Outer mitochondrial membrane permeabilization during apoptosis triggers caspase-independent mitochondrial and caspase-dependent plasma membrane potential depolarization: a single-cell analysis

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Summary

Little is known about the temporal relationship between mitochondrial and plasma membrane potential changes and outer mitochondrial membrane permeabilization during apoptosis. Confocal imaging of breast carcinoma and HeLa cells stably transfected with cytochrome-C-GFP demonstrated that mitochondria rapidly depolarized after the release of the fusion protein into the cytosol. Of note, mitochondria did not completely depolarize but established a new steady-state level that could be further dissipated by treatment with the protonophore carbonyl cyanide p-trifluoromethoxy-phenylhydrazone. Treatment with the FoF₁-ATP-synthase inhibitor oligomycin likewise induced a collapse of this steady-state level, suggesting that FoF₁-ATP-synthase reversal maintained mitochondrial potential after outer mitochondrial membrane permeabilization. Treatment with a broad spectrum caspase inhibitor failed to inhibit the partial depolarization of mitochondria during apoptosis, yet potently abolished the activation of effector caspases detected by fluorescence resonance energy transfer analysis in the same experiment. Interestingly, the onset of mitochondrial depolarization was always coupled with a depolarization of the plasma membrane potential. This was associated with the degradation of the regulatory Na⁺/K⁺-ATPase β-subunit, and both events were blocked by caspase inhibition. Our results demonstrate that outer mitochondrial membrane permeabilization coordinates the depolarization of both membrane potentials during apoptosis.

Key words: Apoptosis, Mitochondrial membrane potential, Plasma membrane potential, Confocal imaging, Mitochondrial respiration

Introduction

Mitochondria produce ATP and control cellular energetics (Nicholls and Ward, 2000). However, mitochondria also play an important role in the initiation and execution of apoptosis, an activity that is controlled by Bcl-2 family proteins (Harris and Thompson, 2000; Lemasters et al., 1999; Zamzami and Kroemer, 2001). During apoptosis, the pro-apoptotic Bcl-2 family proteins Bax and Bak are activated and undergo a conformational change. Active Bax and Bak insert into the outer mitochondrial membrane, oligomerize and trigger an increase in mitochondrial permeability. Several proteins that normally reside in the mitochondrial intermembrane and intracristal space are subsequently released. These include cytochrome C (cyt-C), apoptosis-inducing factor (AIF), Smac/DIABLO and Omi/HtrA2 (Du et al., 2000; Faccio et al., 2000; Liu et al., 1996; Susin et al., 1999; Verhagen et al., 2000). These proteins trigger or facilitate the formation of a caspase-activating complex, the apoptosome. This complex is composed of cyt-C, Apaf-1, dATP and procaspase-9 (Li et al., 1997; Zou et al., 1997). Apoptosome formation results in the activation of effector caspases that are responsible for most of the biochemical and morphological changes during apoptosis.

Confocal time-lapse imaging experiments in cells expressing a cyt-C-green-fluorescent-protein (GFP) fusion protein have suggested that the majority of cyt-C is released rapidly and efficiently during apoptosis (Heiskanen et al., 1999), a process that occurs in one large step (Goldstein et al., 2000) or two or more steps (Luetjens et al., 2001; Scorrano et al., 2002). A change in mitochondrial ultrastructure may be required to guarantee a complete release of cyt-C, because large quantities may normally reside in the inaccessible intracristal space (Luetjens et al., 2001; Scorrano et al., 2002). Although many studies have focused on the role of cyt-C release in the activation of the caspase cascade, loss of large quantities of cyt-C will also directly affect mitochondrial respiration, ATP production and free radical production, resulting in an organelle dysfunction program (Adachi et al., 1997; Cai and Jones, 1998; Luetjens et al., 2000; Mootha et al., 2001). Single-cell imaging experiments have also shown that the mitochondrial membrane potential (ΔΨM) depolarizes...
after or concomitant with the release of a cyt-C-GFP fusion protein (Heiskanen et al., 1999; Waterhouse et al., 2001), a process that has been described as caspase dependent (Bossy-Wetzel et al., 1998; Waterhouse et al., 2001). However, we and others have previously demonstrated that mitochondria are able to transiently maintain a membrane potential after the release of cyt-C (Bossy-Wetzel et al., 1998; Deshmukh et al., 2000; Krohn et al., 1999), even in the absence of caspase inhibitors (Bossy-Wetzel et al., 1998; Krohn et al., 1999). However, interpretations of fluorescence changes of voltage-sensitive probes used in these type of studies are hampered by the fact that these probes are also sensitive to changes in plasma membrane potential (∆Ψ_m) (Ehrenberg et al., 1988; Ward et al., 2000). Mitochondrial depolarization, loss of ATP and increased free radical formation could indeed lead to a disturbance of cellular ion homeostasis and subsequent ∆Ψ_m depolarization. In the present study, we therefore analyzed ∆Ψ_m and ∆Ψ_p changes at the single-cell level during apoptosis employing breast carcinoma and HeLa cells expressing a cyt-C-GFP fusion protein.

Materials and Methods

Materials

Recombinant human tumor necrosis factor-α (TNF-α), cycloheximide (CHX), antimycin A, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and embryo-tested paraffin oil were purchased from Sigma (Deisenhofen, Germany). Staurosporine (STS) was from Alexis (Grüningen, Germany) and oligomycin from Serva (Heidelberg, Germany). The broad-spectrum caspase inhibitor Z-Val-Ala-Asp(O-methyl)-fluoromethylketone (z-VAD-fmk) was purchased from Bachem (Heidelberg, Germany). Tetramethyl rhodamine methyl-ester (TMRM) and bis-(1,3-dibutylbarbituric acid)trimethine oxonol [Dibac4(3)] were from MobiTec (Göttingen, Germany).

Cell culture and transfection

Generation and characterization of human breast carcinoma MCF-7 cells stably expressing caspase-3 (MCF-7/Casp-3) and a cyt-C-GFP fusion protein have been described (Luetjens et al., 2001). We have previously shown that cyt-C-GFP is imported into mitochondria and co-released with endogenous cyt-C after selective outer membrane permeabilization with digitonin. Subcellular fractionation experiments confirmed the concomitant release of endogenous cyt-C and cyt-C-GFP from mitochondria during apoptosis. Generation and characterization of HeLa D98 cells expressing the cyt-C-GFP fusion protein was performed as described for the MCF-7/Casp-3 cells. For detection of effector caspase activation by fluorescence resonance energy transfer (FRET) analysis, MCF-7/Casp-3 or HeLa D98 cells were transiently transfected with plasmid pmyc-CFP-DEVD-YFP DNA (0.6 μg) (Rehm et al., 2002; Tyas et al., 2000) and 6 μl Lipofectamin™ reagent (Life Technologies) per ml serum-free RPMI medium. Cells were cultivated on 35 mm glass-bottom culture dishes (Willco BV, Amsterdam, The Netherlands).

Time-lapse confocal fluorescence microscopy

Cyt-C-GFP, TMRM and Dibac4(3) fluorescence was monitored and quantified confocally using an inverted Olympus IX70 microscope attached to a confocal laser scanning unit equipped with a 488 nm argon laser and a 60× oil objective (Fluoview; Olympus, Hamburg, Germany). Fluorescence transmitted the first dichroic mirror with more than 90% transmission above 505 nm was divided with a second dichroic mirror at 550 nm and detected after transmission of a 510-540 nm bandpass filter [GFP or Dibac4(3)] or a 565 nm high pass emission filter (TMRM). There was no TMRM fluorescence detectable in the GFP or Dibac4(3) channel. The crosstalk between the average pixel intensity of GFP in the TMRM channel was less than 10% of the average pixel intensity in the GFP channel. The maximum change owing to GFP fluorescence in the TMRM channel occurred during the release of the cyt-C-GFP fusion protein. The resulting change was within the standard deviation of the average pixel intensity of single cells in the TMRM channel (approx. 5%). Fluorescence was detected from a 0.7 μm thick confocal section (full width half maximum).

The membrane-permeant, cationic probe TMRM distributes across cellular membranes according to the Nernst equation and accumulates in the negatively charged mitochondrial matrix (Ehrenberg et al., 1988). TMRM has little effects on the respiratory chain activity at the concentration used in the present study (30 nM), and has a lower membrane-bound fraction than TMRE (Scaduto et al., 1999). Dissipation of the inner mitochondrial membrane H+ gradient using 10 μM FCCP resulted in a transient peak in the TMRM fluorescence intensity in less than 50% of control MCF-7/Casp-3 cells, suggesting that TMRM was used below the quenching limit (Ward et al., 2000). Saturation of mitochondrial TMRM fluorescence was reached at 250 nM extracellular probe concentration. For ∆Ψ_p measurements, we used the anionic probe Dibac4(3). The Nernstian behaviour of this probe allows measurements of slow ∆Ψ_p changes by confocal microscopy (Dall’Asta et al., 1997). Dibac4(3) fluorescence was detected with the 510-540 nm bandpass filter and showed a negligible overlap into the TMRM channel.

For time-lapse imaging, culture dishes were mounted onto the microscope stage that was equipped with a temperature-controlled inlay (HT200, Minitüb, Tiefenbach, Germany). In control experiments constant fluorescence values were monitored for 24 hours in case of cyt-C-GFP, 18 hours in the case of TMRM and 12 hours in the case of Dibac4(3). For induction of apoptosis, cells were incubated with 100 ng/ml TNF-α plus 1 μg/ml CHX or 3 μM staurosporine (STS) directly on the stage after 1 hour of equilibration with 30 nM TMRM and subsequent equilibration with 1 μM Dibac4(3) for 1 hour when indicated. The medium was enriched with 10 nM HEPS (pH 7.4) and thoroughly mixed to ensure a proper distribution of the drugs. To prevent evaporation the media was covered with embryo-tested paraffin oil. Dibac4(3) measurements were carried out in serum-free RPMI buffered with 10 mM HEPS because of the extensive binding of this probe to proteins. Image data were obtained using Fluoview 2.0 software (Olympus) and Kalman filtered from three scans for each image.

Image processing and remodeling of TMRM fluorescence kinetics

The quantitative analysis of the fluorescence images was performed using the UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the internet by anonymous FTP from maxrad6.uthscsa.edu). For analysis of TMRM uptake in single cells, the fluorescent mitochondrial regions were segmented from the non-fluorescent regions. After background subtraction the average fluorescence intensity in less than 50% of control MCF-7/Casp-3 cells, suggesting that TMRM fluorescence was released. The membrane-permeant, cationic probe TMRM distributes across cellular membranes according to the Nernst equation and accumulates in the negatively charged mitochondrial matrix (Ehrenberg et al., 1988). TMRM has little effects on the respiratory chain activity at the concentration used in the present study (30 nM), and has a lower membrane-bound fraction than TMRE (Scaduto et al., 1999). Dissipation of the inner mitochondrial membrane H+ gradient using 10 μM FCCP resulted in a transient peak in the TMRM fluorescence intensity in less than 50% of control MCF-7/Casp-3 cells, suggesting that TMRM was used below the quenching limit (Ward et al., 2000). Saturation of mitochondrial TMRM fluorescence was reached at 250 nM extracellular probe concentration. For ∆Ψ_p measurements, we used the anionic probe Dibac4(3). The Nernstian behaviour of this probe allows measurements of slow ∆Ψ_p changes by confocal microscopy (Dall’Asta et al., 1997). Dibac4(3) fluorescence was detected with the 510-540 nm bandpass filter and showed a negligible overlap into the TMRM channel.

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The TMRM kinetics from individual cells were fitted with the

\[ y = \frac{A_1-A_2}{1 + e^{-(x-x_0)/dx}} + A_2, \]

with dx determining the time from polarization to depolarization, A_1 passing through 0.7 μm thick confocal section (full width half maximum).
the maximum, A2 the minimum and x0 the time when \((A1-A2)/2\) is reached.

Remodeling of TMRM kinetics was performed according to Ward et al. (Ward et al., 2000) under the assumption of the Nernstian behavior of the dye. Briefly, the TMRM concentration in mitochondria at a given extracellular concentration results from both the \(\Delta V_M\) and the \(\Delta V_P\).

\[
[T\text{MRM}]_{\text{Mitochondria}} = [T\text{MRM}]_{\text{Cytoplasm}} e^{-\frac{F\Delta V_P}{RT}}
\]

and

\[
[T\text{MRM}]_{\text{Cytoplasm}} = [T\text{MRM}]_{\text{Medium}} e^{-\frac{F\Delta V_P}{RT}}
\]

leads to

\[
[T\text{MRM}]_{\text{Mitochondria}} = [T\text{MRM}]_{\text{Medium}} e^{-\frac{F(\Delta V_P+\Delta V_C)}{RT}}.
\]

In these equations, \(T\) represents the temperature in °K, \(F\) is the Faraday and \(R\) the Rydberg constant. The remodeling takes the mitochondrial volume fraction (5%), the diffusion constant of TMRM across cellular membranes (0.01 per second) and the quenching limit of TMRM (700 μM) into account. Values were calculated as described previously (Poppe et al., 2001).

Epifluorescence microscopy

CFP-DEVD-YFP-expressing cells equilibrated with 30 nM TMRM were treated with STS or STS plus z-V AD-fmk and then placed in a heated (37°C) chamber (Minitüb) mounted on a Nikon TE 300 microscope stage (Nikon, Düsseldorf, Germany). Fluorescence was observed using a 20× S-Fluor objective. The microscope was equipped with a polychroic mirror and filterwheels in the excitation and emission light path containing the appropriate filter sets (polychroic mirror with more than 90% reflexion from 411 to 438 nm, between 491 and 506 nm, and between 593 and 627 nm; CFP: excitation 436±10 nm, emission 480±20 nm; YFP: excitation 500±20 nm, emission 525±20 nm; FRET: excitation 436±10 nm, emission 535±30 nm; TMRM: excitation 500±20 nm, emission 570 nm long pass filter; AHI Analysentechnik, Tübingen, Germany). Images were recorded using a CCD camera (Visicam, Visitron Systems, Puchheim, Germany). The imaging setup was controlled by MetaMorph software (Universal Imaging, West Chester, PA). During control experiments bleaching of the probes was negligible.

Kinetics of FRET disruption

Images were processed using MetaMorph software (Universal Imaging, West Chester, PA). CFP/YFP emission ratios were obtained by dividing the integrated fluorescence intensity values of single cells (Rehm et al., 2002). To compare individual cells, time courses of the emission ratios were scaled by defining the baseline ratio before the onset of FRET disruption as one. Changes in TMRM uptake of individual cells were monitored by total intensity in the TMRM-sensitive channel after subtraction of the YFP overlap. The baseline of total intensity was defined as 100% in cells 1 hour after the equilibration with 30 nM TMRM.

Preparation of whole cell extracts and western blotting

Cells were collected at 200 g for 5 minutes and washed with phosphate-buffered saline (PBS). The cell pellet was resuspended in lysis buffer [62.5 mM Tris HCl pH 6.8, 10% (v/v) glycerin, 2% (w/v) sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml pepstatin A, 1 μg/ml leupeptin and 5 μg/ml aprotinin]. Cell homogenates were centrifuged at 15,000 g and 4°C for 15 minutes. Protein content was determined with the Pierce Micro-BCA Protein Assay (KMF, Cologne, Germany). An equal amount of protein (20 or 40 μg) was loaded onto SDS-polyacrylamide gels. Proteins were separated at 120 V for 1.5 hours and then blotted to nitrocellulose membranes (Protein BA 83; 2 μm; Schleicher & Schuell, Dassel, Germany) in transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol (v/v) and 0.01% SDS]. The blots were blocked with 5% non-fat dry milk in TBST (15 mM Tris-HCl pH 7.5, 200 mM NaCl and 0.1% Tween-20) at room temperature for 2 hours. Membranes were incubated with a mouse monoclonal anti-Na+/K+-ATPase α-1 subunit antibody (clone C464.6, 1:2000, Upstate Biotechnology, Lake Placid, NY), a mouse monoclonal anti-Na+/K+-ATPase β-1 subunit antibody (clone C464.8, 1:2000, Upstate Biotechnology), a rabbit polyclonal anti-caspase-3 antibody (H-277, 1:1000, Santa Cruz Biotechnology, CA), a mouse monoclonal anti-poly(ADP ribose) polymerase (PARP) antibody (clone C2-10, 1:2000, Pharmingen Becton Dickinson, Hamburg, Germany), or a mouse monoclonal anti-α-tubulin antibody (clone D3 1A; 1:5000, Sigma, Missouri). Membranes were washed with TBST six times for 10 minutes and incubated with anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies (Promega, Madison, WI) for one hour. Blots were washed and developed using the ECL chemiluminescence detection reagent (Amersham Pharmacia, Buckinghamshire, UK). Membranes were stripped in standard stripping buffer (2% SDS, 62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol, pH 6.8) at 60°C for 20 minutes, washed twice in TBST for 10 minutes and reprobed.

Data analysis and statistics

For quantification of changes in TMRM and Dibac3(5) uptake, all cells within a given field were analyzed that (a) demonstrated cyt-C-GFP release or TMRM fluorescence loss and (b) could be monitored for at least 60-90 minutes afterward. Data are given as means±s.e.m. For statistical comparison, ANOVA and subsequent Tukey test were employed (SPSS 10.0, SPSS Inc., Chicago, USA). P values smaller than 0.05 were considered to be statistically significant.

Results

Outer mitochondrial membrane permeabilization during apoptosis triggers a permanent decrease in mitochondrial TMRM uptake

Apoptosis was induced in MCF-7/Casp-3 cells stably expressing a cyt-C-GFP fusion protein, and the kinetics of cyt-C-GFP release were monitored by confocal fluorescence microscopy. Cells were exposed to the protein kinase inhibitor staurosporine (STS) (activation of the mitochondrial pathway) or to tumor necrosis factor-α plus cycloheximide (TNFα/CHX) (activation of death receptors). As reported previously, individually cells released cyt-C-GFP at different time points after addition of the pro-apoptotic agents (Goldstein et al., 2000; Heiskanen et al., 1999; Luetjens et al., 2001). The majority of the cyt-C-GFP fusion protein was released in one single step that was completed within 10 minutes (Fig. 1A,B). The cyt-C-GFP redistribution was indicated by a decrease in the s.d. of the average pixel intensity owing to the homogeneous distribution of cyt-C-GFP released. Confocal imaging of the kinetics of cyt-C-GFP release and TMRM uptake in MCF-7/Casp-3 cells suggested that \(\Delta V_M\) depolarized after the release of the cyt-C-GFP fusion protein until a new steady-state level was reached \(\langle\Delta V_M\rangle\). To calculate average kinetics, the time of cyt-C-GFP release was set to zero for all cells evaluated (Fig. 1C). In cells treated with
Release of cyt-C-GFP triggers a decrease in mitochondrial TMRM uptake. (A) MCF-7/Casp-3 cells stably expressing cyt-C-GFP were equilibrated with 30 nM TMRM and treated with 3 μM STS. Cyt-C-GFP release was followed by a reduction in mitochondrial TMRM uptake. Bars, 20 μm. (B) Individual traces of two typical cells treated with 3 μM STS plus 200 μM z-VAD-fmk. Note the absence of apoptotic shrinkage in z-VAD-fmk-treated cells despite cyt-C-GFP release. (C) Mean traces of 16 cells in four independent experiments calculated from single cell kinetics by setting the time of onset of cyt-C-GFP release to zero. (D,E) Transmission light and GFP fluorescence images of cells treated with 3 μM STS for the indicated time periods in the absence (D) or presence (E) of 200 μM z-VAD-fmk. Bars, 20 μm. Note the absence of apoptotic shrinkage in z-VAD-fmk-treated cells despite cyt-C-GFP release. (F) z-VAD-fmk-insensitive decrease in TMRM uptake in HeLa D98 cells stably expressing cyt-c-GFP. Cells were treated with 3 μM STS in the presence of 200 μM z-VAD-fmk. Similar results were obtained in 14 cells in two separate experiments. (G,H) Simultaneous monitoring of DEVDase activity and TMRM uptake in single MCF-7/Casp-3 cells transiently transfected with the FRET probe CFP-DEVD-YFP and treated with 3 μM STS or 3 μM STS plus 200 μM z-VAD-fmk. The increase in the CFP/YFP ratio indicates the time course of the cleavage of the FRET probe. Similar kinetics of FRET probe cleavage and TMRM uptake could be detected in two independent transient transfection experiments per treatment. Traces in H have different time scales to demonstrate the absence of FRET probe cleavage up to 4 hours after completion of the TMRM fluorescence changes.

3 μM STS, the average TMRM fluorescence intensity decreased within 60-90 minutes. In cells treated with TNF-α/CHX, the kinetics of cyt-C-GFP redistribution were similar to those after treatment with STS (Fig. 1D). The decrease in TMRM uptake did not recover during the time course of the experiments (up to 4 hours after cyt-C-GFP release), suggesting that cells failed to recover from ΔΨM depolarization during the time course of the experiments. We also observed a permanent decrease in TMRM uptake in STS-treated, stably transfected HeLa D98 cells after release of the cyt-c-GFP fusion protein (data not shown).

The decrease in mitochondrial TMRM uptake during apoptosis is not sensitive to a broad-spectrum caspase inhibitor

We subsequently addressed the question of whether the decrease in TMRM uptake required the activation of caspases. MCF-7/Casp-3 cells expressing cyt-C-GFP were treated simultaneously with 3 μM STS and 200 μM of the broad-spectrum caspase inhibitor z-VAD-fmk. Although the onset of the cyt-C-GFP release was delayed in cells treated with STS plus z-VAD-fmk (STS: 206±11 minutes, STS+z–V AD-fmk: 311±32 minutes, n=16 cells each in eight separate experiments, P<0.05), the individual release kinetics were not altered by caspase inhibition (Fig. 2A,B) (Goldstein et al., 2000; Luetjens et al., 2001). We could also detect a decrease in mitochondrial TMRM uptake in z-VAD-fmk-treated cells after the release of cyt-C-GFP. Similar to the results obtained in cells treated with STS alone, the depolarization reached a steady-state level after 60 minutes (Fig. 2B,C). However, the average TMRM intensity at this steady state was significantly higher than in cells treated with STS alone (see also Fig. 5D). We could not detect any recovery of TMRM uptake in the presence of z-VAD-fmk up
to 90 minutes after the release of the cyt-C-GFP fusion protein (Fig. 2B,C). In select experiments, cells were monitored up to 6 hours after cyt-C-GFP release and also revealed no recovery of \( \Delta \Psi_M^{\text{cyt-C}} \) in the presence of z-VAD-fmk. By contrast, z-VAD-fmk potently protected cells that had released cyt-C-GFP from apoptotic morphological changes induced by STS, including cell shrinkage and blebbing (Fig. 2D,E). Treatment with z-VAD-fmk also completely inhibited the
processing of caspase-9 as well as caspase-3 and 7 into active subunits as detected by western blot analysis (data not shown). These findings were not confined to MCF-7/Casp-3 cells, since a z-VAD-fmk-independent decrease in mitochondrial TMRM uptake was also observed in HeLa D98 cells stably transfected with cyt-C-GFP (Fig. 2F).

The z-VAD-fmk-independent decrease in mitochondrial TMRM uptake during apoptosis was also observed in a series of FRET experiments in which we simultaneously monitored activation of DEVDases and changes in TMRM uptake by epifluorescence microscopy. MCF-7/Casp-3 cells were transfected with a recombinant FRET probe comprising cyan fluorescent protein (CFP), a linker peptide containing a caspase-cleavage site (DEVD) and yellow fluorescent protein (YFP). The DEVD linker peptide is cleaved upon activation of DEVD-prefering caspses, resulting in a loss of the FRET effect and an increase in the CFP/YFP emission ratio (Tyas et al., 2000). Treatment with STS resulted in a rapid activation of DEVDases in MCF-7/Casp-3 cells as described previously (Rehm et al., 2002), and a simultaneous decrease in the average pixel intensity of the TMRM fluorescence (Fig. 2G). The kinetics of TMRM fluorescence decrease detected in these epifluorescence microscopy experiments resembled those seen in the confocal imaging experiments. Cells treated with STS and z-VAD-fmk also showed a sudden decrease in TMRM uptake, but the activation of DEVDases was potently blocked (Fig. 2H). Similar results were obtained in HeLa D98 cells stably transfected with the recombinant FRET probe (3 μM STS-treated cells: decrease in TMRM uptake that was accompanied by FRET disruption in 19 out of 19 events; 3 μM STS plus 200 μM z-VAD-fmk-treated cells: decrease in TMRM uptake that was accompanied by FRET disruption in 0 out of 18 events; data from three separate experiments per treatment).

**Effect of FCCP or oligomycin on the steady-state level of ΔΨ%

STS-treated cells that had released cyt-C-GFP showed a significantly reduced, but stable, TMRM uptake, suggesting that cells had established a new ΔΨM (ΔΨM<sup>cyt-C</sup>). Treatment with the protonophore FCCP was able to further dissipate mitochondrial TMRM uptake, both in the absence (Fig. 3A) or presence (data not shown) of 200 μM z-VAD-fmk. The ability of mitochondria to maintain ΔΨM<sup>cyt-C</sup> after outer mitochondrial membrane permeabilization could be due to a reversal of the FO F1-ATP-synthase, consuming ATP and thereby generating ΔΨM<sup>cyt-C</sup>. We treated cells with the FO F1-ATP-synthase inhibitor oligomycin (5 μM) 60 minutes after the addition of STS but prior to the release of the cyt-C-GFP fusion protein. In these cells, the release of cyt-C-GFP was always followed by a decrease in mitochondrial TMRM uptake to background levels within a 60 minute time period (Fig. 3B-D). Interestingly, cellular necrosis frequently followed the total decrease in TMRM fluorescence, as indicated by a sudden decrease in the GFP fluorescence intensity (Fig. 3B, indicated by arrows). The average time to cellular necrosis was 83±14 minutes after the cyt-C-GFP release. Addition of oligomycin to cells that already had released cyt-C-GFP and maintained a stable ΔΨM<sup>cyt-C</sup> led to a rapid and complete dissipation of TMRM uptake (Fig. 3E).
intact cells, $\Delta \Psi_p$ is largely maintained by the activity of the Na$^+$/K$^+$-ATPase. Immunoblot analysis of whole cell extracts of MCF-7/Casp-3 cells treated with 3 μM STS showed a significant degradation of the regulatory β-subunit of Na$^+$/K$^+$-ATPase. The degradation correlated with the accumulation of the caspase-derived 85 kDa cleavage product of PARP (Fig. 4F), and the processing of procaspase-3 into active subunits (Fig. 4G). By contrast, a degradation of the α-subunit of Na$^+$/K$^+$-ATPase could not be detected. The degradation of the β-subunit of Na$^+$/K$^+$-ATPase and the activation of
procaspase-3 were potently blocked in cells treated with STS plus z-VAD-fmk (Fig. 4G).

Simulation of TMRM fluorescence changes due to outer mitochondrial membrane permeabilization in a virtual cell

To evaluate quantitatively the contribution of $\Delta \Psi_M$ and $\Delta \Psi_P$ to the changes in TMRM fluorescence after outer mitochondrial membrane permeabilization, we employed a method introduced by Ward et al. (Ward et al., 2000). In this approach, TMRM fluorescence changes are calculated on the basis of Nernst calculations of fluorescence in the extracellular, cytoplasmic and mitochondrial compartments, taking the mitochondrial volume fraction, diffusion constant of TMRM across cellular membranes and the quenching limit of TMRM into account (see Materials and Methods). Similar to our previously reported findings in STS-treated hippocampal neurons and medulloblastoma cells (Poppe et al., 2001), we observed that $\Delta \Psi_M$ hyperpolarized from $-150$ mV to $-160$ mV in the MCF-7/Casp-3 cells within 2 hours of addition of STS (data not shown). The remodeling therefore starts with $\Delta \Psi_M=-160$ mV and $\Delta \Psi_P=-70$ mV. The increase in the Dibac4(3) intensity from 100±3% to 182±20% indicated a depolarization of $\Delta \Psi_P$ to $-58\pm4$ mV calculated with the Nernstian equation (Dall’Asta et al., 1997). In STS-treated cells, we obtained a good fit to our experimentally determined

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**Fig. 4.** Simultaneous depolarization of mitochondrial and plasma membrane potential during STS-induced apoptosis.

(A) Changes in TMRM and Dibac4(3) uptake in a typical MCF-7/Casp-3 cell after and exposure to valinomycin (10 nM, $\Delta \Psi_M$ depolarization) and ouabain (100 µg/ml, $\Delta \Psi_P$ depolarization). Similar traces were recorded in 10 cells in two experiments.

(B) Individual traces of the average fluorescence intensity of two typical MCF-7/Casp-3 cells equilibrated with the positively charged TMRM and the negatively charged Dibac4(3) after treatment with 3 µM STS. (C) Mean values of Dibac4(3) and TMRM fluorescence kinetics were calculated by setting the time of onset of decrease in TMRM fluorescence to zero. Data represent 11 individual cells from three independent experiments. (D) Inhibition of caspases protects MCF-7/Casp-3 cells from STS-induced $\Delta \Psi_P$ depolarization. Individual traces of two typical cells treated with 3 µM STS and 200 µM z-VAD-fmk. (E) Mean values of 12 cells treated with 3 µM STS and 200 µM z-VAD-fmk in two independent experiments.

(F) Time course of Na$^+$/K$^+$-ATPase $\beta$-subunit degradation during STS-induced apoptosis. Cells were treated with STS and whole cell extracts were analyzed by immunoblotting.

(G) Treatment with z-VAD-fmk inhibits the degradation of the Na$^+$/K$^+$-ATPase $\beta$-subunit and the activation of effector caspase-3. Cells were treated with 3 µM STS, 3 µM STS plus 200 µM z-VAD-fmk or vehicle. Whole cell extracts were prepared after 8 hours of treatment and analyzed by immunoblotting.
TMRM kinetics, remodeling a $\Delta \Psi_p$ depolarization of 10 mV ($t_{1/2}=30$ minutes) and a $\Delta \Psi_M$ depolarization of 32 mV ($t_{1/2}=30$ minutes) (Fig. 5A). The mitochondrial TMRM fluorescence in a virtual cell treated with STS and z-VAD-fmk was remodeled with a $\Delta \Psi_M$ depolarization of 32 mV ($t_{1/2}=17$ minutes) in the absence of $\Delta \Psi_p$ depolarization (Fig. 5B). The remodeled TMRM signal after the depolarization of $\Delta \Psi_M$ equilibrates to a value of 30% in a virtual cell treated with STS plus z-VAD-fmk, compared to the value of 33.9±5.5% that we determined experimentally in our confocal imaging experiments (Fig. 5D). The remodeled equilibrated TMRM signal of a virtual cell treated with STS alone reached a value of 21.2% of the initial fluorescence, compared to the experimentally determined value of 20.9±2.9% (Fig. 5D). Finally, the effect of oligomycin was successfully remodeled assuming a $\Delta \Psi_M$ depolarization of 65 mV ($t_{1/2}=30$ minutes) and a $\Delta \Psi_p$ depolarization of 10 mV ($t_{1/2}=30$ minutes) as detected with Dibac4(3) (data not shown) (Fig. 5C,D).

**Discussion**

The present study demonstrates several important findings that increase our understanding of the sequence of events and causal relationship of cell death pathways activated after outer mitochondrial membrane permeabilization. (i) The release of soluble intermembrane proteins during apoptosis triggers a permanent $\Delta \Psi_M$ depolarization in MCF-7/Casp-3 and HeLa D98 cells that is not sensitive to the broad spectrum caspase inhibitor z-VAD-fmk. (ii) After the release, $\Delta \Psi_M$ reaches a new steady-state level, $\Delta \Psi_M^{\text{cyt-C}}$. In the presence of z-VAD-fmk, $\Delta \Psi_M^{\text{cyt-C}}$ is stable over several hours and maintained by FcF1-ATP-synthase reversal. (iii) $\Delta \Psi_p$ also depolarizes in MCF-7/Casp-3 cells during apoptosis. Similar to the release of cyt-C-GFP, it occurs in individual cells at different time points after induction of apoptosis. In apoptotic cells, $\Delta \Psi_p$ depolarization was coupled with $\Delta \Psi_M$ depolarization. Finally, (iv) $\Delta \Psi_p$ depolarization was strictly caspase dependent and associated with the degradation of the regulatory Na+/K+ -ATPase β-subunit.

$\Delta \Psi_M$ depolarization during apoptosis

Cyt-C transports electrons between complex III and cytochrome C oxidase. Outer mitochondrial membrane permeabilization and subsequent release of large quantities of cyt-C will slow down this electron flow, leading to an impairment of mitochondrial respiratory chain activity and a reduced H+ export from the mitochondrial matrix. Indeed, $\Delta \Psi_M$ depolarization is considered a hallmark of apoptosis, and ATP levels are known to decrease considerably during this process (Adachi et al., 1997; Cai and Jones, 1998; Lemasters et al., 1999; Mootha et al., 2001). In isolated mitochondria, re-addition of small amounts of cyt-C restored $\Delta \Psi_M$ and

Fig. 5. Remodeling of TMRM fluorescence changes in a virtual cell. (A) Remodeling of TMRM fluorescence in a STS-treated cell in the absence of caspase inhibitor z-VAD-fmk. TMRM fluorescence changes were remodeled with a $\Delta \Psi_M$ depolarization of 32 mV and a $\Delta \Psi_p$ depolarization of 10 mV as detected with Dibac4(3). The calculated average intensity of TMRM is plotted in black (scale on the left). The modeling of $\Delta \Psi_M$ and $\Delta \Psi_p$ kinetics is plotted in dark and light gray, respectively (scale on the right). Experimentally determined values from cells treated with 3 μM STS are shown for comparison (open squares). (B) Remodeling of a cell treated with 3 μM STS and 200 μM z-VAD-fmk. TMRM fluorescence changes were remodeled with a $\Delta \Psi_M$ depolarization of 32 mV in the absence of $\Delta \Psi_p$ depolarization. (C) Remodeling of TMRM fluorescence changes in cells treated with 3 μM STS and 5 μM oligomycin. TMRM fluorescence changes were remodeled with a $\Delta \Psi_M$ depolarization of 65 mV and a $\Delta \Psi_p$ depolarization of 10 mV as detected with Dibac4(3) (data not shown). Necrosis was remodeled to occur 83 minutes after the release of cyt-C and was simulated with a total depolarization of both potentials at that time. (D) Experimentally determined values for the steady-state level of TMRM fluorescence intensity after cyt-C-GFP release. The values were calculated using the sigmoidal Boltzmann equation as fit function for the single-cell kinetics of TMRM fluorescence from the experiments presented in Figs 1, 2 and 3. (*P<0.05 compared to STS-treated cells).
ATP production after outer mitochondrial membrane permeabilization (Mootha et al., 2001), suggesting a direct role for the loss of cyt-C in these bioenergetic alterations. \( \Delta \Psi_M \) depolarization can be observed very shortly after the quantitative release of a cyt-C-GFP fusion protein (Fig. 1) (Heiskanen et al., 1999; Waterhouse et al., 2001). The majority of cyt-C (85-90%) is believed to reside in the intracristal space and may not be directly accessible for release (Ott et al., 2002; Scorrano et al., 2002). It has been suggested that the complete release of cyt-C requires a reorganization of cristae and that small amounts of cyt-C (but presumably also cyt-C-GFP) are released prior to this (Luetjens et al., 2001; Scorrano et al., 2002). Of note, \( \Delta \Psi_M \) depolarization could only be detected after the complete release of cyt-C-GFP, suggesting that an earlier release of smaller quantities of cyt-C was not sufficient to trigger \( \Delta \Psi_M \) depolarization. It is possible that cyt-C-GFP is less effectively associated with the inner membrane and is thus lost more readily upon outer membrane permeabilization. However, \( \Delta \Psi_M \) clearly depolarized rapidly after the release of the fusion protein. It is also unlikely that cyt-C-GFP substitutes for the electron transport function of endogenous cyt-C, since previous experiments have estimated that the cyt-C-GFP fusion protein represents less than 0.5% of total cellular cyt-C (Goldstein et al., 2000; Luetjens et al., 2001). Cyt-C-GFP can therefore be considered a reliable indicator of the functional consequences of a complete outer mitochondrial membrane permeabilization and the accompanying loss of endogenous cyt-C.

We could demonstrate that \( \Delta \Psi_M \) stabilizes to a new potential, \( \Delta \Psi_M^{cyt-C} \), after the release of cyt-C-GFP, even in the absence of caspase inhibitors. These results are consistent with our previous observations in cultured rat hippocampal neurons, which demonstrated that mitochondria could still be depolarized with FCCP at a time point when nuclear condensation and fragmentation was clearly evident (Krohn et al., 1999). Previous studies using isolated mitochondria and permeabilized hepatocytes have demonstrated that the stabilization of \( \Delta \Psi_M \) after the release of cyt-C is caused by FoF1-ATP-synthase reversal, since treatment with the FoF1-ATP-synthase inhibitor oligomycin induced a rapid \( \Delta \Psi_M \) depolarization (Madesh et al., 2002; Polster et al., 2001). However, in both studies cytosolic cyt-C may have been washed out or diluted in the extracellular buffer solution to an extent that the concentration of cyt-C was too low to maintain mitochondrial respiration. Clearly, the energetics in cells with an intact plasma membrane may differ considerably from isolated mitochondria or permeabilized cells. Using an approach similar to our study, Waterhouse and co-workers (Waterhouse et al., 2001) proposed that diffusion of released cyt-C back into the mitochondrial respiratory chain maintained a \( \Delta \Psi_M \) after outer mitochondrial membrane permeabilization. Our study provides experimental and theoretical evidence for the concept that FoF1-ATP-synthase reversal maintains \( \Delta \Psi_M^{cyt-C} \) also in intact cells. Treatment with oligomycin either prior to or after the release of the cyt-C-GFP fusion protein resulted in a rapid and complete depolarization of \( \Delta \Psi_M \), and the experimentally determined TMRM fluorescence changes could be remodeled in a virtual cell assuming FoF1-ATP-synthase reversal. Likewise, Rego et al. (Rego et al., 2001) could convincingly remodel the kinetics of TMRM fluorescence changes induced by oligomycin in STS-exposed neural cells, assuming that FoF1-ATP-synthase reversal maintained \( \Delta \Psi_M^{cyt-C} \), demonstrating the power of this approach. We have also observed that the complex III inhibitor antimycin A rapidly depolarized \( \Delta \Psi_M \) in intact cells to a level similar to \( \Delta \Psi_M^{cyt-C} \), but had no effect on \( \Delta \Psi_M^{cyt-C} \) in cells that had released cyt-C-GFP (data not shown). There is also evidence that cyt-C may be released out of apoptotic cells, hence limiting the availability of cyt-C for mitochondrial respiration (Luetjens et al., 2000; Renz et al., 2001). It is therefore unlikely that the \( \Delta \Psi_M \) of cells that have released cyt-C is maintained for significant time periods by diffusion of cyt-C back to the mitochondrial inner membrane.

According to our simulation, the 80% decrease in average TMRM fluorescence intensity after the release of cyt-C-GFP reflects a depolarization of 32 mV assuming a \( \Delta \Psi_M \) of \(-160 \) mV prior to the release. These data suggest that FoF1-ATP-synthase reversal contributes to the stabilization of \( \Delta \Psi_M^{cyt-C} \) at a potential of \(-128 \) mV. Indeed, it has previously been shown that a \( \Delta \Psi_M \) depolarization of 10 mV with the complex III inhibitor antimycin A was sufficient to trigger FoF1-ATP-synthase reversal (Ward et al., 2000). However, TMRM may also behave in a non-Nernstian manner owing to membrane and matrix protein binding (Scaduto et al., 1999). Hence, quantification of \( \Delta \Psi_M \) depolarization on the basis of TMRM uptake may lead to an overestimation of \( \Delta \Psi_M^{cyt-C} \). The synthase activity is also reduced with a decrease in \( \Delta \Psi_M \) over the inner mitochondrial membrane (Dimroth et al., 2000; Matsuayama et al., 2000). Increased lactate production and cytosolic acidification is likely to occur in cells in which \( \Delta \Psi_M^{cyt-C} \) is maintained by glycolytic ATP production. Both events have been shown to occur during apoptosis (Matsuayama et al., 2000; Tiefenthaler et al., 2001). Interestingly, \( \Delta \Psi_M \) changes during apoptosis are sensitive to Bcl-2 overexpression but independent of caspase activation (Matsuayama et al., 2000) (see Discussion).

The switch from mitochondrial to glycolytic ATP production may be facilitated in energized cells such as neurons or in tumor cells with higher basal level of glycolysis. The energetic state of cells that have released cyt-C resembles the state of cells depleted of mitochondrial DNA (\( \rho^0 \) cells), which also lack mitochondrial respiration and maintain their \( \Delta \Psi_M \) via FoF1-ATP-synthase reversal. Of note, apoptosis in \( \rho^0 \) cells is not significantly inhibited (Jacobson et al., 1993), suggesting that glycolysis may provide sufficient amounts of ATP for the execution of apoptosis. Of note, pretreatment with oligomycin not only leads to a total breakdown of \( \Delta \Psi_M^{cyt-C} \) but also to subsequent necrosis. It has previously been demonstrated that oligomycin treatment is able to switch the cell death mode from apoptosis to necrosis (Eguchi et al., 1997; Leist et al., 1997). Our results now demonstrate that cyt-C release is also important for the occurrence and timing of necrosis in energetically deprived cells.

Role of caspases in \( \Delta \Psi_M \) and \( \Delta \Psi_p \) depolarization

Simultaneous monitoring of (i) cyt-C-GFP and TMRM fluorescence changes, (ii) DEVdase activity and TMRM uptake, and (iii) Dibac4(3) and TMRM uptake in MCF-7/Casp-3 and HeLa D98 cells demonstrated that the depolarization of \( \Delta \Psi_M \) during apoptosis was insensitive to z-VAD-fmk. Supporting the concept of FoF1-ATP-synthase reversal in maintaining \( \Delta \Psi_M^{cyt-C} \), we did not detect any \( \Delta \Psi_M \) recovery, even if the activation of the major effector caspases
was potently blocked with a broad spectrum caspase inhibitor. Waterhouse and co-workers (Waterhouse et al., 2001) previously reported that HeLa cells treated with z-VAD-fmk showed a rapid recovery of $\Delta V_M$ depolarization after the release of cyt-C-GFP. It is unlikely that these contrasting results are a consequence of cell type differences, since we were also not able to detect a recovery of $\Delta V_M$ in z-VAD-fmk-treated HeLa cells, despite a complete inhibition of DEVDase activity (see Results). In the present study, we could demonstrate that $\Delta V_P$ depolarizes simultaneously to $\Delta V_M$, but in a z-VAD-fmk-sensitive manner. We could also demonstrate that $\Delta V_P$ depolarization contributed to the resulting TMRM signal. The influence of $\Delta V_P$ depolarization on the uptake of potential-sensitive cationic dyes critically depends on the concentration and type of dye and is particularly pronounced when concentrations are used that saturate mitochondrial uptake (‘quenching mode’) (Ward et al., 2000). In the study of Waterhouse and co-workers (Waterhouse et al., 2001), potential effects of $\Delta V_P$ depolarization on the uptake of voltage-sensitive probes into mitochondria were not taken into account, and experiments were performed with tetramethyl rhodamine ethyl-ester, which has a significantly higher partition coefficient in mitochondrial membranes than TMRM (Scaduto et al., 1999). Of note, our simulation of $\Delta V_M$ and $\Delta V_P$ changes in a virtual cell – taking the depolarization of $\Delta V_P$ detected with Dibac(3) into account – confirmed our results detected with TMRM quantitatively. Interestingly, z-VAD-fmk-treated cells that had released cyt-C-GFP showed a stable $\Delta V_M$ whereas the $\Delta V_P$ did not exhibit a depolarization of $\Delta V_P$ and were viable for several hours. However, these cells may also eventually die, a process that may involve the complete removal of mitochondria by autophagy or a final $\Delta V_M$ depolarization after the activation of the permeability transition pore (Chang and Johnson, 2002; Xue et al., 2001).

Ion gradients across the plasma membrane are not stable during apoptosis (Bortner and CIDŁowski, 1999; Bortner et al., 2001; Maeno et al., 2000). Our single-cell experiments demonstrate that – similar to the release of cyt-C-GFP – the time point of $\Delta V_P$ depolarization was set individually for each cell during apoptosis. The broad-spectrum caspase inhibitor z-VAD-fmk not only blocked $\Delta V_P$ depolarization but also inhibited apoptotic cell shrinkage and the degradation of the regulatory $\beta$-subunit of Na$^+$/K$^+$-ATPase, suggesting that these were caspase-dependent events. Previous studies have shown that the intracellular Na$^+$ concentration increases prior to a loss of plasma membrane integrity (Bortner et al., 2001) and that cell shrinkage in apoptotic Jurkat cells is accompanied by a net efflux of ions due to an inactivation of Na$^+$/K$^+$-ATPase (Mann et al., 2001). The latter authors also observed that Na$^+$/K$^+$-ATPase subunits were degraded in populations with reduced volume during apoptosis. Interestingly, it has previously been shown that apoptosis is associated with a Bcl-2-sensitive activation of outward K$^+$ currents (Ekhterae et al., 2001; Yu et al., 1997). Because active cellular volume regulation requires Na$^+$/K$^+$-ATPase activity, both events may act synergistically in the induction of cell shrinkage. Cleavage products of the Na$^+$/K$^+$-ATPase $\beta$-subunit could not be detected in the present study, but it should be noted that the N-terminal cytoplasmic domains of the known $\beta$-subunits do not contain canonical caspase cleavage sites. This suggests that caspases may not directly cleave these subunits. The $\beta$-subunit of the Na$^+$/K$^+$-ATPase, however, regulates the K$^+$ affinity of the enzyme at the extracellular site (Eakle et al., 1994; Shainskaya and Karlish, 1996). A truncation of the N-terminal cytoplasmic domain of the $\beta$-subunit has been shown to severely impair K$^+$ affinity and to increase ouabain-sensitive leak currents (Abriel et al., 1999; Hasler et al., 1998). Apart from a role in cell shrinkage during apoptosis, caspase-mediated degradation of Na$^+$/K$^+$-ATPase and other energy-consuming enzymes such as PARP (Ha and Snyder, 1999) may also help to transiently maintain cytosolic ATP levels sufficient for the execution of apoptosis in cells that have released cyt-C. Indeed, remodeling of TMRM fluorescence changes in the absence or presence of z-VAD-fmk revealed a slower $\Delta V_M$ depolarization when caspase activation was not blocked.

Our data demonstrate that outer mitochondrial membrane permeabilization during apoptosis triggers a z-VAD-fmk-sensitive $\Delta V_P$ depolarization, as well as a z-VAD-fmk-independent $\Delta V_M$ depolarization, and hence coordinates the activation of distinct, apoptotic and necrotic cell death pathways.

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