3 -induced $[Ca^{2+}]_c$ rise was enhanced and the $[Ca^{2+}]_m$ rise was abolished by the uncoupler (Figure 1C, right, black traces). Under these conditions, tcBid failed to change the

$[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ responses (red traces), suggesting that tcBid did not affect Ca^{2+} release from the ER and that the above-described effects of tcBid resulted from changes in mitochondrial Ca^{2+} handling. Furthermore, a change in the IP_3 sensitivity could not account for any effects of tcBid, since a maximal dose of IP_3 (10 μ M) was used in all studies. One might also speculate whether the electron transport chain inhibition (Figure 1B) could account for the effects of tcBid on the $[Ca^{2+}]$ signal. However when we supplemented the medium with cytochrome *c* (20 μ M) to rescue the electron transport chain activity or we added a complex III inhibitor, antimycin A (5 μ M), tcBid could still accelerate the decay of the IP_3 -induced $[Ca^{2+}]_c$ signal ($n = 2$, not shown). Most recently, tcBid has also been reported to trigger reorganization of the IMM, and this process was inhibited by cyclosporin A (Scorrano *et al.*, 2002). Thus it is important to note that in our study, the effect of tcBid on the IP_3 -induced calcium signals was not inhibited by cyclosporin A (5 μ M, $n = 2$).

Taken together, these observations suggest that tcBid selectively permeabilized the OMM and the effect of tcBid on the mitochondria yielded enhanced delivery of the IP_3 -driven $[Ca^{2+}]_c$ signal to the mitochondria. Since tcBid caused a small depolarization in our experiments (Figure 1B), the $\Delta\Psi_m$ component of the driving force of Ca^{2+} uptake was decreased rather than increased. This decrease in the driving force of the mitochondrial Ca^{2+} uptake was significant, because when we used a low dose of uncoupler (FCCP, 20 nM) to establish a depolarization comparable with the effect of tcBid, we noted significant inhibition of mitochondrial Ca^{2+} uptake during IP_3 -induced Ca^{2+} mobilization ($n = 3$, not shown). Thus, the enhanced IP_3 -induced $[Ca^{2+}]_m$ signal in tcBid-pre-treated cells could be due to the fact that the permeabilized OMM optimized delivery of the localized Ca^{2+} release to the mitochondrial Ca^{2+} uptake sites located in the IMM. This could augment the $[Ca^{2+}]$ gradient between the two sides of the IMM, increasing the driving force of Ca^{2+} uptake. In addition, Ca^{2+} could exert an allosteric control on the Ca^{2+} uptake sites, increasing the Ca^{2+} permeability of the IMM. It was also possible that tcBid released a negative modulator of the sites that mediate Ca^{2+} uptake through the IMM.

In addition to the studies with tcBid, we also investigated whether Bax, another proapoptotic Bcl-2 family protein, induces cytochrome *c* release and increases the IP_3 -induced $[Ca^{2+}]_m$ signals. The oligomeric form of Bax (500 nM) evoked cytochrome *c* release in permeabilized RBL-2H3 cells, but >30 min incubation was required to obtain a release similar to that evoked by tcBid ($n = 3$, data not shown). Because the local Ca^{2+} coupling between ER and mitochondria is not preserved for so long after cell permeabilization, we could perform a 5–7 min pre-incubation with Bax before addition of IP_3 . Under these conditions, only a relatively small cytochrome *c* release and no increase in the IP_3 -induced $[Ca^{2+}]_m$ signal were observed ($n = 3$, data not shown).

tcBid fails to affect the $[Ca^{2+}]_m$ response evoked by sustained and global $[Ca^{2+}]_c$ signals

During IP_3 -induced Ca^{2+} release, due to local interactions between ER and mitochondria, subdomains of the mitochondrial surface could be exposed to a high $[Ca^{2+}]$

Fig. 2. No effect of tcBid on delivery of Tg- and Ca²⁺-induced sustained [Ca²⁺]_c signals to the mitochondria. Cytosolic [Ca²⁺]_c was followed with rhod2/FA added to the medium (lower panel) and [Ca²⁺]_m was measured using compartmentalized fura2FF (upper panel). tcBid (200 nM, red) or solvent (black) was added to the permeabilized cells 5 min before treatment with either Tg (2 μM) or CaCl₂ (10 μM, 10Ca) in the absence or presence of uncoupler (Unc; FCCP/oligomycin 5 μg/ml of each). These data are representative of three different experiments.

microdomain that decays very quickly to the level of the measured global [Ca²⁺]_c as the ER becomes depleted. Thus, high Ca²⁺ permeability of the OMM could be critical in activation of mitochondrial Ca²⁺ uptake by the short-lasting high Ca²⁺ microdomain. However, enhancement of the permeability of the OMM by tcBid may be less important for mitochondrial Ca²⁺ uptake evoked by sustained [Ca²⁺]_c elevations. We observed that the Tg-induced gradual depletion of the ER store resulted in a slow [Ca²⁺]_c rise (Figure 2, left, lower part) associated with a delayed and very small [Ca²⁺]_m increase (Figure 2, left, upper part). Neither the [Ca²⁺]_c, nor the [Ca²⁺]_m response was modified by tcBid (Figure 2, left). We also established a sustained [Ca²⁺]_c elevation to ~3 μM by addition of 10 μM CaCl₂ (Figure 2, right). Again, tcBid pre-treatment did not augment the [Ca²⁺]_m response (Figure 2, right, upper part). Furthermore, when a [Ca²⁺]_c rise similar to the IP₃-induced elevation of global [Ca²⁺]_c (~0.7–1 μM) was established by addition of 2–3 μM CaCl₂, only a slow and small [Ca²⁺]_m rise occurred that was not increased by tcBid (*n* = 3, not shown). Thus, the basal Ca²⁺ permeability of the OMM was sufficient to allow activation of the Ca²⁺ uptake sites in the IMM by the sustained [Ca²⁺]_c increases. Since tcBid failed to augment the Tg- and Ca²⁺-induced Ca²⁺ rise, it is unlikely that tcBid promoted the effect of IP₃ by releasing a negative modulator of the Ca²⁺ uptake sites.

tcBid enhances the net mitochondrial Ca²⁺ uptake evoked by IP₃

The [Ca²⁺]_m response reflects the combined effects of the mitochondrial Ca²⁺ uptake, intramitochondrial Ca²⁺ buffering and Ca²⁺ efflux from the mitochondria. To evaluate the net mitochondrial Ca²⁺ uptake in the case of each stimulus (IP₃, Tg and Ca²⁺ addition), we calculated the difference of the [Ca²⁺]_c signals obtained in the presence and absence of uncoupler (Figure 3A). During IP₃-induced Ca²⁺ release, a net mitochondrial Ca²⁺ uptake was apparent immediately and increased further for a short time period (left, black trace). In the presence of tcBid, the

Fig. 3. tcBid enhances the net mitochondrial Ca²⁺ uptake evoked by IP₃-induced Ca²⁺ release. (A) Net mitochondrial Ca²⁺ uptake evoked by IP₃ (10 μM), Tg (2 μM) and CaCl₂ (10 μM, 10Ca) in the absence (black traces) and presence of tcBid (200 nM, red traces). To calculate net mitochondrial Ca²⁺ accumulation, the IP₃-induced [Ca²⁺]_c signal was subtracted from a parallel experiment carried out in the presence of uncoupler. The traces represent mean values calculated for experiments performed with 3–7 different cell cultures (IP₃, seven; Tg, three; and 10Ca, three), and each experiment was carried out in duplicate. (B) Rate of mitochondrial Ca²⁺ uptake during IP₃-induced Ca²⁺ mobilization in the absence (black) or presence of tcBid (red). Decay of the global [Ca²⁺]_c signal (60 s) was normalized to the initial peak value as follows: decay rate (%/min) = {([Ca²⁺]_{c-peak} – [Ca²⁺]_{c-60 s}) / [Ca²⁺]_{c-peak}} × 100. Calculations were carried out with the data obtained in the absence (left) and presence of uncoupler. The data represent means ± SE from seven different experiments.

IP₃-induced Ca²⁺ uptake was larger than in naive cells, in particular, the sustained phase was augmented and prolonged (red trace). With regard to evaluation of the time course of the IP₃ effect, it is also worthy of note that the initial upstroke is very sensitive to the temporal alignment of the two records of [Ca²⁺]_c used for the calculation (+ and – uncoupler), and so evaluation of the initial phase is not as accurate as that of the sustained phase. In the case of Tg and Ca²⁺, the net mitochondrial Ca²⁺ uptake gradually increased over a relatively long time period and no effect of tcBid appeared (middle and right, black versus red traces). Thus, permeabilization of the OMM by tcBid augmented only the IP₃-induced mitochondrial Ca²⁺ uptake. The effect of tcBid on the mitochondrial Ca²⁺ uptake was quantitated by calculation of the decay rate of the IP₃-induced [Ca²⁺]_c rise that was recorded as shown in Figure 1C, left (Figure 3B). On average, the mitochondrial Ca²⁺ uptake rate was increased from 11.1 ± 1.3 to 37.7 ± 5.5%/min in tcBid-pre-treated cells (*n* = 7, *P* < 0.005).

tcBid augmented both the [Ca²⁺]_m rise and mitochondrial Ca²⁺ uptake during IP₃-induced Ca²⁺ mobilization, but the maximal effect on [Ca²⁺]_m took place in <20 s, whereas the effect on net mitochondrial Ca²⁺ uptake developed for >30s. Based on the above studies and on the Mn²⁺ quench measurements (Figure 4), during the initial phase of Ca²⁺ release, a relatively small increase in IMM Ca²⁺ permeability and mitochondrial Ca²⁺ uptake was

Fig. 4. tcBid facilitates the effect of IP₃ on permeability of the mitochondrial Ca²⁺ uptake sites. (A) Fluorescence quenching was initiated by addition of 50 μM MnCl₂ (Mn²⁺) to fura2FF-loaded permeabilized cells. Permeabilized cells were pre-treated with tcBid (200 nM for 5 min, traces b and d) or solvent (traces a and c). The traces show: no IP₃ addition (a and b), IP₃ added (c and d) 10 s after Mn²⁺ (left), 10 s (290 s, middle left), 30 s (270 s, middle right) and 60 s (240 s, right) before Mn²⁺, respectively. Fura2FF fluorescence (F) was normalized to the initial fluorescence (F₀). The free Mn²⁺ (~3 μM, buffered by ATP) exceeds the K_d for Mn²⁺ binding to fura2FF by 2–3 orders of magnitude, which ensures essentially stoichiometric quench of compartmentalized fura2FF as Mn²⁺ enters the mitochondrial matrix. In the lower row, the IP₃-dependent component of the Mn²⁺ quench responses was obtained by subtraction from a parallel quench response in the absence of IP₃ (–tcBid, a–c; +tcBid, b–d), and non-linear regression (single exponential) fits were calculated for the first 30 s after Mn²⁺ addition (solid lines). (B) Pool size and rate constant calculated for the IP₃-dependent Mn²⁺ quench obtained in the absence (grey) and presence of tcBid (red). Calculations were carried out using the traces shown in (A, lower row). Thus, the pool size of the IP₃-dependent Mn²⁺ quench is expressed as a fraction of the initial fluorescence (ΔF/F₀). The rate constant was calculated for a 30 s period (from 300 to 330 s, Mn²⁺ added at 300 s) and is expressed as s⁻¹. Bi-exponential kinetics appeared to provide a better fit to a few recordings of Mn²⁺ quench in control cells pre-treated with IP₃ for 30 and 60 s, but for calculation of the rate constants we used the single exponential kinetic that gave an excellent fit to most of the traces. The symbols show the results with two different cell cultures; each experiment was carried out in duplicate.

sufficient to augment the [Ca²⁺]_m response to IP₃, whereas the sustained increase in Ca²⁺ permeability and mitochondrial Ca²⁺ uptake did not enhance the [Ca²⁺]_m signal further. An important consideration is that the mitochondrial Ca²⁺ efflux mechanisms exhibit very low activity in RBL-2H3 cells as compared with cardiac myotubes and hepatocytes (Csordás *et al.*, 1999; Pacher *et al.*, 2000). Thus a sustained modest increase in mitochondrial Ca²⁺ influx may result in a progressive increase in the net mitochondrial Ca²⁺ uptake. However, the sustained increase in mitochondrial Ca²⁺ uptake was not associated with a progressive increase in [Ca²⁺]_m. One clue to this is that up-regulation of mitochondrial Ca²⁺ buffering has been reported during Ca²⁺ uptake to the mitochondria (David, 1999). We speculate that the sustained component of mitochondrial Ca²⁺ uptake failed to increase [Ca²⁺]_m further due to an increase in the Ca²⁺ buffer species. For example, mitochondrial Ca²⁺ uptake is associated with phosphate uptake that may account for a gradual increase in Ca²⁺ buffering capacity.

We also noted that in tcBid-treated cells, the IP₃-dependent net mitochondrial Ca²⁺ uptake was sustained (>30 s), even though the net Ca²⁺ efflux from the ER decayed in <5 s (calculated as the first derivative of the [Ca²⁺]_c signal, not shown) and the high Ca²⁺ microdomain could not be maintained after cessation of the Ca²⁺ release. However, Ca²⁺ has been shown to exert a slow allosteric control on mitochondrial Ca²⁺ uptake sites *in vitro* (Kröner, 1986) as well as *in vivo* (Hajnóczky *et al.*, 1995). Recent evidence also suggests that ryanodine receptors may mediate mitochondrial Ca²⁺ uptake in cardiac muscle (Beutner *et al.*, 2001), supporting the view that Ca²⁺-gated Ca²⁺ channels may serve as Ca²⁺ uptake sites in the IMM (Litsky and Pfeiffer, 1997). Thus the initial [Ca²⁺]_c rise could establish sensitization/activation of the Ca²⁺ uptake sites, allowing for some Ca²⁺ uptake after the dissipation of the high [Ca²⁺]_c microdomain. Furthermore, the tcBid-induced permeabilization of the OMM could evoke a large increase (>3-fold) of this effect.

tcBid facilitates the effect of IP₃ on permeability of the mitochondrial Ca²⁺ uptake sites

To follow the effect of tcBid on the net conductance of the mitochondrial Ca²⁺ uptake sites throughout extended periods of IP₃ exposure, we developed a method using Mn²⁺ as a Ca²⁺ surrogate to examine the permeability of the mitochondrial Ca²⁺ uptake sites. In RBL-2H3 cells, we could load the mitochondria with fura2FF that shows several hundred-fold higher affinity towards Mn²⁺ than Ca²⁺ and was almost completely quenched by Mn²⁺. It has also been shown that Mn²⁺ can enter the mitochondria through the Ca²⁺ uptake sites (Vinogradov and Scarpa, 1973; Gunter and Pfeiffer, 1990; Gunter *et al.*, 1994). The Mn²⁺ quench experiments were carried out using an excitation wavelength of 357 nm, where fura2FF is insensitive to [Ca²⁺] changes. Addition of Mn²⁺ to the permeabilized cells resulted in a prompt quench of released cytosolic fura2FF (~20%), followed by a slow basal quench of the compartmentalized dye (Figure 4A, traces a). Approximately 60% of the compartmentalized fura2FF was quenched rapidly when IP₃ was added (Figure 4A, left, trace c). However, IP₃ failed to induce

Mn²⁺ quench if: (i) the ER Ca²⁺ store was depleted by Tg; (ii) the $\Delta\Psi_m$ was dissipated by FCCP or antimycin; or (iii) the IMM Ca²⁺ uptake sites were inhibited by ruthenium red (2 μ M) prior to IP₃ addition (not shown). These data show that the IP₃-dependent Mn²⁺ quench was triggered by the Ca²⁺ release and that Mn²⁺ acted as a Ca²⁺ surrogate, passing through the Ca²⁺ uniporter. Since Mn²⁺ quenches fura2FF in an essentially stoichiometric manner until the dye becomes saturated, the rate of Mn²⁺ quench can be used as a measure of the net permeability properties of the mitochondrial Ca²⁺ uptake sites. To examine the permeability properties of the Ca²⁺ uniporter after various periods of IP₃ exposure, Mn²⁺ was added to permeabilized cells pre-incubated in the presence of IP₃ for 10, 30 and 60 s, respectively (Figure 4A, left to right). As illustrated by the c traces (shown in black), the rate and magnitude of the Mn²⁺ quench displayed a monotonic decrease with the time of IP₃ pre-treatment, suggesting that the IP₃-induced enhancement of the Mn²⁺ permeability decayed gradually.

Pre-treatment with tcBid did not change the Mn²⁺ quench rate in the absence of IP₃ (Figure 4A, left, trace b versus trace a), suggesting that permeabilization of the outer membrane by itself did not affect Ca²⁺ entry through the IMM. However, the IP₃-dependent Mn²⁺ quench of compartmentalized fura2FF was enhanced by tcBid (traces d versus traces c) particularly at 30 and 60 s after IP₃ addition (Figure 4A, right). To visualize the tcBid-induced changes better, the IP₃-dependent component of Mn²⁺ quench was obtained for each time point by subtraction of the trace recorded in the presence of IP₃ from a parallel quench response in the absence of IP₃ and, subsequently, non-linear regression analysis was carried out (Figure 4A, lower panel: -tcBid, a-c; + tcBid, b-d). The IP₃-induced Mn²⁺ quench followed single exponential kinetics, and the pool size as well as the rate constant was increased by tcBid (Figure 4B). These results provide direct evidence that permeabilization of the OMM by tcBid facilitated the activation of the Ca²⁺ uptake sites in the IMM during IP₃-induced Ca²⁺ release. The tcBid-induced sustained increase in IP₃-dependent Mn²⁺ quench provides a mechanism that underlies the sustained stimulation of IP₃-dependent Ca²⁺ uptake to the mitochondria (Figure 3). Facilitation of the transmission of the Ca²⁺ signal from the IP₃ receptors to the IMM by tcBid enhances rapid activation of the Ca²⁺ uptake sites. Furthermore, a slower decay from the relatively high initial activity results in prolonged stimulation of the mitochondrial Ca²⁺ uptake. The OMM permeabilization by tcBid is likely to facilitate the Ca²⁺ uptake sites by optimizing the exposure of the allosteric Ca²⁺-binding site to the high local [Ca²⁺]_c rise, since reproduction of the IP₃-induced global [Ca²⁺]_c increase (~0.7–1 μ M) by addition of 2–3 μ M CaCl₂ yielded only a very small increase in the Mn²⁺ quench rate (data not shown).

Localization of the holes in OMM controls the IP₃-induced mitochondrial calcium signal

Release of apoptotic proteins is likely to occur through OMM pores distributed throughout the entire mitochondrial surface. However, to support a local Ca²⁺ signal transmission, the OMM pores should be positioned precisely between the IP₃ receptors and IMM Ca²⁺ uptake sites at the interfacing domains of the ER and mitochon-

Fig. 5. Local calcium signalling is less sensitive than cytochrome *c* release to tcBid, suggesting a role for targeting of the OMM pores to the ER–mitochondrial interface. (A) Dose–response for cytochrome *c* release evoked by tcBid. Cytosolic samples were generated by rapid filtration of the cells. The data are representative of two different experiments. (B) Dose–response for tcBid-induced potentiation of the IP₃-dependent [Ca²⁺]_m signal (upper) and for enhancement of the mitochondrial Ca²⁺ uptake rate (lower). The data represent means $\pm$ SE from 3–5 experiments.

dria. Assuming that the number of sites of OMM permeabilization is proportional to the concentration of tcBid but each pore permeates cytochrome *c* and Ca²⁺, one can speculate whether depletion of cytochrome *c* requires less tcBid than facilitation of delivery of the IP₃-induced Ca²⁺ signal to the mitochondria. As shown in Figure 5A, 2 nM tcBid evoked substantial cytochrome *c* release and 5 nM caused an almost maximal release response. In contrast, no enhancement of the IP₃-induced [Ca²⁺]_m rise or stimulation of mitochondrial Ca²⁺ uptake was elicited by 2 and 5 nM tcBid (Figure 5B). The promotion of IP₃-induced mitochondrial calcium signalling was noted at 20 nM tcBid and maximal response was achieved using 200 nM tcBid (Figure 5B). Thus, despite the relatively small size of Ca²⁺, facilitation of Ca²⁺ signal propagation to the mitochondria requires more extensive permeabilization of the OMM than cytochrome *c* release. This is consistent with the idea that a few, randomly distributed pores are sufficient to allow release of mitochondrial factors from the intermembrane space to the cytosol, whereas strategic localization of the OMM pores to the space between interacting ER and IMM Ca²⁺ transporters is required to support recognition of the highly localized and transient ER Ca²⁺ release by the mitochondria.

Taken together, the present experiments showed that exposure to tcBid enhanced the OMM permeability without destroying the IMM barrier. Since we observed bi-directional passage of cytochrome *c* through the OMM, it is likely that the OMM also permitted free translocation

of Ca^{2+} in tcBid-treated cells. Our data also showed that exposure to tcBid promoted Ca^{2+} signal propagation to the mitochondria. This did not reflect an effect of tcBid on the ER Ca^{2+} store, since tcBid failed to affect IP_3 - or Tg-induced Ca^{2+} release from the ER (Figures 1C and 2) and augmented the IP_3 -induced $[\text{Ca}^{2+}]_m$ signal when ER Ca^{2+} uptake was inhibited (Figure 1C). Then we evaluated whether permeabilization of the OMM or another effect on the mitochondria accounts for tcBid-induced facilitation of mitochondrial Ca^{2+} signalling. For example, tcBid might release a negative modulator of the Ca^{2+} uptake sites from the intermembrane space, but we showed that tcBid did not affect the propagation of global and sustained $[\text{Ca}^{2+}]_c$ signals to the mitochondria (Figure 2). It was also unlikely that tcBid augmented the driving force of mitochondrial Ca^{2+} uptake as the $\Delta\Psi_m$ was slightly attenuated in tcBid-treated cells (Figure 1B). Thus we thought that stimulation of local Ca^{2+} delivery to the mitochondria was due to the permeabilization of the OMM or to a shortening of the distance between ER and Ca^{2+} uptake sites in the IMM. However, electron microscopic and electron tomography analysis did not show a tcBid-induced change in the shape of the mitochondrial surface (von Ahsen *et al.*, 2000) and the Tg-induced mitochondrial Ca^{2+} signalling was not affected by tcBid (Figures 2 and 3), arguing against the idea that tcBid brought the ER and mitochondria closer to each other. Thus we propose that facilitation of mitochondrial Ca^{2+} signalling took place because tcBid evoked selective permeabilization of the OMM. This model challenges the dogma that the OMM is freely permeable to Ca^{2+} . However, the 'leaky OMM' was envisaged based on studies that were carried out with isolated mitochondria, and the OMM permeability could have changed during the preparation of mitochondria. Furthermore, the local $[\text{Ca}^{2+}]_c$ signals delivered from IP_3 receptors to the mitochondria in the cells decay very quickly, underscoring the requirement for a very effective transfer between IP_3 receptors and the Ca^{2+} uptake sites in the IMM. Since tcBid seems to interact selectively with the OMM at the contact sites (Lutter *et al.*, 2000), we postulate that the contact site regions at the ER-mitochondrial interface may be particularly important in local Ca^{2+} signalling. In the contact site regions, the tight association between the OMM and IMM narrows the gap between IP_3 receptors and mitochondrial Ca^{2+} uptake sites. Finally, evaluation of the decay phase of the IP_3 -induced $[\text{Ca}^{2+}]_c$ signal and the Mn^{2+} quench measurements allowed us to establish the kinetics of the Ca^{2+} uptake sites during IP_3 -induced Ca^{2+} release (Figures 3 and 4). The initial large activation was augmented in the presence of tcBid but, more strikingly, the deactivation was slowed down, suggesting that the initial exposure to the high Ca^{2+} microdomain controlled the lifetime of the activated conformation of the Ca^{2+} uptake sites.

Conclusions

Our study demonstrated that transport of Ca^{2+} through the OMM limits propagation of Ca^{2+} spikes to the mitochondria. Although the Ca^{2+} permeability of the OMM is sufficient to ensure optimal activation of the Ca^{2+} uniporter during sustained $[\text{Ca}^{2+}]_c$ signals, the OMM attenuates exposure of the Ca^{2+} uptake sites to the short-lasting, high $[\text{Ca}^{2+}]_c$ microdomains generated by the neighbouring IP_3

receptors. Under physiological conditions, Ca^{2+} transport through the OMM is likely to be mediated by the pores formed by the VDACs (Mannella, 1992). Thus, changes in the level of expression, spatial distribution or activity of VDACs may affect the delivery of Ca^{2+} spikes to the mitochondria. Recently, Rosario Rizzuto's group provided direct evidence that overexpression of the VDAC facilitates Ca^{2+} signal propagation to the mitochondria (E.Rapizzi, P.Pinton, G.Vandecasteele, G.Szabadkai, K.E.Fogarty and R.Rizzuto, submitted). In line with our results, overexpression of the VDAC promoted IP_3 receptor-driven mitochondrial Ca^{2+} signalling but failed to affect the $[\text{Ca}^{2+}]_m$ rise evoked by capacitative Ca^{2+} entry or global $[\text{Ca}^{2+}]_c$ elevation (E.Rapizzi, P.Pinton, G.Vandecasteele, G.Szabadkai, K.E.Fogarty and R.Rizzuto, submitted). Since the IP_3 receptor-driven mitochondrial Ca^{2+} uptake is an important regulator of mitochondrial ATP production and also exerts feedback control on cytosolic Ca^{2+} signalling, VDAC-dependent changes in the local Ca^{2+} control between ER and mitochondria may affect many cellular functions. During apoptosis, if tcBid-induced permeabilization of the OMM does not cause dissipation of the $\Delta\Psi_m$ (Waterhouse *et al.*, 2001), facilitation of mitochondrial Ca^{2+} uptake may result in mitochondrial Ca^{2+} overload. This may evoke PTP-mediated Ca^{2+} -induced Ca^{2+} release that in turn recruits the neighbouring mitochondria (Ichas *et al.*, 1997), providing a mechanism that may also contribute to the coordinated execution of the mitochondrial phase of cell death (Pacher and Hajnóczky, 2001). Future studies will address whether tcBid interacts with VDACs to increase the OMM Ca^{2+} permeability or if channel-forming Bcl-2 family proteins create the molecular structures that support an increase in Ca^{2+} transport at the contact sites.

Materials and methods

Recombinant protein

Caspase 8-cleaved Bid (tcBid) and oligomeric Bax were produced as described earlier (Desagher *et al.*, 1999; Antonsson *et al.*, 2001).

Fluorometric measurements of $[\text{Ca}^{2+}]_c$, $[\text{Ca}^{2+}]_m$, Mn^{2+} quench and $\Delta\Psi_m$ in suspensions of permeabilized cells

RBL-2H3 cells were cultured, loaded with fura2FF for measurements of $[\text{Ca}^{2+}]_m$, permeabilized with digitonin using a protocol that preserves the functional integrity of the calcium coupling between ER and mitochondria, and incubated as described earlier (Csordás *et al.*, 1999; Csordás and Hajnóczky, 2001). Briefly, fura2FF-loaded cells (5.5×10^6 cells/ml) were permeabilized in an intracellular medium (ICM) composed of 120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4 , 20 mM Tris-HEPES, 2 mM MgATP, 5% dextran and 1 $\mu\text{g}/\text{ml}$ each of antipain, leupeptin and pepstatin at pH 7.2 supplemented with 25–35 $\mu\text{g}/\text{ml}$ digitonin [Sigma, 50% (w/w)] for 5 min at 35°C, followed by washout of the released cytosolic fura2FF (125 g for 4 min). Cell permeabilization was evaluated by Trypan Blue exclusion and, after 5 min incubation, >95% of the cells were Trypan positive. Compartmentalized fura2FF has been shown to occur in the mitochondria of RBL-2H3 cells (Csordás *et al.*, 1999). Permeabilized cells were resuspended in ICM supplemented with 2 mM succinate and 0.25 μM rhod2/FA and maintained in a stirred thermostated cuvette at 35°C. Rhod2/FA was added to monitor $[\text{Ca}^{2+}]$ in the intracellular medium that exchanges readily with the cytosolic space. Fluorescence was monitored in a multiwavelength excitation dual-wavelength emission fluorimeter using 340 nm, 380 nm excitation and 500 nm emission for fura2FF, and 540 nm excitation and 580 nm emission for rhod2. In every experiment, five data triplets were obtained per second. Calibration of the Ca^{2+} signals was carried out at the end of each measurement as described previously (Csordás and Hajnóczky, 2001). In the Mn^{2+} quench experiments, excitation of fura2FF was also

carried out at 357 nm (five data points/s), where the fura2FF fluorescence was insensitive to Ca²⁺ changes. At the end of each Mn²⁺ quench measurement, a high concentration of Mn²⁺ (500 μ M MnCl₂) was added in the presence of ionomycin to quench compartmentalized fura2FF completely. Before normalization to the initial fluorescence, the residual signal autofluorescence of the cells was subtracted from the fluorescence signal. Fluorimetric measurements of $\Delta\Psi_m$ were carried out as described previously (Szalai *et al.*, 1999). Briefly, suspensions of cells were incubated in permeabilization medium in the presence of 800 nM JC-1 in a fluorimeter cuvette. Fluorescence was monitored using 490 nm excitation/535 nm emission for the monomeric form and 570 nm excitation/595 nm emission for the J-aggregate of JC1 (five data points/s). $\Delta\Psi_m$ is shown as the ratio of the fluorescence of J-aggregate and monomer forms of JC1.

Detection of cytochrome c release by western blotting

At the end of the fluorimetric measurements of $\Delta\Psi_m$ in suspensions of permeabilized cells, cytosol was separated from the membranes by centrifugation at 10 000 g for 10 min (data shown in Figure 1). Alternatively, suspensions of the permeabilized cells were rapidly filtered (0.45 μ m pore size cellulose acetate membrane; Whatman) using a syringeless filter device (data shown in Figure 5). This approach allowed us to obtain cytosol in <10 s. Supernatant or membrane or filtrate proteins (25 μ g) were resolved on a 15% SDS-polyacrylamide gel and western blotting was carried out for cytochrome c. To evaluate cytochrome c release occurring during cell permeabilization with digitonin, equal volumes of the supernatants obtained using 0, 35 and 100 μ g/ml digitonin were loaded onto the gel (Figure 1A, right). Mouse monoclonal anti-cytochrome c antibody was used with goat anti-mouse peroxidase conjugate for detection. Bound antibody was detected by enhanced chemiluminescence using the Supersignal reagent (Pierce).

Western blots are representative of three experiments. Every fluorimetry recording shown herein represents the mean response of $\sim 10^7$ cells. Furthermore, every recording was repeated at least once using the same cell preparation, and the difference between the parallels was very small in comparison with the differences between control and tcBid described in this study. For each agent (IP₃, Tg, 10Ca), measurements of the effect on control and tcBid-treated cells were obtained using the same cell preparation, and each pair was repeated in 2–7 cell preparations. The data combined from separate experiments are shown as mean $\pm$ SE. Significance of differences from the relevant controls was calculated by Student's *t*-test.

Acknowledgements

We would like to thank Drs Suresh K. Joseph and John G. Pastorino for critical reading of the manuscript. This work was supported by grants from the NIH and American Cancer Society (to G.H.). G.H. is a recipient of a Burroughs Wellcome Fund Career Award.

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Received August 9, 2001; revised February 12, 2002;
accepted March 7, 2002