The properties of the mitochondrial megachannel in mitoplasts from human colon carcinoma cells are not influenced by Bax

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Abstract This paper explores the relationship between Bax and the mitochondrial permeability transition pore (PTP). Isolated human colon tumor (HCT116) Bax$^-$ mitochondria exposed to recombinant Bax exhibited a slow, cyclosporin A-sensitive swelling, but only at [Bax] > 200 nM. The amount of Bax incorporated was much higher than that found in organelles isolated from HCT116 Bax$^+$ staurosporine- or etoposide-treated apoptotic cells, casting doubts on the significance of the putative PT induction for apoptosis. Bax did not influence the electrophysiological properties of an approximately 1 nS channel ascribed to the Ca^{2+}-dependent mitochondrial permeability transition pore. These observations indicate that the PTP is independent of Bax.

Keywords: Permeability transition pore; Mitochondria; Apoptosis; bax; Patch-clamp

1. Introduction

We have recently reported data strongly suggesting that Bax and Bak have little relevance for the Ca^{2+}-induced mitochondrial permeability transition (PT) [1]. The presence or absence of Bax (and Bak) protein, either as a peripheral or integral membrane protein, had no detectable effect on the behavior of mitochondria in a set of widely used assays of Ca^{2+}-induced permeabilization. In apparent contrast, some researchers have reported that exposure of isolated mitochondria to purified Bax induces cyclosporin A (CSP)-inhibitable swelling, attributed to the activation of the permeability transition pore (PTP) [2,3]. On the other hand, in similar experiments others found Bax-induced cytochrome *c* release without significant swelling [4–6] or regardless of the expression or lack of cyclophilin D (CypD), believed to be a component of the PTP [7,8].

Concentrations of Bax above 0.1 μM and as high as 4 μM have been used to induce (or not) swelling of isolated mitochondria [2–6]. We [1] have previously observed the behavior of human colon cancer (HCT116) mitochondria exposed to a much lower concentration, namely 20 nM Bax (approx. 0.4 μg/mg mitochondrial protein). The issue arises of what Bax concentration range ought to be employed in vitro to mimic conditions in vivo. Estimates of the amount of Bax in cells are few. Polster and colleagues [9] found ≈0.7–1.1 μg Bax/mg mitochondrial protein in healthy neural cells. Others [4,10] have concluded that tumor cells may contain 0.1–1 μM Bax. Cellular Bax is located at the ER and in the cytoplasm, and what matters here is how much of it becomes incorporated into a given amount of mitochondrial membranes during the apoptotic process or in vitro. Isolated mitochondria exposed to a large volume of diluted Bax may well incorporate more Bax than mitochondria exposed to a small volume of concentrated Bax. One goal of the present work was therefore to determine whether high doses of exogenous Bax would induce Cyclosporin A-sensitive swelling of HCT116 Bax$^-$ mitochondria, and whether the amount incorporated under these circumstances would be comparable to that incorporated by the mitochondria of apoptotic cells.

Biochemical and electrophysiological data have also been presented which suggest that Bax is a component of the PTP complex [11,12]. We have come to the opposite conclusion [1]. However, our experiments were based on the classical techniques used to study the PT, and involved mitochondrial suspensions. Experiments of this type by themselves can only report on the opening of a permeability pathway allowing the influx of osmotically active components from the medium. It was therefore conceivable that the presence of Bax, while not necessary for the formation of a PTP, would confer some specific properties to it, for example increasing its conductance or modifying its voltage dependence. To investigate this possibility we turned to electrophysiological experiments, capitalizing on our previous work with rat liver mitoplasts, which had led to the identification of an easily recognizable ''megachannel'' as the electrophysiological manifestation of the PTP. In this latter system, the PTP appears as a 0.9–1.3 nS (150 mM KCl) Ca^{2+}-induced, substate-rich ''mitochondrial megachannel'' (MMC) [13–15] (see also [16,17]). A rapidly gating

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Abbreviations: CSP, cyclosporin A; CypD, cyclophilin D; HCT, human colon tumor; MMC, mitochondrial megachannel; RLM, rat liver mitochondria; PTP, permeability transition pore; PS, phosphatidylserine; pS, picoSiemen; STS, staurosporine; VDAC, voltage-dependent anion channel (porin)

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substate with a conductance about one-half that of the fully open channel is characteristic, and has been considered as a "signature" of the channel.

We report here that the same high-conductance channel activity was observed in the membrane of Ca^{2+}-exposed mitoplasts isolated from HCT116 cells, regardless of Bax expression. Furthermore, the properties of these channels did not depend on whether the parent Bax^+cells had been exposed to apoptosis-inducing agents or not. These results strengthen the notion that Bax is not a structural or regulatory component of the "classical" Ca^{2+}-induced PTP.

2. Materials and methods

Cell culture, purification of recombinant proteins, assessment of cytochrome *c* release, isolation of mitochondria from Bax-proficient and –deficient HCT116 cells, alkaline extractions and Western blots were performed as previously described [1]. Fluorescence imaging was performed using an Olympus IX71 microscope with an MT20 light source, and Cell$^{R©}$ software. Pyridine nucleotides were excited at 380 nm, and fluorescence was collected in the 500–550 nm range. Phosphatidylserine exposure was observed with the same apparatus after Annexin-V-Fluos (Roche) binding, following producer-suggested procedures. To assess Bax-induced swelling, mitochondria (1 mg protein/mL) were suspended in 300 mM sucrose, 10 mM HEPES/K^+, 5 mM succinate/K^+, 1.25 μM rotenone, 1 mM P_i/K^+, pH 7.4, and the desired amounts of Bax, pre-incubated for 30 min at 30 °C with equimolar amounts of t^c-Bid, were added. t^c-Bid promotes insertion and oligomerization of Bax in mitochondrial and liposomal membranes [18]. Patch-clamp experiments on HCT116 mitoplasts were carried out essentially as previously reported for rat liver ones [19]. Mitoplasts were obtained by swelling the mitochondria in 30 mM Tris–Cl. Alternatively, mitochondria suspended in experimental medium (150 mM KCl, 0.5 mM $CaCl_2$, 1 mM P_i, 20 mM HEPES, pH 7.35, unless otherwise specified) supplemented with 5 mM succinate were placed in the patch-clamp chamber and allowed to swell spontaneously (having undergone the permeability transition) over about 30 min. The mitoplasts attached to the chamber bottom were washed extensively with the experimental medium and seals were established under symmetrical ionic conditions. For selectivity determination experiments, after the presence of channel activity was ascertained, bath [KCl] was increased to 500 or 600 mM, other components remaining constant. In all cases connection to the Ag/AgCl ground electrode was via a 1 M KCl agar bridge. In inhibition experiments the desired volume (μL range) of concentrated CSP solution (ethanol) was added, and the bath contents were thoroughly mixed by withdrawing and re-adding aliquots with a Gilson pipette. Voltage was controlled by an Axopatch 200 unit, and Axon pClamp software was used for voltage control and data analysis. The voltages reported in this paper are those applied to the patch-clamp pipette interior. Current (cations) flowing from the pipette to the ground electrode is considered as positive and plotted upwards.

3. Results

3.1. Swelling induction by exogenous Bax

To determine whether high concentrations of Bax would induce swelling, we have performed experiments adding 250 and 500 nM (approx. 5 and 10 μg/mg mitochondrial protein; $N = 3$ each condition) purified Bax, preincubated for 30 min at 30 °C with the same concentration of t^c-Bid, to Bax^- HCT116 mitochondria in suspension buffer, and monitoring light scattering. While 20 nM Bax does not cause swelling [1], these higher concentrations do induce a CSP-sensitive absorbance decrease, although at a slow rate (Fig. 1A). To better evaluate the significance of these observations we have compared by Western

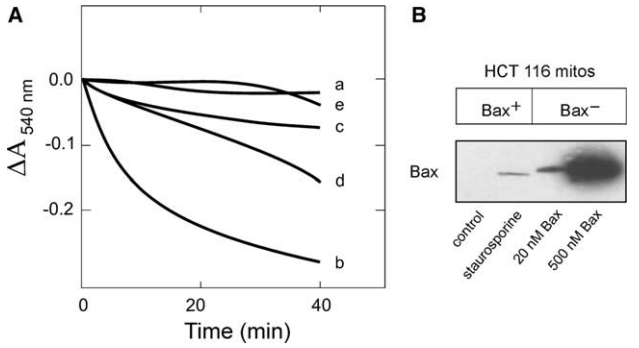

Fig. 1. High [Bax]-induced swelling of mitochondria from Bax-less HCT116 cells. (A) Light scattering (swelling) traces. At time 0 additions of: (a) nothing (control); (b) Ca^{2+} 200 μM; (c) Bax 250 nM; (d) Bax 500 nM; (e) Bax 500 nM and CSP 5 μM. (B) Exemplary Western blot. Mitochondria were isolated from Bax-expressing or Bax deficient HCT116 cells, as indicated. All samples were subjected to alkaline extraction before solubilization and loading onto the SDS–PAGE gel. The same amount of protein (50 μg) was loaded in all lanes. "Staurosporine": the cells were exposed to 1 μM STS for 2 h, and the mitochondria were then isolated; "20 nM Bax": isolated mitochondria were exposed to 20 nM Bax + t^c-Bid as described in Section 2; "500 nM Bax": the mitochondria used for the experiment in trace d of panel A were recovered by centrifugation and used for the Western blot after alkaline treatment.

blot the amount of Bax that inserts into membranes in these experiments with the amount to be found after alkaline treatment in the membranes of mitochondria isolated from HCT116 Bax^+ cells exposed to staurosporine (STS), and in the membranes of mitochondria exposed to 20 nM Bax plus t^c-Bid (Fig. 1B). Treating HCT116 Bax^+ cells with 1 μM STS for 2 h, the conditions relevant for Fig. 1B result in well-developed apoptosis, as documented by extensive alteration of the mitochondrial network (associated with Bax migration to mitochondria [20–22]), cytochrome *c* release, and phosphatidylserine (PS) exposure (Fig. 2). The amount of Bax incorporated in experiments involving exposure to 500 nM Bax is enormously higher than that to be found in mitochondria exposed to 20 nM Bax, which already is higher than that observed in the experiments involving induction of apoptosis by STS (Fig. 1B).

3.2. Properties of the mitochondrial megachannel

To address the possibility of an alteration of the biophysical properties of the PTP/MMC channel, we examined the electrophysiological activity of mitoplasts obtained from mitochondria isolated from HCT116 Bax^+ and Bax^- cells. The presence/absence of Bax in the mitochondrial fraction and its incorporation into the membrane of the mitochondria of cells exposed to pro-apoptotic stimuli have been reported in [1]. With mitoplasts from either Bax-expressing or Bax-less cells in the majority of cases the patch displayed no activity or only activity by "small" (<200 pS) channels, even though the [Ca^{2+}] of the bath was 0.5 mM, a condition favoring the appearance of the MMC. However, in about 14% of attempts on mitoplasts from both Bax-deficient (19 out of 123) and Bax-proficient (26 out of 216) cells activities very similar to the rat liver mitochondria (RLM) MMC were recorded.

The properties of the HCT116 MMC turned out not to depend on Bax expression by the parent cells, and below we refer to both types of preparations if not otherwise

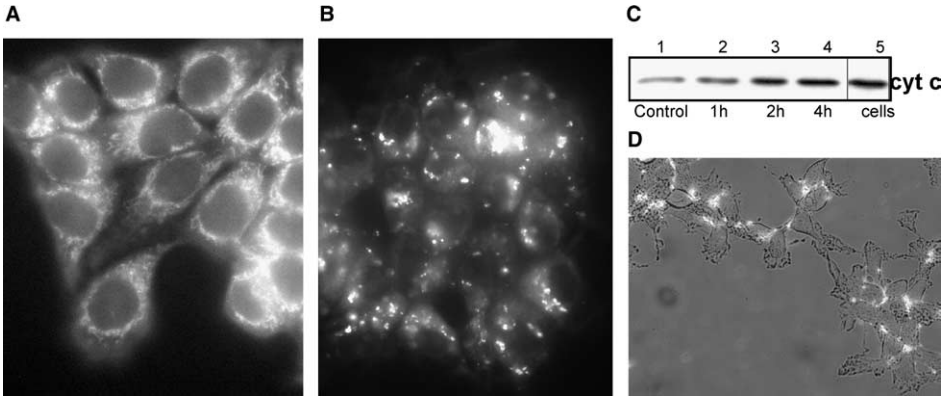

Fig. 2. HCT116 Bax$^+$ cells treated with STS for 2 h are committed to apoptosis. (A) and (B) The mitochondria as revealed by pyridine nucleotide fluorescence (excitation wavelength: 380 nm). (A) Control (untreated) cells. (B) After exposure to 1 μM STS for 2 h. (C) Cytochrome *c* release. Western blot. Lanes 1–4: cytosolic fractions of cells exposed to 1 μM STS for the indicated periods. Lane 5: cell lysate. 50 μg of total protein was loaded in each lane. (D) PS exposure assessed by Annexin-V-Fluos fluorescence after 2 h of 1 μM STS treatment. Annexin-V-Fluos fluorescence is rendered in white. Control (untreated) cells exhibited no fluorescence (not shown).

specified. The trademark "half-conductance" flickering substate was often exhibited (Fig. 3A and B). The full conductance was the same as that of the RLM MMC, i.e., about 1 nS, with variability in the 0.9–1.3 nS range. Like its murine counterpart, this channel was moderately voltage-dependent; it closed sluggishly (on a timescale of several seconds to minutes) and often only partially upon application of voltages above approx. 20 mV of either sign (Fig. 3C). As exemplified in Fig. 3D for Bax$^-$ mitochondria, brief closures were more frequent at pipette-negative voltages. This behavior is identical to that observed with HCT116 Bax$^+$ (not shown) and rat liver mitoplasts.

The channels exhibited variable selectivity (see Section 4). Determinations were conducted by voltage-clamping, under asymmetrical ionic conditions, single-channel patches (examples in Fig. 4; the high conductances are accounted for by the salt concentrations used). *I–V* relationships were obtained by applying voltage ramp protocols and/or constructed by measuring prominent current levels at various applied potentials. Both procedures yielded at least two distinct *I–V* relationships. One exhibited a reversal potential (E_{rev}), variable from experiment to experiment, at $V_{pipette} \leqslant 0$ mV, corresponding to weakly anionic or no selectivity ($P_{Cl}/P_K = 1.8$ in the experiment of Fig. 4A and B). The other, lower, conductance was instead somewhat cation-selective ($P_K/P_{Cl} = 2.8$ for the expt. in Fig. 4B). Transitions between these conductance levels took place often, and were detectable as changes in the sign of the current flowing in the circuit at 0 applied potential (Fig. 4A). As discussed below, we interpret these observations to indicate that the same channel can occupy at least two distinct conductance states, having different selectivities. Somewhat to our surprise, CSP turned out to be an inefficient inhibitor of the channel, if used alone, even at very high concentrations (up to 20 μM). The drug was mostly ineffective: in 4 out of 11 experiments a delayed partial inhibition was, however, observed, and one of these experiments is shown in Fig. 4D. This result is discussed below.

To test whether these conductances might be altered – or if new activities might appear – in apoptotic cells, patch-clamp experiments were also performed on mitoplasts obtained from Bax$^+$ HCT116 cells exposed to 50 μg/mL etoposide overnight or to 1 μM STS for 2 or 24 h (Fig. 2; [1] and references therein). These treatments resulted in integration of Bax in the mitochondrial membrane, cytochrome *c* release and eventually apoptosis (Fig. 2 and not shown; [1]) but they did not cause an increase of the frequency of appearance of the mitoplast channels, nor changes in their properties (not shown; 9 observations out of 85 tight seals).

4. Discussion

Our experiments (Fig. 1) show that the application of Bax in the 5–10 μg/mg mitochondrial protein range to isolated HCT116 Bax-less mitochondria can result in a sluggish but CSP-sensitive swelling, i.e., presumably, in the induction of the PT. This is in agreement with some of the reports in the literature [2,3]. At the same time, however, serious doubts are cast on the significance of this observation by the fact that the amount of Bax incorporated by the mitochondria under these circumstances is enormously higher than that found to be inserted into mitochondria from apoptotic cells. To our knowledge, no such comparison had been previously presented.

The conductance, substates, voltage-dependence, selectivity and gating properties of the approx. 1 nS channels described above are strongly reminescent of the properties of the analogous high-conductance pores observed in RLM and in HepG2 mitochondria, and make it most likely that these channels represent the same biochemical entity as the latter, i.e. the PTP. In agreement with the reluctance of suspended HCT116 mitochondria to undergo Ca^{2+}-induced permeabilization [1], and at variance with the observations on RLM, the MMC appeared in only a small fraction of patches. This may well be related to the neoplastic origin of the cells. Among the possible explanations are the high content of Bcl-x_L [1], and the high glycolytic activity of cancer cells, which is coupled to a PT-hindering association of hexokinase to voltage-dependent anion channel (porin) (VDAC) (e.g. [23]). The MMC appeared in a similarly low (10–20%) proportion of patches of mitoplasts from another cancer cell line (HepG2) [17]. While this low frequency of observation prevented a full-fledged statistics-based

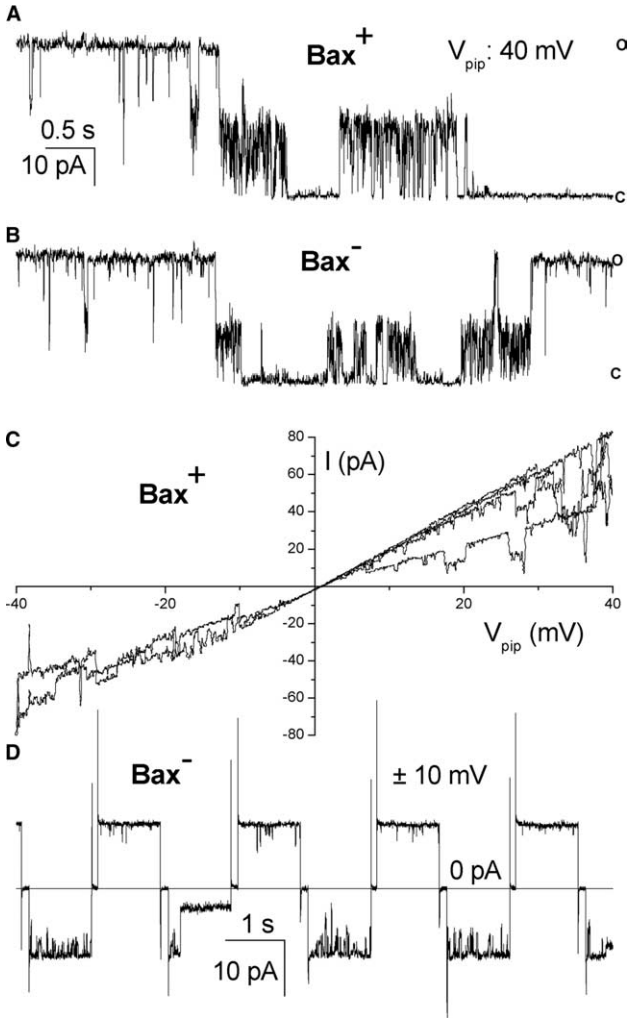

Fig. 3. The HCT116 MMC. (A) and (B) Typical MMC recordings from mitoplasts of (A) Bax-expressing or (B) Bax-less cells. The flickering "half-conductance substate" is displayed. c: closed. o: open. Symmetrical 150 mM KCl medium. Pipette voltage: +40 mV. Sampling rate: 1 kHz. Low-pass filter: 200 Hz. (C) and (D) Voltage dependence: (C) An I–V curve recorded from a Bax^+ mitoplast by driving the pipette potential in the sequence $0 \rightarrow +40 \rightarrow -40 \rightarrow +40 \rightarrow 0$ at 1.5 mV/s. Sampling rate: 100 Hz. Filter: 20 Hz. (D) Current record from a Bax^- mitoplast. 1-second. 10 mV voltage pulses of alternating polarity, separated by 100 ms intervals at 0 mV, were applied. Sampling rate: 1 kHz. Filter: 200 Hz.

comparison, the data allow the conclusion that the presence/absence of Bax has no major impact on the biophysical properties of the pore.

The selectivity properties of these channels deserve comment. In Fig. 4B and C, the lower, cation-selective conductance may in principle correspond either to a substate of one channel (anion-selective in its higher conductance state), or to a background conductance due to the presence of other channels, distinct from the one accounting for the anion-selective higher conductance. In the former case, "jumps" of the current value from one curve to the other would correspond to transitions of the channel from its anion-selective to its cation-selective state or vice versa. Under the latter hypothesis, they would be due to openings or complete closures of a hypothetical anion-selective-only channel operating on top of a cat-

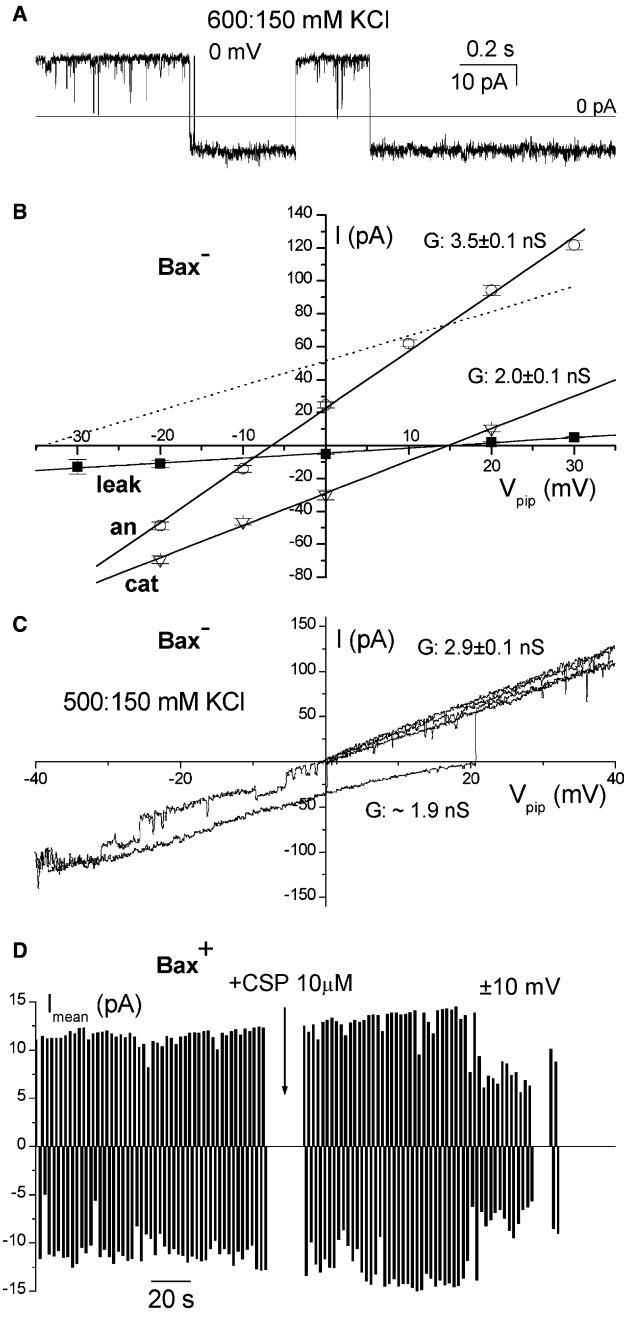

Fig. 4. Selectivity and insensitivity to CSP of the HCT116 MMC. (A) The channel exhibits spontaneous transitions between anion-selective (positive current) and cation-selective (negative current) states at 0 applied voltage. [KCl] was 600 mM in the bath and 150 mM in the pipette. Sampling frequency: 5 kHz. Filter: 1 Hz. (B) I–V curves for the three major current levels observed in the same experiment as in (A) (data points and solid lines). No leak subtractions were performed. The solid lines are the best fits describing the anion-selective conductance state (an), the cation-selective one (cat), and the background conductance (leak). The dotted line plots $y_{hyp}(x) = y_{an}(x) - y_{cat}(x)$. See Section 4 for details and explanations. (C) I–V plot from another experiment with a Bax^- mitoplast, with 500 (bath) vs. 150 (pipette) mM KCl. Reversal potentials are close to 0 and +20 mV. Sampling: 100 Hz. Filter: 20 Hz. (D) Lack of inhibition of the high-conductance channels of HCT116 mitochondria by CSP. The same voltage protocol as in Fig. 1D was applied to a Bax^+ mitoplast. The mean of the current recorded during each 1-second pulse is plotted vs. time. CSP was added when indicated.

ion-selective background. The data allow us to conclude that the former hypothesis is correct.

This can be illustrated more easily with reference to an example such as that of Fig. 4A and B. In this experiment an I–V relationship could be determined for the "leak" conductance (curve marked "leak" in Fig. 4B), allowing leak subtraction. The real I–V curve of an hypothetical anion-selective-only channel ($i_{hyp} = g_{hyp}(V - E_{r,hyp})$, Fig. 4B, dotted line) would have to be such as to produce the observed higher-conductance I–V relationship ($I_a = G_a(V - E_{r,a})$, curve "an" in Fig. 4B) when summed to the I–V relationship resulting from the presence of "leaks" and hypothetical cation-selective-only channel(s), that is, to the curve labeled "cat" in Fig. 4B ($I_{cat} = G_{cat}(V - E_{r,cat})$). When the reversal potential for the hypothetical anion-selective channel of the example at hand is calculated ($E_{r,hyp} = [G_a E_{r,a} - G_{cat}E_{r,cat}]/[G_a - G_{cat}]$), it turns out to be −34 mV, very close to the theoretical Nernstian potential for chloride of −36 mV (600 vs. 150 mM KCl in the experiment). Such a pronounced selectivity in a channel with nS conductance is very unlikely. Thus the two I–V curves most probably correspond to two states of the same channel. This conclusion is reinforced by the observation of direct transitions to the anion-selective state from the non-conducting state and vice versa. If the patch contained two independent channels, for such transitions to take place both channels would have to open or close simultaneously, which can be ruled out on statistical grounds. Thus the conclusion seems warranted that the megachannels possess one or more cation-selective states in addition to unselective or weakly anion-selective states. VDAC, the mitochondrial porin, has very similar properties [24–26], and is thought to be one of the components of a supramolecular complex forming the PTP (e.g. [27,28]). Our data thus support this model.

The insensitivity to CSP of the approx. 1 nS conductances apparently contrasts with both the sensitivity of Ca^{2+}-induced swelling of HCT116 mitochondria [1] and that of the very similar MMC of RLM [13]. CSP inhibition of the PTP is a complex phenomenon, which shows different characteristics in different systems, and despite its widespread use CSP has often been reported to be ineffective or to provide only a transient effect ([28–31] and references therein). The protective effect of CSP may be lost if the mitochondria undergo severe depolarization [29]. Some authors have concluded that CypD-regulated and -unregulated forms of the PTP exist, suggesting that the PT may progress from a Ca^{2+}-dependent and CSP-sensitive to a Ca^{2+}-independent and CSP-insensitive phenomenon as the strength of induction increases [30,32]. The PTP of CypD-deficient mouse mitochondria is insensitive to CSP [33], and CypD, a matrix protein, may be lost under the conditions of patch-clamp experiments. Practically all the inhibition-hindering conditions mentioned apply in the case of patch-clamp experiments: [Ca^{2+}] was high (0.5 mM), cofactors such as ADP or Mg^{2+} had been lost, and the membrane had been completely depolarized. The discrepancy between rat liver and HCT116 mitochondria remains to be rationalized, but we may note that it mirrors the reluctance of PT induction in the latter as compared to the former.

In conclusion, our data indicate that Bax, a key pro-apoptotic protein, has little to do with the Ca^{2+}-induced permeability transition pore.

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