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Mitochondrial Outer Membrane Permeability Change and Hypersensitivity to Digitonin Early in Staurosporine-induced Apoptosis*

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We have shown here that the apoptosis inducer staurosporine causes an early decrease in the endogenous respiration rate in intact 143B.TK$^-$ cells. On the other hand, the activity of cytochrome *c* oxidase is unchanged for the first 8 h after staurosporine treatment, as determined by oxygen consumption measurements in intact cells. The decrease in the endogenous respiration rate precedes the release of cytochrome *c* from mitochondria. Moreover, we have ruled out caspases, permeability transition, and protein kinase C inhibition as being responsible for the decrease in respiration rate. Furthermore, overexpression of the gene for Bcl-2 does not prevent the decrease in respiration rate. The last finding suggests that Bcl-2 acts downstream of the perturbation in respiration. The evidence of normal enzymatic activities of complex I and complex III in staurosporine-treated 143B.TK$^-$ osteosarcoma cells indicates that the cause of the respiration decrease is probably an alteration in the permeability of the outer mitochondrial membrane. Presumably, the voltage-dependent anion channel closes, thereby preventing ADP and oxidizable substrates from being taken up into mitochondria. This interpretation was confirmed by another surprising finding, namely that, in staurosporine-treated 143B.TK$^-$ cells permeabilized with digitonin at a concentration not affecting the mitochondrial membranes in naive cells, the outer mitochondrial membrane loses its integrity; this leads to a reversal of its impermeability to exogenous substrates. The loss of outer membrane integrity leads also to a massive premature release of cytochrome *c* from mitochondria. Most significantly, Bcl-2 overexpression prevents the staurosporine-induced hypersensitivity of the outer membrane to digitonin. Our experiments have thus revealed early changes in the outer mitochondrial membrane, which take place long before cytochrome *c* is released from mitochondria in intact cells.

It has become clear over the last several years that mitochondria play a central role in the cell death program initiated by most inducers of apoptosis. Several key components of the apoptotic machinery, *i.e.* cytochrome *c*, AIF (apoptosis-inducing factor), SMAC/Diablo, and HtrA2/Omi, are released from mitochondria in cells undergoing apoptosis (1–5). Although the importance of mitochondrial changes leading to the release of these apoptogenic molecules is well established, the detailed mechanism(s) involved in this phenomenon, and in particular, the possible role of dysfunctions of the oxidative phosphorylation apparatus and of oxidative stress, have yet to be clarified. The observation that mitochondrial DNA-less (ρ°) cells can be induced to undergo apoptosis (6, 7) has led to the conclusion that the apoptotic program does not require a functional oxidative phosphorylation apparatus. However, these experiments did not exclude the possibility that, in normally respiring cells, oxidative phosphorylation alterations do play an important role in the early phase of apoptosis and that this role can be bypassed by the profound biochemical changes accompanying the establishment of ρ° cells. Indeed, several mitochondrial functional changes, such as impaired mitochondrial adenine nucleotide exchange (8) and permeability transition pore (9) opening with membrane potential collapse (10), have been suggested to play an important role in apoptosis. Moreover, the level of cellular ATP can determine whether a cell undergoes necrosis or apoptosis (11). We considered the possibility that investigating the respiratory activity in cells induced to undergo apoptosis might reveal changes in mitochondria that precede, and may contribute to, the release of cytochrome *c* and other apoptogenic molecules. Our investigations on 143B.TK$^-$ osteosarcoma cells induced to undergo apoptosis with staurosporine have indeed revealed two early changes in mitochondria that result from initiation of the apoptosis program. These are 1) a decrease in the permeability of the outer membrane, pointing to a closure of the voltage-dependent anion channel (VDAC),[1] and 2) a hypersensitivity of the outer mitochondrial membrane to digitonin, suggesting that a change in lipid organization or composition has taken place.

EXPERIMENTAL PROCEDURES

Cell Culture—143B.TK$^-$, a human osteosarcoma-derived cell line (ATCC CRL 8303) (12–15), and hereafter referred to as 143B, was grown in Dulbecco's modified Eagle's medium supplemented with 5 or 10% fetal bovine serum. For induction of apoptosis, semiconfluent dishes of cells were exposed for different times to 1 μM staurosporine. For experiments involving respiration measurements, cells were plated 3 days before the experiment, and the medium was changed in the afternoon or evening of the day before.

Bcl-2 Transfection—A human Bcl-2α cDNA (16) was subcloned from SFFV-Bcl-2 n1 into the neomycin resistance marker-containing plasmid pcDEF3 (17) and then used for transfection of 143B cells by calcium phosphate precipitation (18). Stably transfected clones were selected in medium containing 0.5 mg/ml G418 sulfate (Geneticin; Invitrogen).

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[1] The abbreviations used are: VDAC, voltage-dependent anion channel; Bis-I, bisindolylmaleimide GF 109203X; CsA, cyclosporine A; DAPI, 4,6-diamidino-2-phenylindole; DNP, dinitrophenol; PBS, phosphate-buffered saline; zVADfmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine; 143B, 143B.TK$^-$ cells; TD, Tris deficient (in Ca^{2+}, Mg^{2+}).

Nuclear Apoptosis Assay—The cells were grown in a 6-cm Petri dish; 0.5 volume of phosphate-buffered saline (PBS; 0.145 M NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.65), and formaldehyde, to a final concentration of 4%, was added directly to the medium in the dish. After 10 min at room temperature, bovine serum albumin was added to 0.1%, and the cells were collected by scraping them with a rubber policeman and then transferred into a centrifuge tube. The cells were washed once with PBS, 0.1% bovine serum albumin and then resuspended in 1 ml of the same solution and stained with 1 μg/ml of the double-stranded DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature, with occasional agitation. The cells were collected by centrifugation, resuspended in 10 μl of PBS, mixed with 10 μl of a 50:50 mixture of glycerol and 2× PBS, and mounted on a slide for viewing in a fluorescence microscope.

Oxygen Consumption Measurements in Intact Cells—The respiration rate was measured in an oxygraph (Yellow Springs Instruments, model 5300) in a suspension of naive or staurosporine-treated cells at 3–6 × 10^6 cells/ml in TD buffer (25 mM Tris-HCl, pH 7.4–7.5 (25 °C), 137 mM NaCl, 10 mM KCl, 0.7 mM Na_2HPO_4) at 37 °C. Previous work from this laboratory had shown that 143B cells respire in TD buffer at the same rate as in Dulbecco's modified Eagle's medium lacking glucose (19). After obtaining a stable rate, the uncoupler dinitrophenol (DNP) was added at a concentration of 17–25 μM, and the resulting respiration rate was recorded. After blocking the electron flux upstream of cytochrome *c* oxidase with 20 nM antimycin A, the cytochrome *c* oxidase-dependent oxygen consumption rate isolated from the upstream segment of the respiratory chain was measured by using the membrane-permeant electron donor *N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine (TMPD) at 400 μM and ascorbate, as primary reducing agent, at 10 mM (19). The rate of TMPD-driven O_2 consumption was corrected for the autooxidation rate of TMPD, measured on the same day, as determined in a separate run in TD in the absence of cells. Oxygen consumption rates were expressed in nmol of oxygen consumed/min/mg of cellular protein. The rates of the treated samples were calculated as percentages of the rates of untreated samples, determined on the same day.

Oxygen Consumption Measurements in Digitonin-permeabilized Cells—Digitonin permeabilization was carried out by a modification of a previously described procedure (20–22). For measurements of glutamate/malate- and succinate-driven respiration rates, 2–10 × 10^6 cells were harvested by trypsinization, washed in 5 ml of Respiration Buffer I (modified from Ref. 23: 30 mM HEPES, pH 7.1, 75 mM sucrose, 20 mM glucose, 5 mM potassium P_i, 40 mM KCl, 0.5 mM EDTA, 3 mM $MgCl_2$), centrifuged, and resuspended in 0.15 ml of the same buffer per 10^6 cells. A fresh 1000-fold dilution of 10% (w/v) digitonin was then made in Respiration Buffer I, an equal volume of the 0.01% digitonin was added to the cell suspension, and the mixture was incubated for 2 min at room temperature. A concentration of 0.005% digitonin permeabilized, in general, 100% of untreated 143B cells and 97–100% of cells treated with staurosporine. Permeabilization was stopped by the addition of 8–10 volumes of Respiration Buffer I containing 0.3% bovine serum albumin. After confirming by trypan blue uptake that at least 95% of the cells were permeabilized, the cells were centrifuged, resuspended in 150 or 300 μl of Respiration Buffer I, and transferred into one or two oxygraph chambers, respectively, each containing about 1.4 ml of the same buffer. Respiration measurements were carried out in the presence of 1 mM ADP. Glutamate and malate were added at 5 mM each. Alternatively, 40–200 nM rotenone and 10 mM succinate were added. Both the coupled and 17–25 μM DNP-uncoupled rates were determined. Cytochrome *c*, when used, was 115 μM. To measure TMPD-driven rates, permeabilization was carried out, and respiration rates, measured in an alternative buffer, Respiration Buffer II (0.25 M sucrose, 20 mM Hepes-KOH, pH 7.1, 2 mM potassium phosphate, 10 mM $MgCl_2$, 1 mM ADP (20)) because the autooxidation rate of TMPD is lower in Respiration Buffer II than in Respiration Buffer I. For these measurements, 20 nM antimycin, 0.4 mM TMPD, and 10 mM ascorbate were used. The TMPD-dependent respiration rate was determined after subtraction of the autooxidation rate, measured on the same day. Respiration rates were expressed as nmol/min/mg. Rates of treated samples were compared with those of untreated samples determined on the same day.

In one experiment aimed at testing the possibility of transactivation (from cell to cell), the cells were suspended at one-seventieth of the usual concentration during permeabilization. Because of the high dilution, 10 times as much digitonin per cell (but only one-seventh the usual concentration) had to be used to effect permeabilization of the plasma membrane. After 2 min of incubation at room temperature and the addition of bovine serum albumin to quench the action of the digitonin, the cells were centrifuged, resuspended, and added to the oxygraph chamber.

Confocal Immunofluorescence Microscopy—Cells grown on coverslips were fixed and stained for immunofluorescence as detailed (24). In brief, the cells were double-labeled with mouse anti-cytochrome *c* monoclonal antibody 6H2.B4 (Pharmingen) and rabbit anti-Hsp60 antiserum (Stressgen Biotechnologies Corp.). The secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse IgG and lyssamine-rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). The preparations were analyzed on a Zeiss 310 laser scanning confocal microscope. In some experiments, the cells were centrifuged onto glass coverslips and then fixed and treated as described above. Cells with punctate cytochrome *c* staining (green) that overlapped with Hsp60 staining (red) were counted as cells with mitochondrial cytochrome *c* staining, whereas cells with diffuse nuclear and cytosolic cytochrome *c* staining and punctate mitochondrial Hsp60 staining were counted as cells with extramitochondrial cytochrome *c*.

Preparation of Mitochondrial Fraction—Cells were harvested from ~40 T-175 flasks by trypsinization and washed thrice in PBS. For cell breakage, the cells were resuspended in mitochondria isolation medium consisting of 0.25 M sucrose, 10 mM Hepes-KOH, pH 7.4, 1 mM EGTA, and 0.5% bovine serum albumin. The cells were disrupted with a motor-driven Potter-Elvehjem glass-Teflon homogenizer until ~80% of the nuclei had been released. The mitochondrial fraction was isolated by differential centrifugation (25) and subsequently washed and resuspended in mitochondria isolation medium and stored at −80°.

Enzyme Activity Assays—All assays were carried out at room temperature in a split-beam spectrophotometer. The mitochondria-enriched fraction was thawed, adjusted to a mitochondrial protein concentration of 1 mg/ml, and frozen and thawed two more times. For assays of complex I, complex III, and complex I + III activities, the mitochondria were sonicated in a microcentrifuge tube, immersed in ice water, with a stepped microtip using a Branson Digital Sonifier for 60 s in 2-s bursts at 8-s intervals at 20% amplitude. The output was 3 watts.

Complex I activity was assayed in samples containing 30 μg of mitochondrial protein in 50 mM potassium phosphate, pH 7.5, 2 mM KCN, 50 μM NADH. The reaction was started by the addition of 10 μM decylubiquinone, and the change in absorbance at 340 nm was recorded. For both complex III and complex I–III activity measurements, the assay mixtures were as described (26), and the increase in absorbance at 550 nm was recorded.

Cytochrome *c* oxidase activity was measured at 550 nm in samples containing 10 μg of mitochondrial protein, using unsonicated mitochondria, in 40 mM potassium phosphate, pH 6.65, with 0.5% Tween 80 (low peroxide, Sigma) in the cuvette. The initial concentration of reduced cytochrome *c* was 0.04% w/v. The aforementioned assays measured inhibitor-sensitive activities as the reference cuvette in the split beam spectrophotometer contained the reaction mixture plus inhibitor. The inhibitors for complex I, complex III, complex I–III, and cytochrome *c* oxidase activity assays were 2 μM rotenone, 5 μg/ml antimycin, 5 μg/ml antimycin, and 1 mM KCN, respectively.

Citrate synthase activity was measured in unsonicated mitochondria by using buffer and reagent concentrations as described (26). In addition, the cuvette contained 0.5% Triton X-100 to lyse the mitochondria. The change in absorbance at 412 nm was recorded. The reference cuvette contained the reaction mixture lacking oxaloacetate.

Reagents—Benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (zVADfmk) was purchased from Kamiya Biomedicals; bisindolylmaleimide GF 109203X (Bis-I) (27, 28) was purchased from Calbiochem. Cyclosporine A (CsA) was a gift from Sandoz Research Institute, East Hanover, NJ. All other reagents were obtained from Sigma.

Miscellaneous—Protein concentration was determined as described (29).

RESULTS

Early Decrease in Endogenous Respiration Rate in Staurosporine-treated 143B Cells—143B osteosarcoma cells induced to undergo apoptosis by exposure to 1 μM staurosporine exhibited a rapid decline in their respiration rate in the first hour, to ~80% of the normal rate, followed by a slower decline over the next 15 h, to 35% of normal (Fig. 1). This rate was measured in intact cells suspended in buffered saline solution. Thus, respiration in these cells was driven by endogenous substrates. The rate of respiration uncoupled by the addition of DNP decreased with staurosporine exposure in a way that paralleled closely the decrease in endogenous respiration. To measure the respiratory activity of the terminal enzyme of the respiratory chain, cytochrome *c* oxidase, the upstream segment of the respiratory

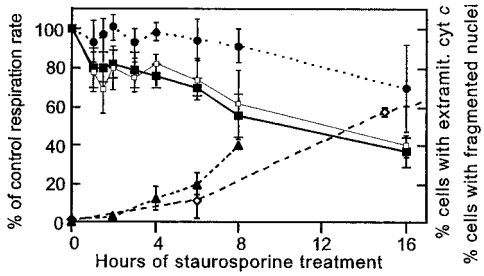

FIG. 1. **Decrease in endogenous respiration rate precedes cytochrome *c* release in intact staurosporine-treated 143B cells.** Respiration rates of intact 143B cells treated with 1 μM staurosporine for different lengths of time were measured as described under "Experimental Procedures" and normalized to the protein content of the samples. Values (averages of 2–11 independent experiments ± one standard deviation) are given as percentages of the rates in untreated cells measured on the same day. (*Filled squares*, endogenous substrate-dependent respiration; *open squares*, uncoupled endogenous substrate-dependent respiration; *filled circles*, TMPD-driven respiration.) The increase with time of the percentage of cells having released cytochrome *c* from mitochondria after staurosporine addition was determined by confocal laser scanning microscopy (*filled triangles*, percentage of cells with extramitochondrial cytochrome *c*). In other experiments, cells stained with DAPI were viewed by fluorescence microscopy, and total cells and cells with fragmented nuclei were counted in several adjacent fields (*open diamonds*, percentage of cells with fragmented nuclei).

chain was first blocked by the addition of antimycin. Respiration was then started by the addition of a cell-permeant reductant of cytochrome *c*, TMPD, and the primary reductant, ascorbate. The TMPD-driven respiration rate, which was typically 1.4–1.7 times higher than the endogenous substrate-dependent rate, was minimally affected (≤5%) during the first 6 h of staurosporine treatment and then slowly declined to about 70% of the control rate by 16 h of cell exposure to the drug.

The Decrease in Respiration Rate Precedes Cytochrome c Release—Cytochrome *c* was released from mitochondria in cells treated with staurosporine, as shown in Fig. 2. The anti-cytochrome *c* antibodies revealed a diffuse extramitochondrial localization of cytochrome *c* in a progressively increasing fraction of the cells during their treatment with the drug, whereas in all untreated cells, cytochrome *c* had a punctate distribution and co-localized with Hsp60, a mitochondrial protein. It appears from Fig. 2 that in many cells, the extramitochondrial cytochrome *c* had a nuclear localization. This phenomenon was reported previously in breast carcinoma cells induced to apoptosis by tumor necrosis factor-α or staurosporine (30) and in NIH-3T3 cells induced to apoptosis by actinomycin D (31), and it was observed in PC12 cells treated with staurosporine.[2] The proportion of cells that showed release of cytochrome *c* was quantified at various times after staurosporine addition. Fig. 1 shows that the percentage of cells having released cytochrome *c* was less than the percentage of decrease in endogenous respiration rate for the first 6 h after staurosporine addition, indicating that the respiration decrease was not dependent on cytochrome *c* release from mitochondria. Furthermore, the fraction of cells that had reached the late apoptosis stage of nuclear fragmentation was determined in DAPI-stained cells (Fig. 1). Under the reasonable assumption that the fraction of cells having released cytochrome *c* included those undergoing nuclear fragmentation, it appears that cells reached this stage only 2–3 h after their mitochondria had released cytochrome *c*.

For the first 8 h after staurosporine addition to 143B cells, the TMPD-driven respiration rate did not decrease significantly (Fig. 1), indicating that cytochrome *c* oxidase activity remained high. On the other hand, by 8 h after staurosporine

[2] E. Ferraro and G. Attardi, personal communication.

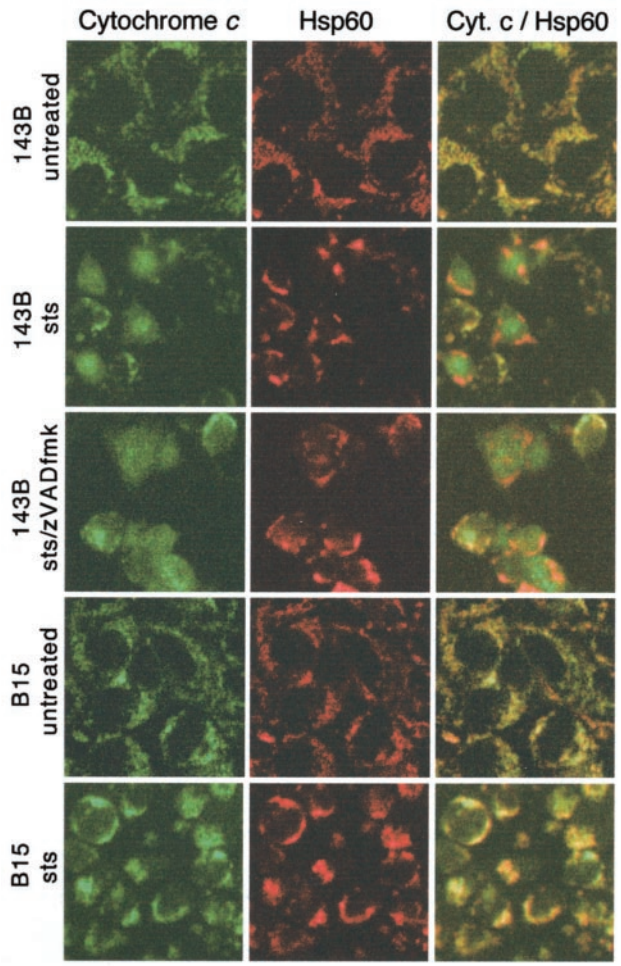

FIG. 2. **Distribution of cytochrome *c* in intact staurosporine-treated and untreated cells and B15 Bcl-2-overexpressing cells.** Untreated 143B and Bcl-2-overexpressing 143B cells (*B15*) and the same cells exposed to staurosporine (*sts*) for 8 h were fixed and double-immunolabeled with antibodies directed against cytochrome *c* and against Hsp60. The *panels* in the *third column* show the merged images of green (*cytochrome c*) and red (*Hsp60*) fluorescence. In one experiment, 143B cells were exposed to zVADfmk during the staurosporine treatment.

addition, mitochondria had already massively released cytochrome *c* in ~40% of 143B cells. This fact indicated that, even after cytochrome *c* had been released, the level of cytochrome *c* available to cytochrome *c* oxidase was still sufficient to maintain normal cytochrome *c* oxidase activity. This is consistent with the finding of Waterhouse *et al.* (32) that the mitochondrial membrane potential could be maintained by respiration after cytochrome *c* release (provided that caspase activity was inhibited).

Tests of Possible Role of Caspases, Permeability Transition, and Protein Kinase Inhibition in Respiration Decrease—To gain some insight into the mechanisms underlying the decrease in endogenous respiration rate in staurosporine-treated cells, several inhibitors were tested for the ability to modify this effect of staurosporine. The caspase inhibitor zVADfmk failed to prevent the decrease in respiration rate (Fig. 3*a*) and, as had been found previously (33), the release of cytochrome *c* from mitochondria (Fig. 2), whereas apoptosis was completely inhibited in these cells (not shown). Thus, it appeared that the respiration decrease was not dependent on the activity of caspases. An inhibitor of the permeability transition, CsA (34–36), present at 10 mM during the staurosporine treatment, also had no significant effect on the decrease in respiration (Fig. 3*b*).

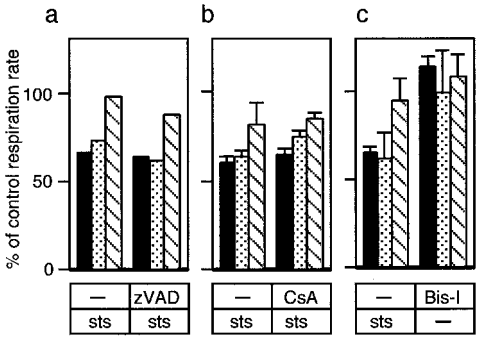

FIG. 3. **Staurosporine-induced decline in respiration rate is not prevented by inhibitors of caspases (zVADfmk) or permeability transition (CsA) or mimicked by protein kinase C inhibitor (Bis-I).** As shown in *a*, 100 μM zVADfmk (*zVAD*) was added to cultures 1 h prior to the addition of 1 μM staurosporine. As shown in *b*, 10 mM CsA was added at the time of staurosporine addition. As shown in *c*, 1 μM Bis-I instead of staurosporine was added to cultures at time 0. Cultures were harvested and assayed for respiration activity 6 h after staurosporine (*a* and *b*) or Bis-I (*c*) addition. *Black bars* represent endogenous substrate-dependent respiration; *stippled bars* represent DNP-uncoupled endogenous substrate-dependent respiration; and *hatched bars* represent TMPD-driven respiration. Values are given as percentages of the rates in untreated cells. Data in *panels b* and *c* are averages of 2 independent experiments ± one standard deviation.

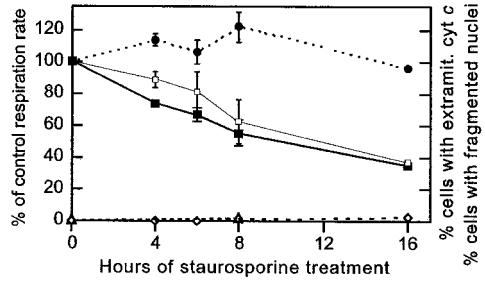

FIG. 4. **Staurosporine-treated B15 Bcl-2-overexpressing cells also exhibit the early decline in endogenous respiration rate.** The respiration rates of intact B15 cells treated with 1 μM staurosporine were measured as described under "Experimental Procedures," normalized to the protein content of the samples, and presented as percentages of the rates in untreated cells, measured on the same day. Except for the 16 h data, values given are averages of 2–4 independent experiments ± one standard deviation. (*Filled squares*, endogenous substrate-dependent respiration; *open squares*, uncoupled endogenous substrate-dependent respiration; *filled circles*, TMPD-driven respiration.) The proportions of cells with extramitochondrial cytochrome *c* (*cyt c*) (*triangles*) and with fragmented nuclei (*diamonds*) are indicated as well.

Treatment of the cells with 20 mM CsA for 6 h or with 10 mM CsA added twice during the staurosporine treatment likewise had no effect (not shown). These results indicated that, barring an unusual failure of the cells to take up the drug or a high capacity to export it, permeability transition did not influence the decrease in respiration rate.

Lastly, an attempt was made to dissociate the respiration rate decline from the apoptotic program. Specifically, we used an analog of staurosporine, Bis-I (27, 28), which, like staurosporine, inhibits protein kinase C (28, 37), but unlike staurosporine, does not induce apoptosis (37). Fig. 3*c* shows that 1 μM Bis-I did not cause a decrease in respiration rate. It was also confirmed that Bis-I did not induce apoptosis in 143B cells (data not shown). Thus, these experiments did not provide any evidence that the respiration decline is not a part of the apoptotic program.

Bcl-2-overexpressing 143B Cells Also Exhibit the Early Endogenous Respiration Decrease—Experiments were carried out to determine whether overexpression of the antiapoptotic protein Bcl-2 (16) would prevent the decrease in respiration rate. 143B cells were stably transfected with a cDNA clone for Bcl-2, and an overexpressing clone, B15, was analyzed for its response to staurosporine. As observed previously for Bcl-2-overexpressing staurosporine-treated HL-60 cells (38), Bcl-2-overexpressing 143B cells did not release cytochrome *c* from mitochondria upon exposure to staurosporine (Fig. 2). Also, as expected, the cells did not undergo apoptosis, as assayed by nuclear fragmentation (Fig. 4). Nevertheless, as Fig. 4 shows, B15 cells exhibited a decrease in both coupled and uncoupled respiration rates of a degree comparable with that observed in 143B cells. Thus, it appeared that the site of Bcl-2 activity was downstream of the respiration rate decrease or in an independent pathway.

Hypersensitivity of the Outer Mitochondrial Membrane to Digitonin is an Early Staurosporine-induced Effect—The endogenous substrate-dependent respiration rate depends on three other respiratory chain complexes besides complex IV: namely, complex I, or respiratory NADH dehydrogenase; complex III, or ubiquinone-cytochrome *c* oxidoreductase; and complex II, or succinate dehydrogenase. Electrons pass from NADH, generated by the oxidation of metabolites, sequentially to complex I, ubiquinone, complex III, cytochrome *c*, complex IV, and finally, oxygen. Electrons from fatty acid oxidation can also enter the respiratory chain via succinate and succinate dehydrogenase. This enzyme transfers electrons to ubiquinone, and subsequently, electron transfer continues as described above. In 143B cells, the endogenous substrate-dependent respiration has been found to be ≥90% inhibitable by rotenone, an inhibitor of complex I (not shown); thus, electrons from endogenous substrates enter the chain almost exclusively through complex I, and very few electrons enter through complex II. Therefore, the observed early decrease in respiration rate in staurosporine-treated cells could be due to a decrease in either complex I or complex III activity or both.

By using digitonin-permeabilized cells and by providing mitochondria with exogenous substrates that feed into complex I or that bypass complex I, one can determine which respiratory enzyme activity is decreased. Fig. 5*a* shows the results of exogenous substrate-driven respiration assays performed on digitonin-permeabilized 143B cells. Surprisingly, all activities, whether driven by glutamate and malate (electrons entering the respiratory chain at complex I), succinate (electrons entering at complex II), or TMPD and ascorbate (electrons entering at complex IV), were reduced by at least 75% within 4 h after staurosporine addition. The relative rates shown in Fig. 5*a* represent the initial rates after the addition of DNP. For each substrate, the respiration rate decreased, in staurosporine-treated cells, during the measurement, over the course of minutes. The difference observed between the dramatic decreases in respiration rates in permeabilized cells and the moderate decreases in intact cells suggested the possibility that digitonin treatment had removed an electron carrier or some other component(s) of the respiratory chain in cells treated with staurosporine. The addition to the oxygraph chamber of 10 μM ubiquinone (oxidized or reduced) had no effect (not shown). On the contrary, the addition of cytochrome *c* restored the ability of the permeabilized staurosporine-treated cells to respire (Fig. 6*a*), suggesting that the electron carrier cytochrome *c* had been lost from mitochondria. In fact, in the presence of added cytochrome *c*, respiration driven by glutamate and malate, by succinate, or by TMPD/ascorbate attained the rates observed in permeabilized untreated cells. To confirm that digitonin treatment of staurosporine-treated 143B cells caused the premature loss of cytochrome *c* from mitochondria, permeabilized treated and untreated cells were incubated with anti-cytochrome *c* and anti-Hsp60 antibodies and examined by confocal fluorescence

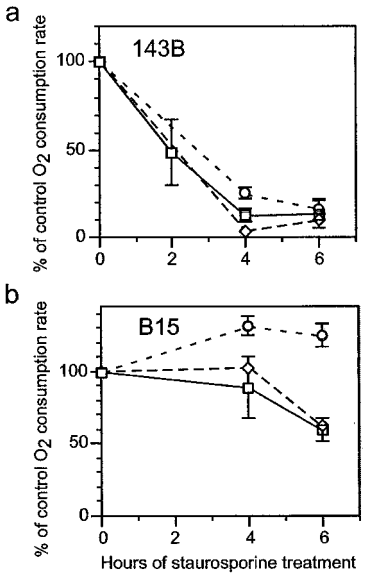

FIG. 5. **Respiration rates in digitonin-permeabilized 143B and B15 cells treated with 1 μM staurosporine.** Respiration measurements were carried out in the presence of DNP. The glutamate/malate- (*squares*), succinate- (*diamonds*), and TMPD/ascorbate- (*circles*) driven rates in staurosporine-treated cells were normalized to the protein content of the samples and expressed as percentages of the rates in untreated cells. The data presented are averages ± one standard deviation of 2–7 independent experiments. *a*, 143B cells; *b*, B15 cells.

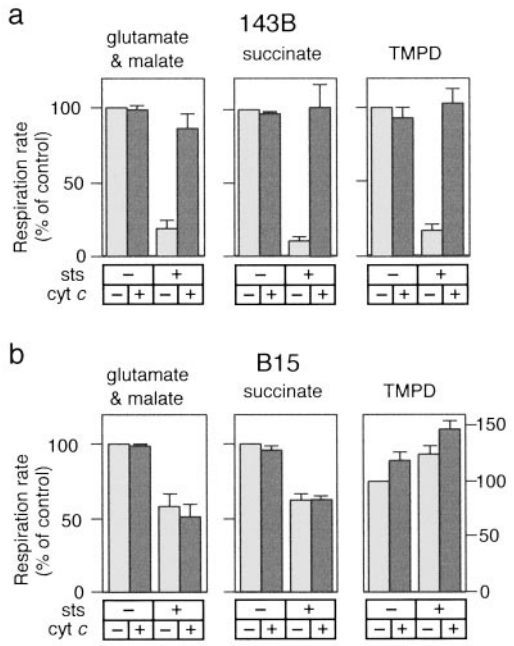

FIG. 6. **Addition of cytochrome *c* (*cyt c*) restores respiration in digitonin-permeabilized 143B cells treated with staurosporine but not in B15 cells.** Cells were exposed to 1 μM staurosporine (*sts*) for 6 h or left untreated and then permeabilized with digitonin. Respiration was stimulated by the addition of glutamate and malate, of succinate, or of TMPD, in all cases in the presence of DNP. Rates (averages of 2–4 independent experiments ± one standard deviation) are given as percentages of rates in untreated permeabilized cells. *a*, 143B cells; *b*, B15 cells. *Light gray bars*, no added cytochrome *c*; *dark gray bars*, added cytochrome *c*.

microscopy. Fig. 7 shows that cytochrome *c* was virtually absent in the permeabilized staurosporine-treated cells, but it was readily visible and co-localized with Hsp60 in the digitonin-permeabilized untreated cells.

The most plausible explanation for the loss of cytochrome *c* from mitochondria of staurosporine-treated and permeabilized 143B cells was that the mitochondrial outer membrane had lost integrity. However, an alternative explanation was that the digitonin permeabilization of the plasma membrane allowed the diffusion of transactivating factors from cells advanced in the apoptotic program to cells less advanced. To test this hypothesis, digitonin permeabilization of staurosporine-treated 143B cells was performed at a very low cell concentration, *i.e.* at one-seventieth of the normal concentration, to dilute any possible transactivating factors, thereby presumably reducing their activity. In this experiment, exogenous substrate-driven respiration rates were found to be decreased as much as in a parallel experiment in which permeabilization was carried out at the usual cell concentration (not shown). This result strongly suggested that the loss of cytochrome *c* from mitochondria was not due to the action of transactivating factors.

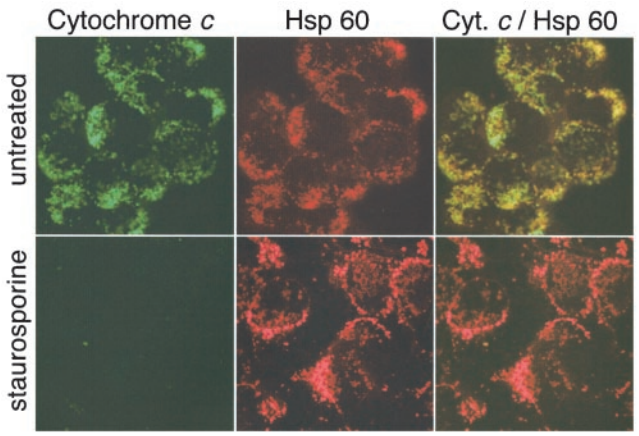

FIG. 7. **Distribution of cytochrome *c* (*Cyt.c*) in digitonin-permeabilized 143B cells.** Unexposed cells or cells exposed to staurosporine for 4 h were permeabilized, centrifuged onto round glass coverslips, fixed, and stained with antibodies directed against cytochrome *c* and against Hsp60. The *panels* on the *right* show the merged images of green fluorescence (cytochrome *c*) and red fluorescence (Hsp60).

Lack of Hypersensitivity to Digitonin in Bcl-2-overexpressing Staurosporine-treated Cells—Quite different results from those described above were obtained with B15 Bcl-2-overexpressing cells treated with staurosporine and permeabilized with digitonin (Fig. 5*b*). In fact, in B15 cells permeabilized after 6 h of exposure to staurosporine, the glutamate- and malate-driven respiration rate was decreased by 40%, and the succinate-driven rate was decreased by 37% (Fig. 5*b*). The decrease in the glutamate/malate-driven respiration rate was thus very similar to the decrease, 30–35%, which had been observed in the endogenous substrate-dependent respiration rate of intact B15 and 143B cells. The TMPD-driven respiration rate in the digitonin-permeabilized B15 cells was not decreased by the staurosporine treatment, as had been observed in intact cells. Furthermore, the addition of cytochrome *c* had no effect on the glutamate/malate- or succinate-driven respiration rates of staurosporine-treated permeabilized B15 cells (Fig. 6*b*). These results thus led to the conclusion that Bcl-2 protected staurosporine-treated cells from the dramatic loss of cytochrome *c* and respiration caused by permeabilization with digitonin. Thus, presumably, Bcl-2 helped to maintain the integrity of the outer mitochondrial membrane.

Respiratory Enzyme Defects Are Not Involved in the Early Endogenous Respiration Decrease—The polarography experiments carried out on B15 cells suggested that both complex I and complex III activities were affected by staurosporine treatment of the cells (Fig. 5*b*). To validate this suggestion and to extend it to 143B cells, spectrophotometric enzyme assays were performed on disrupted mitochondria from 143B cells treated

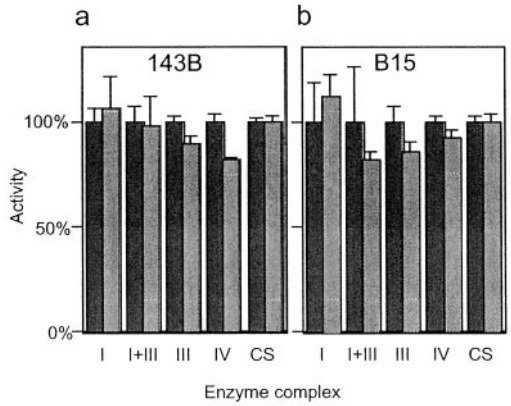

FIG. 8. **Enzyme activities in mitochondria isolated from staurosporine-treated or untreated 143B cells (*a*) and B15 cells (*b*).** *Dark gray bars* represent data after no staurosporine treatment, and *light gray bars* represent data after staurosporine treatment for 6 h at 1 μM. Two mitochondria preparations each were made from control and staurosporine-treated 143B cells, and two preparations each were made from control and staurosporine-treated B15 cells. Measurements were made from both preparations for all assays except for complex IV in 143B cells. Activities were first normalized to citrate synthetase (*CS*) activity in the mitochondria preparations. The activity for each enzyme assay (averages of 4–7 measurements (*a*) or 3–11 measurements (*b*) ± one standard deviation) is presented as a percentage of the activity in mitochondria from untreated cells.

with staurosporine for 6 h, from untreated 143B cells, and likewise, from naive and staurosporine-treated B15 cells. The surprising results shown in Fig. 8 revealed that the activities of complex I, complex III, and complex I + III were not significantly different between untreated and staurosporine-treated cells. This important observation, combined with the finding of complete restoration by cytochrome *c* of the capacity of digitonin-permeabilized staurosporine-treated 143B cells to respire on exogenous oxidizable substrates, indicated that the early decline in endogenous respiration in staurosporine-caused apoptosis was due to an apoptosis-induced outer mitochondrial membrane permeability change, resulting in a block in respiratory substrate uptake.

DISCUSSION

We have reported here an early and progressive inhibition of endogenous substrate-dependent respiration in 143B cells during the first 6–8 h of treatment with the apoptosis inducer staurosporine. TMPD-driven (complex IV-dependent) respiration was not significantly affected during the same period. We have no information on the distribution within the cell population of this respiration loss, *i.e.* whether respiration was affected partially in every cell or whether a fraction of the cells had completely lost endogenous substrate-dependent respiration. The latter alternative seems, however, more likely, on the basis of the available evidence on the intercellular mosaicism of apoptosis-related phenomena (24, 39). In any case, the data clearly showed that this loss of respiration in staurosporine-treated cells preceded the release of cytochrome *c* from mitochondria and the fragmentation of nuclei. Our results differ from those of Hájek *et al.* (24), who reported that, in anti-Fas antibody-treated Jurkat cells, the loss of TMPD-dependent and of endogenous respiration occurred nearly simultaneously and followed cytochrome *c* release from mitochondria. This difference in the sequence of events could be due to the different pathway taking place in Fas-induced apoptosis.

It should be mentioned that an early decline in endogenous substrate-dependent respiration, which appeared to be independent of cytochrome *c* release, has also been observed in a rat cell line of neuronal derivation, PC12, treated with staurosporine.[3] In yet another system, Schulze-Osthoff *et al.* (40) reported a specific decrease in complex I- and complex III-dependent respiration rates in mouse fibrosarcoma cells treated with tumor necrosis factor-α.

A valuable insight into the mechanism underlying the early decrease in endogenous respiration in staurosporine-treated cells was provided by the observation that, although respiration measurements in B15 cells suggested that complex I and complex III activities were decreased, spectrophotometric assays of the respective enzyme activities, namely NADH-ubiquinone oxidoreductase activity and ubiquinol-cytochrome *c* oxidoreductase activity, showed them to be normal in sonicated mitochondria from both 143B and B15 cells. We propose that the basis for this apparent discrepancy in our results is that the outer mitochondrial membrane, during staurosporine-induced programmed cell death, becomes impermeable to metabolites such as glutamate, succinate, and ADP. Consequently, respiration is halted in these cells because of the inability of mitochondria to take up oxidizable substrates. Fig. 9 depicts in schematic form our views of the early changes in mitochondria that have been detected in our experiments. Thus, we interpret our finding that, in intact 143B cells, the overall endogenous substrate-dependent respiration rate was decreased by about 30% in a cell population exposed to staurosporine for 6 h as indicating that, in 30% of the cells, the outer mitochondrial membrane was impermeable to substrates.

The interpretation that we propose to explain the apparent discrepancy between the results of enzyme activity measurements and those of the polarographic measurements has been fully confirmed by the observation of another unusual phenomenon associated with staurosporine-induced apoptosis. As reported previously by Hájek *et al.* (24) for Jurkat cells induced to undergo apoptosis by anti-Fas antibodies, we found that the outer mitochondrial membrane in cells undergoing the apoptotic program was disrupted by the digitonin treatment used to permeabilize cells, in such a way as to allow cytochrome *c* to be released from mitochondria (Fig. 9). In our experiments, the first evidence for this disruption of the outer membrane was that cytochrome *c* added back to digitonin-treated 143B cells stimulated all of the substrate-driven respiration rates. In fact, mitochondria of staurosporine-treated and digitonin-permeabilized 143B cells exhibited a recovery of ~100% of the exogenous substrate- and TMPD-driven respiration rates of digitonin-permeabilized naive cells, when cytochrome *c* was provided. In this situation, the removal of the outer membrane also removed the barrier to substrate uptake. Therefore, there was no evidence of any defect in complex I- or complex III-dependent respiration.

The loss of cytochrome *c* from mitochondria in digitonin-permeabilized cells was subsequently directly confirmed by confocal immunofluorescence microscopy as had been done previously for Jurkat cells treated with anti-Fas antibodies and digitonin (24). This loss of outer mitochondrial membrane integrity occurred at a concentration of digitonin that had no effect on normal mitochondria, indicating that the phenomenon took place in cells in which the outer mitochondrial membrane had been "primed" for apoptosis.

At 4 h after the addition of staurosporine, when permeabilization of 143B cells with digitonin led to loss of cytochrome *c* from mitochondria in at least 75% of the cells, no other parameter of apoptosis progression was positive in such a large proportion of the cell population (Fig. 1). In particular, at 4 h after staurosporine addition, only 12% of intact cells showed an extramitochondrial localization of cytochrome *c*.

[3] E. Ferraro and G. Attardi, unpublished data.

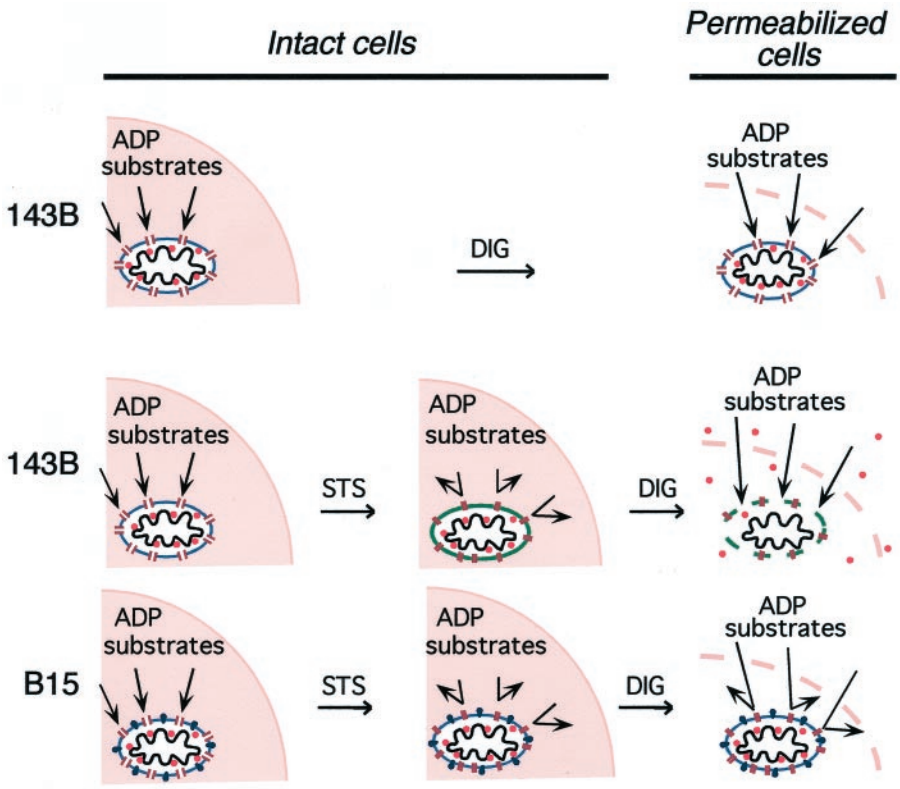

FIG. 9. **Scheme of a model proposed for the interpretation of the experimental results.** In 143B intact cells, respiration is sustained by uptake of ADP and endogenous oxidizable substrates into mitochondria from the cytosol (*pink*), presumably through VDAC (or porin) (*brown*). Digitonin (*DIG*) permeabilization of naive cells causes endogenous ADP and substrates to diffuse out of the cytosol and allows exogenous ADP and oxidizable substrates to pass through the plasma membrane (*pink*) to drive respiration. Staurosporine (*STS*) treatment causes the permeability of the outer mitochondrial membrane to ADP and oxidizable substrates to become dramatically curtailed, presumably via VDAC closure. Staurosporine also causes an alteration in the structure of the outer membrane (shown as a change to *green*) so that now, digitonin treatment of staurosporine-treated cells not only permeabilizes the plasma membrane but also disrupts the outer mitochondrial membrane, thereby allowing cytochrome *c* (*red dot*) to leave mitochondria and allowing substrates and ADP to be taken up into the mitochondrial matrix. In the presence of adequate exogenous cytochrome *c* and oxidizable substrates, respiration is fully restored. In B15 cells, the outer mitochondrial membrane permeability change produced by staurosporine occurs as in 143B cells, but digitonin treatment does not cause disruption of the outer membrane, presumably because this membrane is stabilized by Bcl-2 (in *dark blue*). Oxidizable substrates and ADP still cannot be taken up by mitochondria.

The most likely interpretation of the digitonin hypersensitivity observed in this work is that the outer membrane of mitochondria underwent, very early in apoptosis, a structural change. Such a structural change might have involved a change in lipid composition or distribution that made its integrity sensitive to 0.005% digitonin. This structural change may be the "priming" event for apoptosis referred to by Hájek *et al.* (24). An alternative interpretation of the digitonin effect, namely that the digitonin permeabilization may have caused the release of transacting apoptogenic factors from cells more advanced in the apoptotic program, thereby triggering cytochrome *c* release from mitochondria in the less advanced cells, was substantially excluded in the present work by the result of an experiment we performed in which the digitonin permeabilization was carried out in a highly diluted cell suspension.

An important observation in the present work was that Bcl-2-overexpressing 143B cells exhibited the same early decrease in endogenous respiration after staurosporine treatment. This finding indicated that the change in outer membrane permeability occurred upstream of the step affected by Bcl-2 activity or in an independent pathway. In Bcl-2-overexpressing cells, there was no release of cytochrome *c* from mitochondria, underlining the fact that the decrease in endogenous respiration is not dependent on cytochrome *c* release.

In contrast to 143B cells, mitochondria of the B15 Bcl-2-overexpressing cells exposed to staurosporine for 6 h and permeabilized with digitonin exhibited a 40% decrease in the rate of glutamate/malate-driven respiration, very similar to the ~35% decrease in the rate of endogenous respiration observed in intact cells. These results are consistent with the interpretation that the outer mitochondrial membrane in digitonin-permeabilized B15 cells is intact, and that in ~40% of the cells, it is refractory to uptake of substrates (Fig. 9). The lack of effect of added cytochrome *c* on the respiration rates in digitonin-permeabilized B15 cells provided further support for this interpretation.

What is the change in the outer mitochondrial membrane that occurs early in the program of apoptosis and renders the membrane refractory to uptake of substrates? The passage of substrates, other metabolites, and small molecules through the outer membrane takes place through the VDAC (or porin) (41, 42), an abundant mitochondrial protein. Furthermore, the permeability of VDAC is regulatable (43–45). Thus, the closure of VDAC in a fraction of the cells exposed to staurosporine would restrict respiration driven by oxidizable substrates, such as malate and succinate. On the other hand, the entry of TMPD, which is membrane-permeant, into mitochondria would not be inhibited by a closed VDAC, and TMPD-driven respiration would be expected to be unaffected by VDAC closure. Indeed, in 143B and B15 cells treated with staurosporine for 2–6 h, which exhibited a 20–35% decrease in endogenous respiration rate, the TMPD-driven respiration rate was found to be the same as in untreated cells.

Thompson and colleagues (8, 46) have proposed previously that VDAC closes in apoptosis, on the basis of experiments showing that the outer mitochondrial membrane becomes less

permeable to ADP in lymphoblastoid cells that have been deprived of growth factor IL-3. Our results are in agreement with theirs on this point. However, they reported that overexpression of Bcl-2 or Bcl-X_L prevents this decrease in outer membrane permeability, whereas we find that overexpression of Bcl-2 has no such effect. This discrepancy may be due to differences in the level of expression of Bcl-2 or to differences in the apoptotic pathway in the two systems.

The evidence that VDAC closure, as concerns ADP/ATP exchange, occurs in lymphoblastoid cells induced to undergo apoptosis by interleukin withdrawal (46) provides strong support for the idea that the early loss of exogenous substrate-dependent respiration in staurosporine-treated cells represents another manifestation of VDAC closure. The early decrease in respiration in a different cell type treated with staurosporine (PC12)[3] and the decrease in complex I- and complex III- dependent respiration rates in a mouse cell line treated with tumor necrosis factor-α (40) may likewise be due to VDAC closure.

As concerns the question of whether the hypersensitivity to digitonin detected here in staurosporine-treated cells is related to apoptosis, the previous evidence of a similar phenomenon in a completely different apoptotic system (Fas-induced Jurkat cells) strongly supports the conclusion that this hypersensitivity is an important indicator of an apoptosis-induced priming of the outer mitochondrial membrane. On a technical note, the hypersensitivity to digitonin that we have observed indicates that caution should be used in interpreting results from experiments in which digitonin has been used to permeabilize or lyse cells undergoing apoptosis or in the preparation of a mitochondrial fraction from such cells.

In summary, we have provided evidence that points to changes in the outer mitochondrial membrane that occur very early in the apoptotic program. A comparison of the data in Figs. 1 and 5*a* gives an idea of the order of these changes relative to the hallmark events of apoptosis. After 4 h of exposure of 143B cells to staurosporine, the outer mitochondrial membrane in at least 75% of the cells has undergone a change in structure that makes it hypersensitive to digitonin (Fig. 5*a*). At the same time, endogenous substrate-dependent respiration has decreased by 20–25%, 12% of the cells have released cytochrome *c* from mitochondria, and fewer than 10% of the cells have progressed to the stage of having fragmented nuclei (Fig. 1). These data suggest the following sequence. Very early, after the apoptotic stimulus, the outer membrane of mitochondria undergoes a structural change, becoming primed for apoptosis. This change is detectable so far only *in vitro*, as a hypersensitivity to digitonin. Next, the outer membrane becomes impermeable to oxidizable substrates and ADP, presumably because VDAC closes. This step is followed by cytochrome *c* release from mitochondria, and lastly, by nuclear degradation. It seems likely that staurosporine-treated cells that have released cytochrome *c* from mitochondria represent a subgroup of those in which the outer membrane permeability has been altered (presumably those in which the effect of VDAC closure are more advanced). Vander Heiden *et al.* (46) have suggested that VDAC closure in lymphoblastoid cells deprived of IL-3 leads to hyperpolarization of the mitochondrial inner membrane, swelling of the matrix, and consequent rupture of the outer membrane and release of cytochrome *c*. Further work is needed to clarify which effects of VDAC closure in staurosporine-treated cells may be crucial for the release from mitochondria of apoptogenic molecules, in particular, of cytochrome *c*.

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