The Role of the Mitochondrial Apoptosis Induced Channel MAC in Cytochrome *c* Release

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Permeabilization of the mitochondrial outer membrane is a crucial event during apoptosis. It allows the release of proapoptotic factors, like cytochrome *c*, from the intermembrane space, and represents the commitment step in apoptosis. The mitochondrial apoptosis-induced channel, MAC, is a high-conductance channel that forms during early apoptosis and is the putative cytochrome *c* release channel. Unlike activation of the permeability transition pore, MAC formation occurs without loss of outer membrane integrity and depolarization. The single channel behavior and pharmacology of reconstituted MAC has been characterized with patch-clamp techniques. Furthermore, MAC's activity is compared to that detected in mitochondria inside the cells at the time cytochrome *c* is released. Finally, the regulation of MAC by the Bcl-2 family proteins and insights concerning its molecular composition are also discussed.

KEY WORDS: Mitochondrial apoptosis-induced channel MAC; patch clamp; cytochrome *c*; Bax.

INTRODUCTION

Apoptosis is a phenomenon fundamental to higher eukaryotes and essential to mechanisms controlling tissue homeostasis. The release of cytochrome *c* and other proapoptotic factors from mitochondria is a pivotal early event in the apoptotic cascade of many cell types [reviewed in (Antonsson, 2004; Danial and Korsmeyer, 2004; Green *et al.*, 2004; Reed *et al.*, 2004)]. Once in the cytosol, cytochrome *c* and procaspase 9 bind the cytosolic protein apaf-1 and dATP to form apoptosomes that promote caspase activation and destruction of the cell (Liu *et al.*, 1996; Wang, 2001).

The mechanisms by which proapoptotic factors are released from mitochondria early in apoptosis are not well understood. It has been speculated that a permeability transition pore (PTP) in the inner membrane opens and causes swelling of the matrix space. As the inner membrane has a much greater surface area than the outer membrane, the ensuing swelling ruptures the outer membrane and spills cytochrome *c* and other proapoptotic proteins into the cytosol (Skulachev, 1996; Brenner and Kroemer, 2000; Kroemer and Reed, 2000). However, recent studies of cyclophilin D knockouts show that PTP is principally involved in necrosis and ischemia-reperfusion injury rather than intrinsic apoptosis (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005). Furthermore, release of cytochrome *c* also occurs in the absence of mitochondrial depolarization and without loss of outer membrane integrity in some cell types. These findings suggest the existence of a more selective mechanism of permeabilization, e.g., formation of a pore in the outer membrane (Antonsson *et al.*, 1997; Brenner and Kroemer, 2000; Chipuk *et al.*, 2004; De Giorgi *et al.*, 2002; Green *et al.*, 2004; Pavlov *et al.*, 2001). This alternative mechanism

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Key to abbreviations: MAC: Mitochondrial apoptosis-induced channel; PTP: Permeability transition pore; TOM: Translocase of the outer membrane; VDAC: Voltage-dependent anion-selective channel; IL-3: Interleukin 3.

is supported by the discovery of MAC, mitochondrial apoptosis-induced channel, a novel channel that forms in mitochondria early in apoptosis (Dejean *et al.*, 2005; Pavlov *et al.*, 2001).

MAC, is a high-conductance channel with an estimated mean diameter of >5 nm, large enough to allow the release of the ∼3 nm diameter cytochrome *c* from mitochondria (Guo *et al.*, 2004; Martinez-Caballero *et al.*, 2004; Dejean *et al.*, in press-a; Pavlov *et al.*, 2001) and hence MAC is a putative cytochrome *c* release channel. MAC activity has been detected early in the intrinsic apoptotic pathway about the time of cytochrome *c* release in multiple cell types (mouse embryonic fibroblasts and various clones of the hematopoietic FL5.12 cells and HeLa cells) (Pavlov *et al.*, 2001; Dejean *et al.*, 2005). In contrast, a recent report indicates that MAC forms only at a late stage of the extrinsic apoptotic pathway (Guihard *et al.*, 2004), reinforcing the notion that alternative pathways may also play a role in this commitment step of the cell death program. Note that the difference in the time of onset for MAC formation may be due to differences in the apoptosis inducers, pathways involved, and/or the cell type.

The BCL-2 family of proteins are essential regulators of the apoptotic cascade and has both pro- and antiapoptotic members (Antonsson, 2004; Cory *et al.*, 2003; Danial and Korsmeyer, 2004; Newmeyer and Ferguson-Miller, 2003). Interestingly, many of these proteins are associated with mitochondria. The BCL-2 family has three subfamilies: (1) antiapoptotic proteins represented by Bcl-2 and Bcl-xL; (2) the multidomain, proapoptotic proteins including Bax and Bak; and (3) "BH3 domain only" proteins which are small proapoptotic proteins represented by t-Bid, Bad, and Bik that are only comprised of the BH3 death domain. It has been hypothesized that the cell's decision to live or die is controlled, at least in part, by whichever group(s) is in excess.

"BH3 domain only" proteins are sentinels for cellular damage and function. As initiator cell-death signaling molecules, each "BH3 domain only" protein is most likely coupled to a specific death signal. "BH3 domain only" proteins translate the death signal to the multidomain BCL-2 members by either activating proapoptotic Bax/Bak or inactivating antiapoptotic Bcl-2/Bcl-xL. It has been shown that "BH3 domain only" proteins directly or indirectly activate Bax/Bak to induce apoptosis. In contrast, antiapoptotic Bcl-2 and Bcl-xL prevent activation of Bax/Bak either by direct interaction or by "chelating" the activating "BH3 domain only" molecules (Chittenden *et al.*, 1995; Hirotani *et al.*, 1999). The balance among the three groups is finely regulated by a variety of gene products, including p53 (Chipuk *et al.*, 2004).

There is considerable evidence that members of the BCL-2 family exert their pro- and antiapoptotic effects by regulating the release of cytochrome *c* from mitochondria, but the mechanisms of action are still subject to speculation (Antonsson, 2004; Brenner and Kroemer, 2000; Chipuk *et al.*, 2004; Reed *et al.*, 2004; Saito *et al.*, 2000; Scorrano and Korsmeyer, 2003; Wei *et al.*, 2001).

Regulation of MAC by BCL-2 Family Proteins

There is substantial support of the notion that BCL-2 family proteins regulate the formation of MAC. The detection of MAC activity in interleukin-3 (IL-3) starved FL5.12 cells was eliminated by overexpression of the antiapoptotic protein Bcl-2. However, expression of a mutant Bcl-2(G145E), which does not inhibit apoptosis (Yang *et al.*, 1997), resulted in the same levels of MAC detection as the parental clones (Pavlov *et al.*, 2001). MAC activity appears when Bax levels increase in mitochondria of apoptotic FL5.12 cells. A positive correlation exists between the detection of MAC activity and the level of the proapoptotic protein Bax in the mitochondrial outer membrane (Dejean *et al.*, 2005; Guo *et al.*, 2004; Pavlov *et al.*, 2001). MAC is detected at the time of GFP-Bax translocation into mitochondria and cytochrome *c* release in a clone of HeLa cells treated with staurosporine (Dejean *et al.*, 2005). Furthermore, application of the 'BH3 domain only' protein t-Bid to isolated mitochondria induces both cytochrome *c* release (Cheng *et al.*, 2003; Polster *et al.*, 2001) and MAC activity (unpublished results). While these findings demonstrate regulation of MAC by Bcl-2 family proteins, data now also supports a structural role for Bax in MAC activity.

Oligomeric Bax is a component of MAC in at least some apoptotic cell types. Immunoprecipitation of oligomeric Bax depletes MAC activity from mitochondrial lysates of staurosporine-treated HeLa cells. However, MAC is not detected and cytochrome *c* is not released from staurosporine-treated MEF cell lines lacking both Bax and Bak (Dejean *et al.*, 2005). While there is not yet a direct demonstration of Bak involvement in MAC formation or structure, MAC activity is detected in the Bax knockouts suggesting Bak may also be a constituent of MAC. This finding is consistent with the results of others indicating that Bax and Bak are likely to be functionally redundant with respect to cytochrome *c* release and MAC formation/structure (Cheng *et al.*, 2001, 2003; Dejean *et al.*, 2005; Wei *et al.*, 2001). Nevertheless, the structural role of Bax, and perhaps Bak, in formation of the pore of MAC is unclear at this time.

Some proteins in the BCL-2 family have structures similar to pore-forming toxins and can create ion channels in artificial membranes (Antonsson *et al.*, 1997; Saito *et al.*, 2000; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997). Recombinant oligomeric Bax forms high-conductance channels in lipid bilayers and causes the release of trapped cytochrome *c* and dextrans (Antonsson *et al.*, 2000; Kuwana *et al.*, 2002; Lewis *et al.*, 1998; Polster *et al.*, 2001; Roucou *et al.*, 2002; Saito *et al.*, 2000; Terrones *et al.*, 2004). Importantly, recombinant oligomeric Bax has channel activity that is, as described below, similar to that of MAC (Dejean *et al.*, in press-a; Pavlov *et al.*, 2001). Furthermore, a MAC-like activity is detected in the mitochondria of VDAC-deficient yeast that express hBax (see Table I). These findings suggest oligomeric recombinant Bax and yeast expressing BCL-2 proteins are model systems that should be useful in understanding the formation and function of MAC.

Electrophysiological Characterization of MAC

The mitochondrial apoptosis-induced channel MAC was first detected by directly patch-clamping mitochondria isolated from apoptotic FL5.12 cells, 12 h after IL-3 withdrawal (Pavlov *et al.*, 2001). There was a significant increase in the mean conductance of the mitochondrial outer membrane of apoptotic compared to control cells. This increase in ion permeability, coincident with release of proapoptotic proteins from mitochondria, suggested the formation of a novel pore in the outer membrane. Reconstitution of purified mitochondrial outer membranes of apoptotic FL5.12 cells into liposomes allowed the characterization of the single channel behavior of MAC.

MAC is a heterogeneous high-conductance channel, with multiple sub-conductance levels and single channel transitions of up to 2.5 nS (Fig. 1(A)). Although rapid flickering between conductance states was observed, MAC typically showed a stable open conductance state with relatively infrequent transitions (Dejean *et al.*, 2005; Guo *et al.*, 2004; Pavlov *et al.*, 2001). The activity of MAC is significantly different from the constitutive channels of the mitochondrial outer membrane, Tom and VDAC. The single channel parameters of peak conductance, transition size, selectivity, and voltage dependence for these channels are in Table I and illustrated by the current traces of Fig. 1(A).

MAC exhibits a variable but high conductance (Fig. 1(B)). The mean peak conductance of MAC of apoptotic FL5.12 and HeLa cells is 4.5 and 3.3 nS, respectively (Dejean *et al.*, 2005; Pavlov *et al.*, 2001). There may be several reasons for this heterogeneity of conductance. For example, it may be due to an asynchronous response of individual mitochondria within a cell to apoptotic signals (Heiskanen *et al.*, 1999; Goldstein *et al.*, 2000). Thus, MACs with smaller conductances may reflect the development of channels found in mitochondria earlier in their individual response to an apoptotic signal compared to MACs with larger conductances (i.e., by 12 h of IL-3 withdrawal). Alternatively, the heterogeneity may reflect MAC of different compositions and/or the inherent properties of MAC components. In any case, MAC with larger conductances may provide a transport pathway for cytochrome *c* and potentially other factors out of mitochondria during apoptosis.

MAC is typically a voltage-independent channel (Dejean *et al.*, 2005; Guo *et al.*, 2004; Martinez-Caballero *et al.*, 2004; Pavlov *et al.*, 2001), although it has also been reported to occupy less than peak conductances at higher potentials (Guihard *et al.*, 2004). The channel

Table I. Comparison of the Properties of Mitochondrial Outer Membrane and Bax△C20 Channels

	MAC[a] FL5.12	MAC[b] HeLa	hMAC[c]	Bax△C20[d]	TOM[e]	VDAC[e]
Peak conductance (nS)	4.5 ± 2.4	3.3 ± 1.3	3.4 ± 1.0	5.0 ± 3.0	0.71 ± 0.06	0.68 ± 0.09
Ion selectivity	Cation	Cation	Cation	Cation	Cation	Anion
P_K/P_{Cl}	3.0 ± 0.9	n.d.	4.7 ± 1.3	6.8 ± 1.0	3.6 ± 0.8	0.7 ± 0.1
Voltage dependent	No	No	No	No	Yes	Yes
Pore size (nm)[f]	4.9 ± 1.4	4.2 ± 0.84	4.3 ± 0.7	5.1 ± 1.7	2.0 ± 0.1	2.0 ± 0.2

Note. n.d.: not determined.
[a]MAC of apoptotic FL5.12 cells (Guo *et al.*, 2004; Pavlov *et al.*, 2001).
[b]MAC of apoptotic HeLa cells (Dejean *et al.*, 2005a).
[c]MAC of yeast expressing human Bax (Pavlov *et al.*, 2001; Priault *et al.*, 2003).
[d]Recombinant Bax channel (Dejean *et al.*, 2005a; Pavlov *et al.*, 2001).
[e]VDAC and Tom of FL5.12 cells (Pavlov *et al.*, 2001).
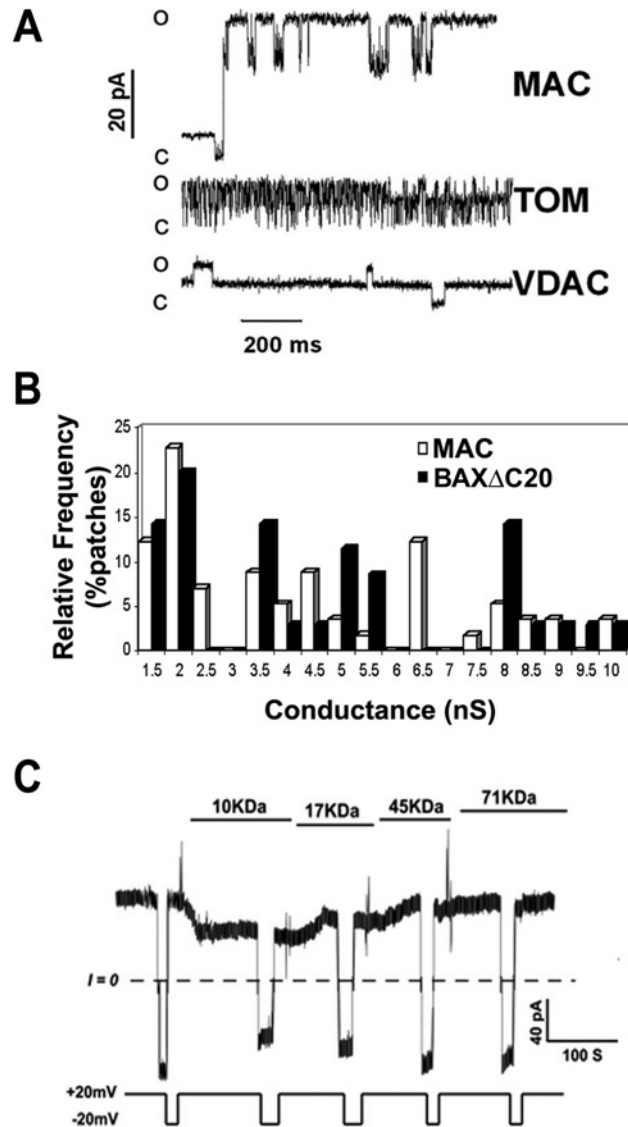[f] Calculated from peak conductance using the method of Hille (Hille, 2001).

Fig. 1. Single-channel behavior of MAC. (A) Current traces at 20 mV allow comparisons of the single channel behavior of MAC, Tom channel and VDAC. O and C indicate open and closed states. MAC activity was reconstituted from mitochondrial outer membranes of FL5.12 cells 12 h after IL-3 withdrawal. (B) Comparison of frequency profile of peak conductances for MAC of apoptotic FL5.12 cells (MAC) and channels formed by oligomeric BaxΔC20. Peak conductances were determined from total amplitude histograms, not leak subtracted, and are shown as a function of detection frequency ($n = 57$ and 35 independent patches displaying MAC and BaxΔC20 channels, respectively). Bin is 0.5 nS. Conductances below 1.5 nS were not considered. (C) Estimation of pore size of MAC by the polymer exclusion method. Current traces are shown of 4 nS MAC of apoptotic FL5.12 cells after sequential bath perfusion with 5% of indicated MW dextrans as the voltage was switched between ±20 mV. The decrease in current caused by 10 and 17 kDa MW dextrans indicates they are permeant, unlike the 45 and 71 kDa polymers. Parts of this figure were reprinted from Pavlov *et al*. (2001), Guo *et al*. (2004), and Dejean *et al*. (2005a).

is slightly cation-selective, which is consistent with the notion that MAC can mediate the release of the cationic protein cytochrome *c* from mitochondria early in apoptosis.

Recombinant oligomeric BaxΔC20 (amino acids 1-172) channels have electrophysiological properties that are similar to those of MAC including multiple conductance levels, a slight cation selectivity, and little voltage dependence (Dejean *et al.*, 2005; Pavlov *et al.*, 2001). MAC and BaxΔC20 channels also show a similar, but not identical, frequency profile of conductance (Fig. 1(B)). Importantly, the pores of MAC and recombinant oligomeric BaxΔC20 appear to be large enough to allow passage of cytochrome *c* to initiate the commitment step of apoptosis. The differences in properties noted between MAC and oligomeric recombinant BaxΔC20 may be the result of a variety of factors including additional components, e.g., lipids or protein (Antonsson, 2004; Kuwana *et al.*, 2002; Polcic and Forte, 2003), method of reconstitution, or deletion of the last 20 amino acids in the recombinant Bax used in these experiments. Interestingly, the channels formed by BaxΔC19 (amino acids 1-173) have similar peak conductances, but unlike BaxΔC20 channels, BaxΔC19 channels are slightly anion selective and voltage independent (Saito *et al.*, 2000).

If MAC forms the pathway for the release of proteins like cytochrome *c* from mitochondria during apoptosis, then the diameter of MAC's pore should be larger than that of cytochrome *c*. Using the method of Hille (Hille, 2001), the pore diameter of MAC with a conductance of ~4.5 nS, and assuming a pore length of 5.5 nm, has been estimated to be ~5 nm. That is Pore Diameter = $2 ((\text{Conductivity} \times \text{Conductance} \times \text{Length})/\pi)^{1/2}$. The estimate of pore diameter increases to >7 nm if access resistance is included.

The pore size of MAC has also been estimated using the polymer exclusion method (Guo *et al.*, 2004), as has been done with other channels (Bezrukov and Kasianowicz, 1997; Grigoriev *et al.*, 2004; Krasilnikov *et al.*, 1992, 1998; Truscott *et al.*, 2001). This approach estimates a diameter for VDAC at 2 nm on one side and 4 nm on the other (Krasilnikov *et al.*, 1996; Rostovtseva *et al.*, 2002). MAC with a conductance of 4 nS is permeable to 10 and 17 kDa, but not 45 and 71 kDa Dextran, since there is no current decrease upon introduction of the larger polymers shown in Fig. 1(C). The polymer exclusion method indicates that MAC with conductances between 1.5 and 5 nS has pore sizes of 2.9–7.6 nm. Hence, the pore diameter of MAC is similar to that calculated from the conductance using the method of Hille.

MAC is a Putative Cytochrome *c* Release Channel

The notion that MAC provides the pathway through the outer membrane for release of cytochrome *c* is supported by data obtained through combining electrophysiology with microscopic, biochemical and molecular approaches. For example, MAC formation and cytochrome *c* release are suppressed in double knockouts of Bax and Bak (Dejean *et al.*, 2005). As described above, the pore size of MAC predicted by both the polymer exclusion method and calculated from the conductance is large enough to allow the passage of cytochrome *c*. Below, further evidence showing MAC is the cytochrome *c* release channel is discussed.

A high-conductance channel activity that resembles MAC has been recorded inside apoptotic neural cells around the time cytochrome *c* is released using a double barrel patch-clamp technique (Jonas *et al.*, 2004, 2005). Immunostaining shows cytochrome *c* is normally localized in a punctate, peri-nuclear pattern typical of mitochondria (Fig. 2(A)). Much of this punctate immunofluorescence is lost after 24 h of serum deprivation, indicating most of the cytochrome *c* is released from mitochondria by this time. As shown in Fig. 2(B), a high-conductance channel activity is present on intracellular membranes in the serum-deprived cells at the time of cytochrome *c* release. In contrast, transitions of less than 200 pS (likely
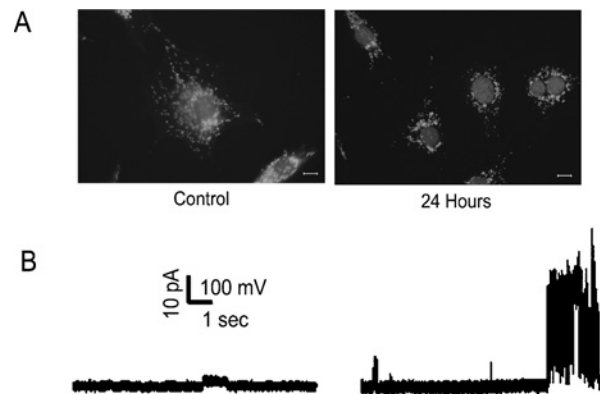

Fig. 2. MAC-like activity is recorded inside apoptotic cells when cytochrome *c* is released. Apoptosis is induced by serum withdrawal in neural cells (CSM14.1). (A) Fluorescence images of cells immunostained with anti-cytochrome *c* antibody (*green*) and nuclei with Hoechst (*blue*) reveals that most of the cytochrome *c* is released by 24 h after serum withdrawal since most of the green punctate fluorescence is lost. Scale bar is 10 microns. (B) Intracellular patch-clamp recordings reveal the onset of MAC-like, high conductance activity on intracellular membranes ~24 h after serum withdrawal (*n* = 7). In contrast, only low conductance transitions are recorded in control cells (*n* = 4).

due to VDAC) are recorded from intracellular membranes of control cells. The mean conductance recorded inside apoptotic cells (751 ± 209 pS) is significantly different from MAC. Nevertheless, the voltage independence, low selectivity, and appearance in the mitochondrial outer membrane around the time of cytochrome *c* release of this novel activity are characteristics consistent with MAC of other systems. However, future studies will be needed for further comparisons.

The temporal association of MAC formation and cytochrome *c* release is strengthened by biochemical findings which show that MAC is detected in mitochondrial outer membranes early in apoptosis of cells, about the time cytochrome *c* is released (Gross *et al*., 1998; Pavlov *et al*., 2001; Guo *et al*., 2004; Dejean *et al*., 2005). A comparison of cytochrome *c* permeability was made between proteoliposomes prepared with mitochondrial outer membranes from FL5.12 cells that were (apoptotic) and were not (control) deprived of interleukin-3 for 12 h. Proteoliposomes made from apoptotic membranes expressed MAC activity and failed to retain cytochrome *c* compared with proteoliposomes of control cells (Pavlov *et al*., 2001). These findings suggest that cytochrome *c* permeability increases early in apoptosis, when MAC is first detected.

Importantly, cytochrome *c* modifies the electrophysiological behavior of MAC in a manner consistent with entrance of cytochrome *c* into the pore of MAC (Fig. 3) (Dejean *et al*., in press-a; Guo *et al*., 2004). Physiological concentrations (0.1–1 mM) of cytochrome *c* reduce the current flow through MAC with conductances between 2 and 5 nS (Guo *et al*., 2004; Gupte and Hackenbrock, 1988). However, the effects of cytochrome *c* are complex and are now classified as Type 1 and Type 2.

The Type 1 behavior is a 4–50% decrease in the conductance of MAC that is voltage dependent, reversible, and associated with an increase in current noise (Fig.3(A) and (B)). These effects are consistent with partitioning of cytochrome *c* into the pore of MAC
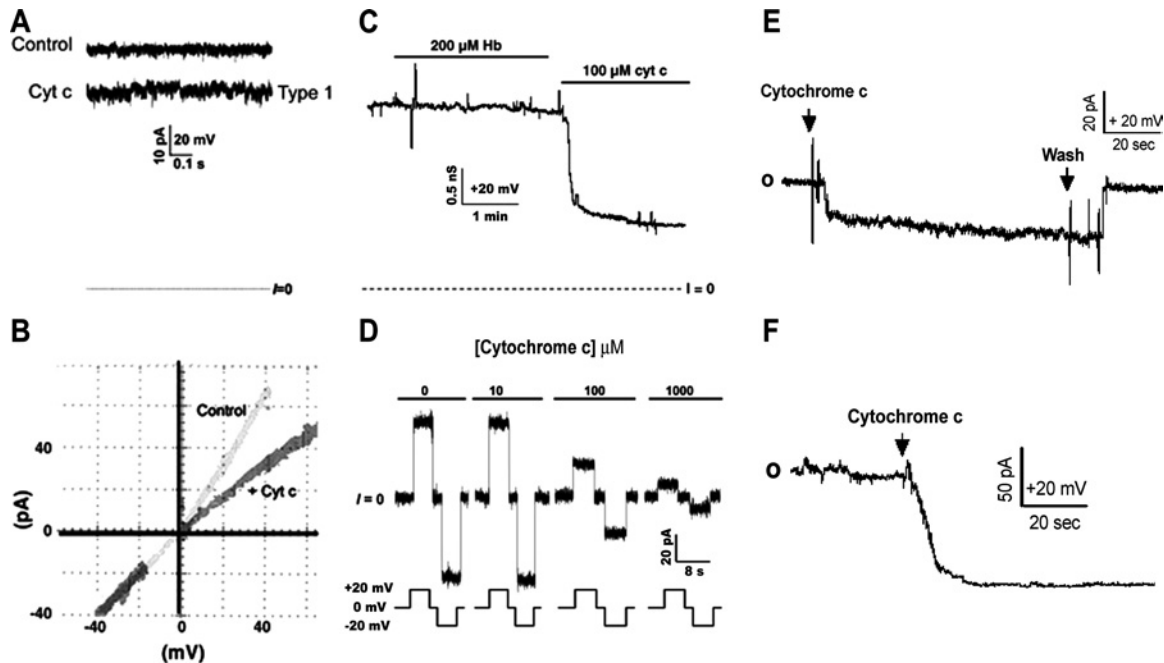

Fig. 3. Effects of cytochrome *c* on MAC and BaxΔC20 channels. (A) Current traces at +20 mV of MAC in the absence (control) and presence of 100 μM cytochrome (Cyt *c*) show a decrease in current and increase in noise corresponding to a Type 1 effect. Sampling was 5 kHz with 2 kHz filtration. (B) Current-voltage relationship for MAC in the presence (+Cyt *c*) and absence (Control) of cytochrome *c* shows rectification at bath positive potentials. (C) A current trace of MAC illustrates that while cytochrome *c* (cyt *c*) induces a rapid Type 2 reduction in MAC conductance, hemoglobin (Hgb) has no effect. Spikes in the current trace are due to perfusion of the bath. (D) Sequential current traces of MAC (initial conductance ~2.6 nS) are shown at indicated voltage and doses of cytochrome *c*. (E) Current trace of a BaxΔC20 channel before and after addition of 100 μM cytochrome *c* (Cyt.c) shows reversal of the effects upon washing (perfusion to remove cytochrome *c*), similar to the Type 1 effect of cytochrome *c* on MAC. (F) Current trace of a BaxΔC20 channel show a Type 2 effect of perfusing 100 μM cytochrome *c* into the bath. Parts of this figure were reprinted from Guo *et al*. (2004) and Dejean *et al*. (2005a).

since they are similar to the effects of other molecules on their translocating channels, e.g., ATP on VDAC and DNA on Hemolysin (Korchev *et al.*, 1995; Rostovtseva and Colombini, 1997; Rostovtseva and Bezrukov, 1998; Akeson *et al.*, 1999; Kasianowicz *et al.*, 2001; Rostovtseva *et al.*, 2002). Hence, the Type 1 effects are strong evidence that cytochrome *c* can enter the pore of MAC, and presumably pass through MAC of larger conductance classes. Interestingly, hemoglobin has no effect on MAC's conductance, indicating this 32 kDa, heme-containing dimer (Margoliash *et al.*, 1961) does not permeate through 2–5 nS MAC. In contrast, the cationic protein ribonuclease A (14 kDa) has the same effects as cytochrome *c* on the current flow through MAC (12.5 kDa) (Guo *et al.*, 2004).

The Type 2 effects of cytochrome *c* likely correspond to a destabilization of the open state of MAC and are rarely seen if the conductance is above 4 nS. The Type 2 characteristics are a 50–90% reduction in conductance that is dose-dependent, not easily reversible, and voltage independent (Fig. 3(C) and (D)). The current noise usually decreases. We speculate that binding sites for cytochrome *c* exist in the structure of 2–4 nS MAC which may be important in synchronization of apoptotic events.

Like MAC, cytochrome *c* and ribonuclease A also modify oligomeric BaxΔC20 channels. These two proteins decrease the current flow through BaxΔC20 channels with conductance below 5.4 nS (Dejean *et al.*, 2005), and the two types of effects of cytochrome *c* on MAC, Type 1 and Type 2, are also observed in BaxΔC20 channels (Fig. 3(E) and (F)). In addition, as was found with MAC, 10 and 17 kDa dextrans block the current flow through BaxΔC20 channels, while hemoglobin has no effect (Dejean *et al.*, in press-a; Guo *et al.*, 2004). Furthermore, proteoliposomes containing BaxΔC20 or BaxΔC19 fail to retain cytochrome *c* (Antonsson *et al.*, 2000; Saito *et al.*, 2000). This findings are consistent with an association of MAC and Bax and with the work of several labs that implicate the channel forming properties of Bax with the release of cytochrome *c* (Antonsson *et al.*, 1997, 2000; Cheng *et al.*, 2001; Kuwana *et al.*, 2002; Polster *et al.*, 2001; Saito *et al.*, 2000; Schendel *et al.*, 1997).

Finally, molecular studies indicate MAC is the cytochrome *c* release channel in early apoptosis that is regulated by Bcl-2 family proteins. Bcl-2 over-expression suppresses MAC activity and prevents cytochrome *c* release (Pavlov *et al.*, 2001). MAC is formed and cytochrome *c* is released in parental and Bax knockout cell lines treated with staurosporine. Importantly and consistent with MAC's putative role, this channel is not detected

and cytochrome *c* is not released in Bax and Bak double knockout cells (Dejean *et al.*, 2005). These observations, taken together, indicate that MAC functions as the cytochrome *c* release channel in early apoptosis.

Pharmacology of MAC

MAC is a potential target for novel therapies because of its putative role in cytochrome *c* release during apoptosis. The pharmacological profile of MAC activity is still limited. However, three inhibitors of MAC have been identified in patch-clamp experiments and include dibucaine, propranolol and trifluoperazine (Fig. 4) (Martinez-Caballero *et al.*, 2004). These three amphiphilic cations inhibit MAC activity in a dose dependent manner as shown in Fig. 4(C). The IC_{50} are 39 μM, 52 μM and 1 μM for dibucaine, propranolol and trifluoperazine, respectively. The mechanisms of blockade for the three compounds are not identical.

Dibucaine acts as a fast blocker, decreasing the current flow through MAC in a reversible manner (Fig. 4(A)). In contrast, the blockade of MAC by propranolol and trifluoperazine is not typically reversible (Fig. 4(B)) and, like the Type 2 effects of cytochrome *c*, probably causes a destabilization of the open state. Dibucaine and propranolol also block cytochrome *c* release from mitochondria induced by recombinant Bax and 'BH3 domain only' proteins like t-Bid (Polster *et al.*, 2003). This finding is consistent with the putative role of MAC in apoptosis and that Bax is a component of MAC (Dejean *et al.*, 2005). In contrast, lidocaine, which is structurally similar to dibucaine, has little effect on MAC. Finally, cyclosporine A, a well-known PTP blocker (Broekemeier *et al.*, 1992; Lenartowicz *et al.*, 1991; Szabo *et al.*, 1992), does not have an effect on MAC activity (Fig. 4(D)) reinforcing the notion that MAC and the PTP are independent (Martinez-Caballero *et al.*, 2004).

None of these pharmacological agents are specific for MAC blockade. Trifluoperazine, propranolol and dibucaine also inhibit mitochondrial protein import, and trifluoperazine and dibucaine block the PTP, although the IC_{50} for MAC is lower than that of the PTP (Hoyt *et al.*, 1997; Pavlov and Glaser, 1998). It has been shown that trifluoperazine and propranolol prevent apoptosis in some cell lines (Freedman *et al.*, 1991; Nieminen *et al.*, 1995) and trifluoperazine and dibucaine also block mitochondrial depolarization induced by glutamate in neurons (Hoyt *et al.*, 1997). This blockade has been interpreted as an inhibition of PTP by these agents. However, it is also possible that the
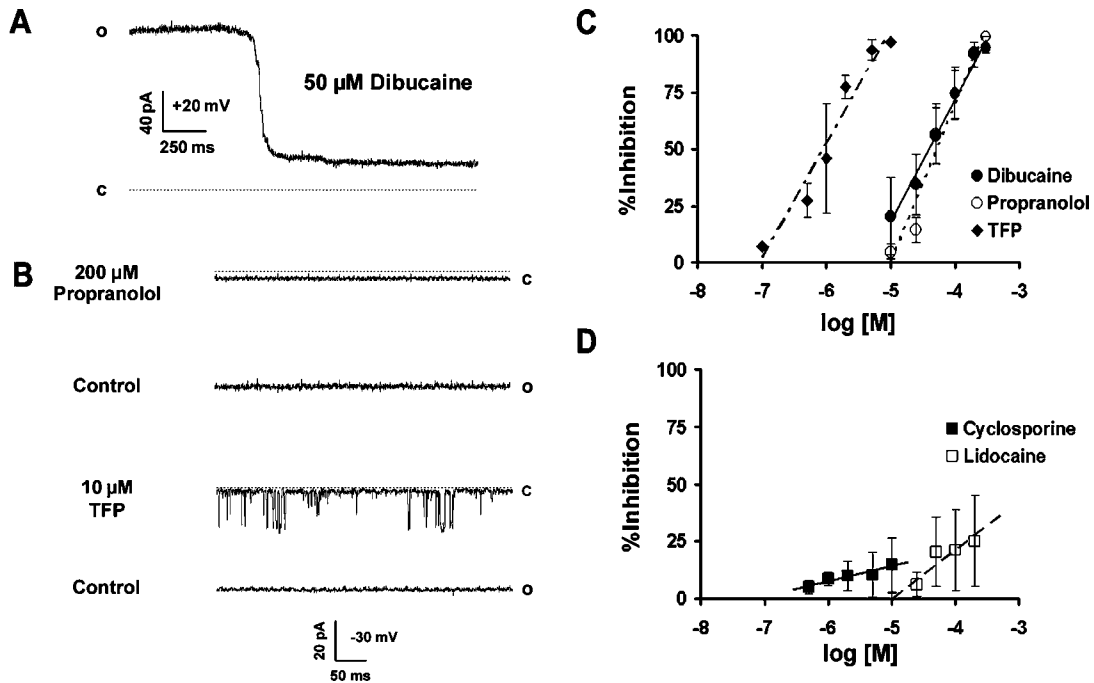
Fig. 4. Pharmacological characterization of MAC. (A) A representative current trace of a MAC from FL5.12 cells, recorded at +20 mV with 2 kHz filtration, shows a fast blockade after perfusion of the bath with 50 μM Dibucaine. O and C indicate open and closed conductance states. (B) Blockade of MAC by propranolol and trifluoperazine. Current traces of two patches are shown in which MAC was recorded at −30 mV before and after perfusion with 200 μM propranolol and 10 μM trifluoperazine (TFP). (C, D) % Inhibition of conductance (% mean conductance with/without drug) as a function of the log concentration (M) of dibucaine, propranolol and trifluoperazine (TFP) (C), and lidocaine and cyclosporine A (D). While dibucaine, propranolol and trifluoperazine block the current flow through MAC with IC$_{50}$ of 39, 52 and 0.9 μM, respectively, lidocaine and cyclosporine A do not. Reprinted from Martinez-Caballero *et al.* (2004).

pleiotropic effects of these compounds on MAC may also be involved. Finally, it has been reported that dibucaine and propranolol have an effect on membrane fluidity (Jutila *et al.*, 1998; Kingston *et al.*, 1993; Varga *et al.*, 1999).

On the Role of Lipids and Proteins in MAC Structure

A major breakthrough in our understanding of the regulation of apoptosis by BCL-2 family proteins came when Bax was identified as a component of MAC, a channel whose activity is completely suppressed by Bcl-2 overexpression. These observations illustrated that Bcl-2 family proteins could accomplish much of their regulation by promoting or inhibiting formation of the putative cytochrome *c* release channel MAC. Cells deficient in both Bax and Bak do not release cytochrome *c* and do not express MAC activity after treatment with death stimuli. Although, Bax was shown to be a component of MAC in some cell types through immunodepletion studies, MAC exists in Bax knockout cells treated to

undergo apoptosis (Dejean *et al.*, in press-a). Presumably, Bak replaces Bax as a component of MAC in these cells as these two proteins are thought to be functionally redundant.

The structural role of the multidomain proapoptotic proteins Bax and Bak in MAC activity is not yet known and no other proteins are clearly implicated in MAC structure. The N-terminus of Bax is thought to have a mitochondrial targeting sequence which is exposed upon "activation" of Bax (Cartron *et al.*, 2003). Hence, the TOM complex, which is the protein import channel in the mitochondrial outer membrane, may play a role in the import of Bax into mitochondria. Furthermore, it has been shown that Bcl-2 interacts with Tom20, one of the receptors of the TOM complex, although Bcl-2 apparently does not use the Tom40 pore for import (Motz *et al.*, 2002). VDAC is also proposed to be a site of action of Bcl-2 (Vander Heiden *et al.*, 2001) and Bax (Pastorino *et al.*, 2002), and one of its isoforms, VDAC2, was found to interact with Bak (Cheng *et al.*, 2003). Nevertheless, VDAC is not required for MAC activity since VDAC is not present in oligomeric fractions from apoptotic HeLa cells

that express MAC activity (Antonsson *et al.*, 2001; Dejean *et al.*, 2005). Moreover a MAC-like activity has been recorded in VDACless yeast mitochondria that express human Bax (Pavlov *et al.*, 2001). Future studies will be needed to identify additional proteinaceous components and proteomics should provide a logical approach.

Lipids may be functional and/or structural elements of MAC and therefore release of cytochrome *c*. The Colombini group has found that some ceramides form high conductance channels in planar bilayers and cause the release of a subset of intermembrane space proteins in isolated mitochondria (Siskind *et al.*, 2002, 2003). Furthermore, it has been hypothesized that Bax may orchestrate the formation of lipidic pores, with some dependence on the presence of the lipid cardiolipin (Basanez *et al.*, 1999; Kuwana *et al.*, 2002). The barrel and stave model of pore formation exemplified by alamethicin is attractive and may represent MAC formation (Baumann and Mueller, 1974). Alternatively, the pore of MAC may correspond to a toroidal model in which the lipid monolayer continuously curves through the pore so that the water-filled pore is actually lined with both lipid headgroups and protein (Yang *et al.*, 2001; Sobko *et al.*, 2004). While speculative, this possibility is supported by the pleiotropic effects of amphiphilic cations like dibucaine and propranolol that are known to modify the fluidity of lipid membranes and block the current flow through MAC and Bax channels (Polster *et al.*, 2003; Martinez-Caballero *et al.*, 2004).

CONCLUSIONS

There is an abundance of evidence supporting a direct involvement of MAC in the release of cytochrome *c*. For example, a MAC-like activity is detected inside dying cells with a double-barrel patch-clamp technique at the time of cytochrome *c* release (Fig. 2). However, other mechanisms may be involved in this commitment step of apoptosis induced in different cell types and by different death stimuli. It has been hypothesized that MAC and PTP function sequentially to maximize cytochrome *c* release (Scorrano and Korsmeyer, 2003). The opening of PTP causes a remodeling of the cristae and breakage of the outer membrane that could complete the release of proapoptotic factors from mitochondria initiated by MAC. However, t-Bid also induces a cristae remodeling that should facilitate release of the same factors (Scorrano *et al.*, 2002). Furthermore, PTP opening was recently shown to primarily participate in necrosis rather than in the apoptosis regulated by the BCL-2 family of proteins (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa

et al., 2005). Alternatively, activation of caspases may in turn cleave normally antiapoptotic factors into additional death factors that modulate outer membrane permeability (Jonas *et al.*, 2004). Two major goals of the future will be to characterize the many faces of MAC underlying cytochrome *c* release and to unravel the signaling pathways that modulate how this channel activity facilitates the timely progression of apoptosis.

ACKNOWLEDGMENTS

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