We investigated the relationship between opening of the permeability transition pore (PTP), mitochondrial depolarization, cytochrome c release, and occurrence of cell death in rat hepatoma MH1C1 cells. Treatment with arachidonic acid or A23187 induces PTP opening in situ with similar kinetics, as assessed by the calcein loading-Co²⁺ quenching technique. However, depolarization, as assessed from the changes of mitochondrial tetramethylrhodamine methyl ester (TMRM) fluorescence, is rapid and extensive with arachidonic acid and slow and partial with A23187. Cyclosporin A-inhibitable release of cytochrome c and cell death correlate with the changes of TMRM fluorescence but not with those of calcein fluorescence. Since pore opening must be accompanied by depolarization, we conclude that short PTP openings are detected only by trapped calcein and may have little impact on cell viability, while changes of TMRM distribution require longer PTP openings, which eventually cause cytochrome c release and cell death. Modulation of the open time appears to be the key element in determining the outcome of stimuli that converge on the PTP.

One of the key events in the course of apoptosis is the release of cytochrome c from mitochondria (1), which is able to activate procaspase 9 (2) and thus downstream caspases that amplify the death process (see Ref. 3 for review). Other mitochondrial proteins can be released as well, including apoptosis-inducing factor (4) and Smac/DIABLO, which promotes apoptosis by inactivating inhibitors of apoptosis proteins (5–7). Cytochrome c remains by far the most studied, and the mechanism(s) underlying its release are the matter of intense investigation and of considerable controversy.

Mitochondria from a variety of tissues can be induced to undergo an inner membrane permeability increase, the PT,¹ which allows diffusion of solutes with molecular mass up to about 1,500 Da. It is widely accepted that this transition is mediated by opening of an inner membrane high conductance channel, the PTP (see Ref. 8 for a recent review). The PTP has been shown to be implicated in ischemic cell death through dysregulation of Ca²⁺ homeostasis and ATP depletion (9–11). Since the PTP is accompanied by swelling as well as by cytochrome c release in vitro (12), it also represents an excellent candidate for the release of intermembrane proteins in the course of apoptosis as well (13, 14). An alternative mechanism of cytochrome c release is outer membrane insertion of truncated BID followed by oligomerization of BAX and/or BAK (15–18 and see Ref. 19 for review). However, it has been reported that insertion of BAX/BAK in the outer mitochondrial membrane can also cause cytochrome c release and cell death through a PT (20, 21), and BNP3 (a BCL-2 family member) can cause a PT and cell death without release of cytochrome c and caspase activation (22). The mechanistic relationships between PT, cytochrome c release, and cell death remain therefore a matter of intense debate. It is conceivable that an overlap may exist between different mechanisms (which often are not mutually exclusive); but it should also be recognized that different pathways may be activated in different paradigms of cell death. Finally, apparent discrepancies may also arise from the interpretation of the results obtained with fluorescent probes (13) and from the fact that detection methods based on cell disruption can cause rather than measure cytochrome c release (23).

In this study we investigated the occurrence of cell death in rat hepatoma MH1C1 cells treated with AA, a potent PTP inducer that is characterized in the accompanying article (24), or with the ionophore A23187. We found that both treatments cause PTP opening in situ with similar kinetics, as assessed by the calcein loading-Co²⁺ quenching technique (25), while depolarization, as assessed from the fluorescence changes of the potentiometric probe TMRM, was rapid and extensive with AA and slow and partial with A23187. A parallel assessment of cell viability and of CsA-inhibitable cytochrome c release with a quantitative in situ method showed that cell death correlates with the TMRM rather than with the calcein fluorescence changes. Since pore opening must be accompanied by depolarization, these findings suggest that relatively short PTP openings are detected only by trapped calcein and have little impact on cell viability, while detectable changes of TMRM distribution require longer PTP openings, which eventually cause cy-

¹The abbreviations used are: PT, permeability transition; PTP, permeability transition pore; AA, arachidonic acid; CsA and CsH, cyclosporin A and cyclosporin H, respectively; TMRM, tetramethylrhodamine methyl ester; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MDR, multidrug resistance.
tochrome c release and cell death. Thus, modulation of the open time appears to be the key element in determining the outcome of stimuli that impinge on the PTP.

MATERIALS AND METHODS

Cell Cultures—MH1C1 rat hepatoma cells were seeded onto uncoated 22-mm (for calcein and annexin-V staining) or 13-mm (for immunofluorescence) diameter round glass coverslips and grown for 2 days in Ham’s F-10 nutrient mixture supplemented with 10% fetal calf serum in a humidified atmosphere of 95% air, 5% CO2 at 37 °C in a Forma tissue culture water-jacketed incubator.

TMRM and Calcein Staining and Imaging—MH1C1 cells were loaded with 10 nM TMRM and incubated as specified in the legend of Fig. 2. The extent of cell and hence mitochondrial loading with potentiometric probes is affected by the activity of the plasma membrane MDR P-glycoprotein, which is inhibited by CsA (13). Treatment with this drug therefore causes an increased mitochondrial fluorescence that can be erroneously interpreted as an increase of the mitochondrial membrane potential (see Ref. 13 for discussion). To prevent this artifact and to normalize the loading conditions, in all experiments with TMRM the medium was supplemented with 1.6 μM CsH, which inhibits the MDR pump (13), but not the PTP (26). MH1C1 cells were loaded with 1 μM calcein-acetoxyester for 30 min at 37 °C in 1 ml of Hanks’ balanced salt solution supplemented with 10 mM Hepes, pH 7.4, and 1 mM CoCl2 (25). Cells were then washed free of calcein and Cs2+ and maintained in Hanks’ balanced salt solution-Hepes. When specified, CsA was added to the cells after probe loading, and fluorescence acquisition was started 30 min later, a protocol that made addition of CsA unnecessary in the experiments with calcein (results not shown). Cellular fluorescence images were acquired with an Olympus IMT-2 inverted microscope, equipped with a xenon light source (75 watts) for epifluorescence illumination and with a 12-bit digital cooled CCD camera (Micronax, Princeton Instruments). For detection of fluorescence, 488 ± 25 nm excitation and 525 nm longpass emission and 565 ± 25 nm excitation and 585 nm longpass emission filter settings were used for calcein and TMRM, respectively. Images were collected with exposure times ranging between 50 and 100 ms using a 40×, 1.3 NA oil immersion objective (Nikon). Data were acquired and analyzed using Meta-morph software (Universal Imaging). Clusters of several mitochondria (10 to 30) were identified as regions of interest, and fields not containing cells were taken as the background. Sequential digital images were acquired every 60 s or every 3 min for the experiments with a time course of 20 and 60 min, respectively, and the average fluorescence intensity of all relevant regions was recorded and stored for subsequent analysis. Mitochondrial fluorescence intensities minus background are reported in Figs. 2–4 after normalization of the initial fluorescence for comparative purposes, and they represent the mean of 10 regions of interest.

Immunodetection of bc1 Complex and Cytochrome c—MH1C1 cells were incubated as detailed in the figure legends and then washed. Cells were fixed for 30 min at room temperature with 3.7% (v/v) ice-cold formaldehyde, permeabilized for 20 min with 0.01% (v/v) ice-cold Nonidet P-40, and incubated for 15 min with a 0.5% solution of BSA and then for 15 min at 37 °C with a mouse monoclonal anti-cytochrome c antibody (PharMingen, clone 6H2.B4) and with an affinity-purified rabbit antibody against the rat bc1 complex (a generous gift of Prof. Roberto Biasson, Padova, Italy). Cells were then sequentially incubated for 15 min at 37 °C with tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG and with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG.

For cytochrome c and bc1 complex detection, red and green channel images were acquired simultaneously using two separate color channels on the detector assembly of a Nikon Eclipse E600 microscope equipped with a Bio-Rad MRC-1024 laser scanning confocal imaging system equipped with 488/522 ± 25 nm bandpass and 568/605 nm longpass filter settings, and a 60×, 1.4 NA oil immersion objective (Nikon). Twenty randomly chosen fields in each coverslip were stored for subsequent analysis.

Fig. 1 shows an example of the quantitative analysis carried out on a control (panel A) and AA-treated MH1C1 cell (panel B). Using the Bio-Rad LaerSharp analysis program a set of lines was drawn across the cells (only two such lines are illustrated in panels A and B for the sake of clarity). Using the appropriate function of the analysis program, the fluorescence intensity of each pixel along the line in both the green and the red channel was measured, and panels A’ and B’ report the fluorescence intensity profiles along the lines drawn in panels A and B, respectively. The localization index is defined as the ratio of the S.D. of the fluorescence intensity divided by the total fluorescence for each channel: (S.D./μ)/μ. A punctate distribution results in a higher S.D., while normalization allows correction for different fluorescence intensities in the two channels. A localization index of 1 indicates that cytochrome c and the bc1 complex have the same distribution, which is expected in normal cells, while an index lower than 1 means that the distribution of cytochrome c is more homogeneous than that of the bc1 complex. In the example of Fig. 1 the localization index is 1 for the cell of panel A and 0.6 for the cell in panel B.

Annexin-V and propidium iodide staining and imaging were carried out exactly as described previously (27).

RESULTS

The experiments of Fig. 2, panel A, report the fluorescence changes of mitochondria loaded with calcein in the presence of Co2+ in intact cells, a method that allows detection of PTP openings in situ (25). Addition of AA resulted in a large decrease of calcein fluorescence that was due to PTP opening (panel A, squares), as indicated here by inhibition of the fluorescence changes by CsA (panel A, circles; see also Ref. 24). A similar experiment was carried out in cells loaded with TMRM, a probe that accumulates within polarized mitochondria where and when it is released upon depolarization (28). It can be seen (panel B) that the addition of AA was followed by a large CsA-sensitive depolarization with the same time course as that displayed by the changes of calcein fluorescence (compare with panel A) and comparable in extent to that observed upon the addition of FCCP to CsA-treated cells (panel B) or to cells that had not been treated with AA (see Fig. 4, panel B).
results indicate that PTP opening by AA causes mitochondrial depolarization in situ, a finding that was also obtained in nominally Ca$^{2+}$-free media (results not shown).

A similar experiment was carried out with the divalent metal ionophore A23187. Fig. 3, panel A, shows that addition of A23187 caused a rapid drop of calcein fluorescence (squares), which was much faster than that caused by AA (compare with panel A in Fig. 2), and essentially complete within about 3 min of the addition of A23817. The fluorescence changes were still due to PTP opening, as indicated by their sensitivity to CsA (circles). Quite unexpectedly, however, the changes of TMRM fluorescence were negligible over the same time frame and slowly decreased by only 20% in about 20 min of incubation (panel B, squares). These TMRM fluorescence changes were also due to the PTP, since they could be blocked by CsA, which instead did not affect the probe response to FCCP (panel B, circles). These experiments demonstrate that PTP openings may occur that are accompanied by negligible changes of TMRM fluorescence and suggest that the time required for redistribution of potentiometric probes like TMRM may be too long (28) to report these PTP openings, which we assume to be of short duration. At variance from the case of AA, the PTP-inducing effects of A23187 disappeared in Ca$^{2+}$-free media (results not shown).

We next tested whether depolarization with the protonophore FCCP was able to cause PTP opening in MH1C1 cells in the absence of inducing agents like AA or A23187. The experiments of Fig. 4, panel A, indicate that no changes of calcein fluorescence could be elicited by FCCP either in the sensitivity to CsA (squares) and of the mitochondrial bc$_1$ complex were studied in individ-
al cells in situ with the quantitative technique described under “Materials and Methods.” It can be clearly appreciated that the addition of FCCP had negligible effects on the cyto-
da of the mitochondrial bc$_1$ complex and of the mitochondrial cytochrome c localization. MH1C1 cells were treated for the indicated times with 0.2 mM AA (squares), 2 μM A23187 (circles), 2 μM FCCP (triangles) or vehicle (0.02% v/v ethanol, diamonds). In the experiments denoted by solid symbols, cells were pre-
treated with 2 μM CsA for 15 min. Cells were fixed and treated with antibodies against the bc$_1$ complex and cytochrome c, and the cyto-
chred). Where indicated (arrows) 2 μM A23187 and 2 μM FCCP were added.
The PTP has been extensively characterized in mitochondrial suspensions (29–31), in individual organelles (32), and at the single channel level (33 and 34 and see Ref. 8 for a comprehensive review). Although its structural features are still a matter of debate (see e.g. Ref. 35), the functional properties of the PTP and the consequences of a PT in vitro are relatively well understood (8). Despite these advances, it remains extremely difficult to make predictions about the occurrence and regulation of the PT in situ, in part because the PTP is detected by indirect means, most often based on the expected changes of the mitochondrial membrane potential, and in part because multiple PTP modulatory factors change at the same time. A striking example is represented by the effects of FCCP. The pore is voltage-dependent, in the sense that depolarization favors PTP opening (36), yet the effect of depolarization may be offset by matrix acidification (37) and by the increase of matrix ADP and Mg$^{2+}$, which all prevent pore opening. The experiments reported here indicate that depolarization with FCCP is not followed by PTP opening in MH1C1 cells, suggesting that PTP inhibitory factors prevail despite depolarization. Two main issues should be considered.

(i) The open-closed transitions of individual channels occur well below the time range of the msec (33), which imposes obvious constraints in measurements based on redistribution of potentiometric fluorescent probes in situ. Indeed, transient PTP openings may not be detected when the probe response time is below the PTP open time, implying that only relatively long lasting PTP openings may be reliably detected by these probes in situ. Furthermore, even TMRM-detectable PTP openings may occur asynchronously in individual mitochondria (32) and would be missed unless single mitochondria are resolved.

(ii) The cellular accumulation of potentiometric probes is not a simple function of the mitochondrial membrane potential. It also depends on net transport across the plasma membrane, which is determined by the plasma membrane potential (which drives the accumulation) and by the activity of the MDR pump (which extrudes the probe). Mitochondrial accumulation will be affected by changes of the plasma membrane potential (38, 39), and inhibition of the MDR pump will inevitably increase the mitochondrial probe accumulation (13). It is unfortunate that both the PTP and the MDR pump are inhibited by CsA (13), a finding that calls into question the interpretation of a large number of experiments where the PTP has been considered as a causative event in cell death (13).

Because of these problems, we have developed a method for in situ PTP detection that is based on trapping of calcine followed by quenching of cytosolic fluorescence by Co$^{2+}$. Since calcine and Co$^{2+}$ cannot cross the mitochondrial inner membrane, PTP opening can be studied as a CsA-sensitive quenching of mitochondrial calcine fluorescence (25). To address the second problem we have included CsH (which inhibits the MDR pump but not the PTP) in our measurements. Given that the MDR pump is already inhibited by CsH, the effects of CsA on the TMRM signal coming from mitochondria must be due to effects on the PTP. A contribution from variations of the plasma membrane potential cannot be excluded easily, yet this was not the major cause of the TMRM fluorescence changes in our protocols, because they were also observed in KCl-based media (results not shown).

**PTP Opening and Cell Death**

**Preliminary Considerations**—The PTP has been extensively characterized in mitochondrial suspensions (29–31), in individual organelles (32), and at the single channel level (33 and 34 and see Ref. 8 for a comprehensive review). Although its structural features are still a matter of debate (see e.g. Ref. 35), the functional properties of the PTP and the consequences of a PT in vitro are relatively well understood (8). Despite these advances, it remains extremely difficult to make predictions about the occurrence and regulation of the PT in situ, in part because the PTP is detected by indirect means, most often based on the expected changes of the mitochondrial membrane potential, and in part because multiple PTP modulatory factors change at the same time. A striking example is represented by the effects of FCCP. The pore is voltage-dependent, in the sense that depolarization favors PTP opening (36), yet the effect of depolarization may be offset by matrix acidification (37) and by the increase of matrix ADP and Mg$^{2+}$, which all prevent pore opening. The experiments reported here indicate that depolarization with FCCP is not followed by PTP opening in MH1C1 cells, suggesting that PTP inhibitory factors prevail despite depolarization. Two main issues should be considered.

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in a nonsynchronized fashion (41). It is noteworthy that even after PTP-dependent large amplitude swelling of the whole mitochondrial population pore closure was followed by shrinkage with full functional recovery provided that cytochrome c was added back (12). This finding indicates that no permanent damage to the inner membrane is caused by even long lasting PTP openings (12).

An alternative possibility is that subtle changes of matrix volume may make more cytochrome c available for selective release (13). Tomographic reconstruction of thick sections of mitochondria after high voltage electron microscopy has indeed revealed that the intercristal spaces, which contain cytochrome c, are pleomorphic structures that communicate with the peripheral (intermembrane) space, and sometimes between themselves, through very narrow tubular regions (42). These findings are in good agreement with earlier work demonstrating that only 10–15% of cytochrome c is available for reduction by outer membrane NADH-cytochrome b5 reductase and that this fraction can be effectively increased by matrix swelling (43). If this compartmentation also occurs in vivo, the matrix-intercristal volume changes caused by a PT could be instrumental to make cytochrome c available for release.

In summary, the present results demonstrate that PTP opening can be a causative event in cytochrome c release in situ irrespective of whether the latter occurs through a selective pathway or because of outer membrane rupture. Further work will be required to define the relevance of this mechanism to endogenous signaling pathways of cell death.

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