

Mitochondrial Precursor Signal Peptide Induces a Unique Permeability Transition and Release of Cytochrome *c* from Liver and Brain Mitochondria

Yulia E. Kushnareva,^{*1} Brian M. Polster,^{†‡1} Patricia M. Sokolove,^{*} Kathleen W. Kinnally,[§] and Gary Fiskum^{*†2}

^{*}Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201; [†]Department of Anesthesiology, University of Maryland School of Medicine, Baltimore, Maryland 21201; [‡]Neuroscience Program, University of Maryland School of Medicine, Baltimore, Maryland 21201 (in partial fulfillment of a Ph.D.); and [§]Division of Basic Sciences, New York University College of Dentistry, New York, New York 10010

Received September 1, 2000, and in revised form November 2, 2000; published online January 11, 2001

This study tested the hypothesis that mitochondrial precursor targeting peptides can elicit the release of cytochrome *c* from both liver and brain mitochondria by a mechanism distinct from that mediated by the classical, Ca²⁺-activated permeability transition pore. Human cytochrome oxidase subunit IV signal peptide (hCOXIV₁₋₂₂) at concentrations from 15 to 100 μM induced swelling, a decrease in membrane potential, and cytochrome *c* release in both types of mitochondria. Although cyclosporin A and bongkrekic acid were without effect, dibucaine, propranolol, dextran, and the uncoupler FCCP were each able to inhibit signal peptide-induced swelling and cytochrome *c* release. Adenylate kinase was coreleased with cytochrome *c*, arguing against a signal peptide-induced cytochrome *c*-specific pathway of efflux across the outer membrane. Taken together, the data indicate that a human mitochondrial signal peptide can evoke the release of cytochrome *c* from both liver and brain mitochondria by a unique permeability transition that differs in several characteristics from the classical mitochondrial permeability transition. © 2001 Academic Press

Key Words: adenylate kinase; cyclosporin A; dibucaine; propranolol; membrane potentials; mitochondrial swelling.

The mitochondrial permeability transition has been the focus of much attention in studies on both necrotic and apoptotic cell death (1). The classical permeability transition pore (PTP)³ is a proteinaceous pore, the opening of which is triggered by Ca²⁺, P_i, and oxidative stress and inhibited by cyclosporin A (CsA), bongkrekic acid (BA), EGTA, adenine nucleotides, and Mg²⁺ (2, 3). The opening of the PTP, resulting in swelling and ultimate rupture of the outer membrane, is a common explanation for the redistribution of proapoptotic proteins such as cytochrome *c* and apoptosis inducing factor (AIF) from the mitochondria to the cytoplasm (4–7). Disruption of mitochondrial functional integrity can alternatively lead to a necrotic cell death in cells heavily dependent on mitochondria for ATP production.

A novel permeability transition initiated by mitochondrial precursor targeting peptides has recently been characterized (8, 9). Specifically, short, positively charged, amphipathic signal peptides that normally target yeast cytochrome *c* oxidase subunit IV to the mitochondrial inner membrane have been shown to induce membrane potential depolarization and swelling in isolated liver mitochondria. Furthermore, the inner membrane pore that is opened in response to signal peptides has a size and pharmacological sensi-

¹ The first two authors contributed equally to this work.

² To whom correspondence should be addressed at Department of Anesthesiology, University of Maryland, Baltimore, MSTF 5.34, 685 West Baltimore Street, Baltimore, MD 21201. Fax: (410) 706-2550. E-mail: gfishum@anesthlab.ummc.umaryland.edu.

³ Abbreviations used: PTP, permeability transition pore; CsA, cyclosporin A; BA, bongkrekic acid; AIF, apoptosis inducing factor; MCC, multiple conductance channel; hCOXIV₁₋₂₂, human cytochrome oxidase subunit IV signal peptide; TPP⁺, tetraphenylphosphonium; TBS-T, Tris-buffered saline containing 0.05% Tween 20; BSA, bovine serum albumin; TIM, translocase of the inner membrane; Alm, alamethicin; PVDF, polyvinylidene difluoride.

tivity that can be clearly distinguished from both the classical PTP and the CsA-insensitive mastoparan-induced pore characterized by Sokolove and Kinnally (8) and Pfeiffer and co-workers (10).

The multiple conductance channel (MCC) is an inner mitochondrial membrane channel that has been characterized electrophysiologically (11). Previous evidence has linked the yeast MCC to mitochondrial protein import machinery (12). Signal peptide induced permeability changes can be blocked by compounds that inhibit both protein import (13) and the MCC (8, 9, 14). This finding, together with the observation that signal peptides modulate the conductance state of the yeast (9, 15) and mammalian MCC (9), strongly suggests that mitochondrial import proteins are involved in the formation of this novel pore. The question of whether cytochrome *c* release occurs following the induction of this pore, however, has yet to be addressed.

Signal peptide-induced permeability changes have previously been examined in isolated liver, yeast, and potato tuber mitochondria under conditions that bear little resemblance to those present in cells (8, 9, 16–20). The classical, Ca^{2+} -induced permeability transition pore exhibits relatively little activity in mitochondria isolated from brain tissue compared to liver mitochondria (21, 22) and its activation is inhibited by the presence of normal cytosolic components, such as Mg^{2+} and adenine nucleotides (23). Nevertheless, Ca^{2+} does induce cytochrome *c* release from brain mitochondria under these conditions by a cyclosporin A-insensitive mechanism. The lack of information on mitochondrial permeability changes induced by signal peptide in the presence of K^+ , adenine nucleotides, and Mg^{2+} and the differences in the Ca^{2+} -triggered permeability transition activity in brain and liver mitochondria merit a further consideration of signal peptide effects on mitochondrial permeability. The present study tested the following hypotheses: (1) Human signal peptide induces mitochondrial permeability changes *in vitro* even in the presence of physiological concentrations of Mg^{2+} and ATP that effectively suppress the Ca^{2+} -activated PTP activity. (2) These changes in the permeability of the mitochondrial inner membrane cause osmotic swelling sufficient to elicit outer membrane disruption and release of cytochrome *c*. (3) The extent of signal peptide-induced permeability changes and cytochrome *c* release differ between liver and brain mitochondria.

EXPERIMENTAL PROCEDURES

Materials. Rat brain mitochondria were isolated according to the procedure of Rosenthal *et al.* (24). In contrast to the most commonly used procedures for brain tissue that yield primarily nonsynaptosomal mitochondria, this procedure yields a combination of both nonsynaptosomal and synaptosomal mitochondria. Rat liver mitochondria were isolated by standard differential centrifugation as previously described (25). Signal and control peptides were synthesized by the Wadsworth Center Biochemistry and Peptide Synthesis Core

using an Applied Biosystem 431A automated peptide synthesizer as previously described (15). The peptides studied were the targeting sequence, ¹MLATRVFSLVVGKRAISTSV₂₂, of human cytochrome oxidase subunit IV (hCOXIV₁₋₂₂) and an artificial sequence, ML₁SR₂QQ₃SR₄QR₅QQ₆SR₇QR₈SR₉ (SynB2), with low amphiphilicity and lacking targeting function (26). Peptides were prepared as dilute (0.75–2.0 mM) stocks in distilled water. Dextran T-10 (MW 10,000) was obtained from Amersham Pharmacia Biotech, tetraphenylphosphonium chloride from Aldrich, and bongkreik acid from Calbiochem. Cyclosporin A (OL 27-400) was the generous gift of Sandoz Research Institute (East Hanover, NJ). Other chemicals were from Sigma Chemical Company, and all reagents were of the highest grade available.

Measurement of mitochondrial swelling and membrane potential as indicators of permeability transition. Assays were carried out at 30°C in a basic assay reagent containing 125 mM KCl, 2 mM P_i , and 20 mM HEPES–KOH, pH 7.0 (KCl media), or in a mannitol/sucrose-based medium containing 210 mM mannitol, 70 mM sucrose, 3 mM P_i , and 10 mM HEPES–KOH, pH 7.4 (mannitol–sucrose media). Where indicated, the assay reagents were supplemented with 4 mM MgCl_2 and 3 mM ATP. In some experiments, 250 μM EGTA and/or 20% Dextran T-10 (w/w in KCl media) were also included. In experiments determining the dose dependence of signal peptide effects or the influence of permeability transition pore inhibitors, mitochondria (0.4 mg/ml) were incubated for 3.5 min in the presence of 1 μM rotenone and then energized with 5 mM succinate. Peptide was added at 5.5 or 6.5 min. In all other experiments mitochondria were incubated in the presence of 1 μM rotenone and 5 mM succinate for 3 min at which time peptide or vehicle control was added. Test inhibitor compounds were added to the assay reagents prior to the mitochondria.

For most experiments, mitochondrial swelling and membrane potential were measured simultaneously in a custom-constructed 2-ml measuring chamber. Membrane potential ($\Delta\Psi$) was indicated by redistribution of tetraphenylphosphonium (TPP^+ , 2 μM), which was measured with a TPP^+ -selective electrode (27). Mitochondrial swelling was monitored by following a decrease in absorbance at 660 nm of the mitochondrial suspension by means of a light-emitting diode and photodetector. The amphipathic cations dibucaine and propranolol were found to interfere with TPP^+ electrode measurements of membrane potential. When these compounds were used, membrane potential was monitored with an LS-3 fluorescence spectrometer (Perkin–Elmer) by measuring fluorescent changes due to the extent of mitochondrial sequestration of the fluorescent cationic dye safranine-O (5 μM , excitation at 485 nm, emission at 586 nm) (28, 29). Swelling was measured independently in the custom-constructed 2-ml measuring chamber as described above.

Determination of cytochrome *c* and adenylate kinase release. Cytochrome *c* and adenylate kinase loss was determined for mitochondria incubated under the conditions described above. Alamethicin treatment was used as a positive control representing maximum release (30). At 3 or 4 min following the addition of peptide or vehicle control, the mitochondria (1 ml of suspension) were pelleted by centrifugation at 13,000g for 2.5 min and the supernatant and pellet were assayed for the presence of cytochrome *c* by immunoblot and for adenylate kinase activity by a modification of the procedure described by Schmidt *et al.* (31). Because of increased viscosity, mitochondria were pelleted at 21,000g for 10 min in experiments when dextran was used. Following centrifugation, a 0.75-ml aliquot of supernatant was carefully removed, supplemented with 15 μl of Protease Inhibitor Cocktail (Sigma), and stored at -70°C . Pellets were frozen at -70°C and resuspended in a 130 mM KCl, 6 mM MgSO_4 , 100 mM Tris/HCl buffer (pH 7.5) containing 1% Triton-X supplemented with 15 μl of Protease Inhibitor Cocktail at the time assays were conducted.

For cytochrome *c* immunoblots, equal aliquots of supernatant samples or 1:5 dilutions of pellet samples were run on 4–12% Bis–Tris

gels (Novex) at 200 V for 35 min. For experiments involving dextran, the amount of dextran in all supernatants was normalized just prior to loading to control for possible effects of dextran on electrophoresis or protein transfer. In control experiments, 20% Dextran T-10 had no effect on the electrophoresis or transfer of 4-ng cytochrome *c* standards. Proteins were electrotransferred to PVDF membranes at 25 V for 1 h; the membranes were rinsed with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and blocked for 1 h in TBS-T supplemented with 1.5% BSA and 1.5% dry milk. Cytochrome *c* was immunostained with primary 7H8 mouse anti-cytochrome *c* antibody (PharMingen, 1:2000 dilution) plus secondary anti-mouse IgG conjugated to horseradish peroxidase (Amersham, 1:4000 dilution). Peroxidase activity was detected using the Enhanced Chemiluminescence detection kit (Amersham) and X-ray film. Band intensities were analyzed densitometrically using the GelExpert system (NucleoTech).

The presence of adenylate kinase was detected in supernatant and pellet samples (100- μ l aliquots for liver, 200- μ l aliquots for brain) by measuring enzyme activity using a DU 7500 spectrophotometer (Beckman-Coulter) at 37°C in a 1.2 ml reaction buffer composed of 130 mM KCl, 6 mM MgSO₄, 100 mM Tris/HCl, pH 7.5, 4 μ l of 0.1 M NADH, 2 μ l of 250 mM ATP, 10 μ l of 50 mM phosphoenolpyruvate, 2.5 μ l of 2 mM rotenone, 5 μ l of 1.5 mM oligomycin, and 10 μ l of a mixture of pyruvate kinase (1000 U/ml) and lactate dehydrogenase (200 U/ml). Triton-X was added to the reaction mixture when assaying supernatant activity to control for the presence of Triton-X used in the resuspension of pellets. The reaction was initiated by the addition of 5 μ l of 0.15 AMP and followed by monitoring a decrease in absorbance at 366 nm that reflects a decrease in the concentration of NADH (31). Linear reaction rates were determined over a 1-min time interval subsequent to AMP addition. For brain mitochondrial samples, an adenylate kinase-independent rate of decrease in absorbance was determined prior to AMP addition and subtracted from the measured rate of enzyme activity. Adenylate kinase-independent decreases in absorbance were negligible in liver mitochondrial samples compared to the enzymatic rates measured. Adenylate kinase release was expressed as activity measured in the supernatant as a percentage of total activity measured in the supernatant plus pellet.

Statistical analysis. A one-way analysis of variance with a Tukey's post-hoc test was utilized to determine statistical differences in adenylate kinase release among groups ($n = 3$ determinations). $P < 0.05$ was considered significant.

RESULTS

Dose dependence of signal peptide-induced permeability changes and cytochrome *c* release. Permeability changes induced by mitochondrial precursor targeting peptides have been reported in isolated rat liver mitochondria suspended in a mannitol-sucrose assay medium (8, 9, 20, 32). However, it has also been reported that increasing the K⁺ ion content in the assay medium inhibited signal peptide-induced swelling and that adenine nucleotides and magnesium ions at concentrations believed to be in the physiological range produced a similar inhibition of signal peptide effects (8). The high cytoplasmic concentration of potassium ions bathing mitochondria within cells as well as the presence of adenine nucleotides and Mg²⁺ raised the question of whether permeability changes initiated by signal peptides can occur under these conditions.

The human precursor targeting peptide for cytochrome oxidase subunit IV is substantially different in

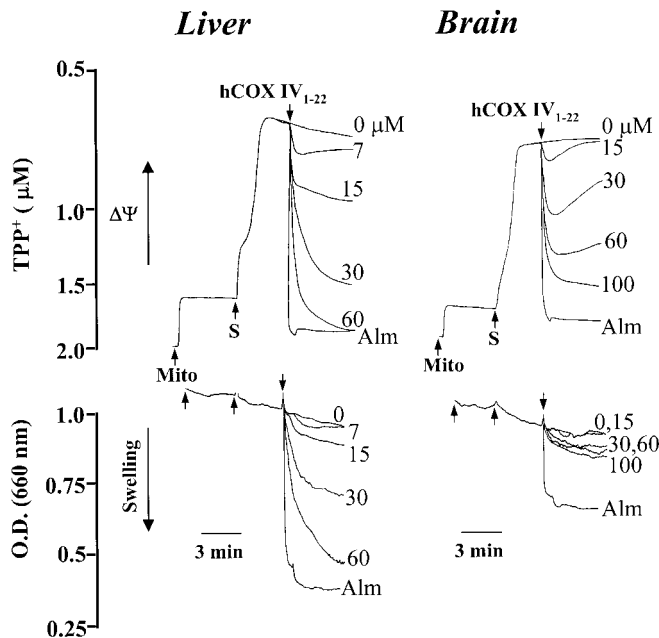


FIG. 1. Signal peptide hCOXIV₁₋₂₂ induces inner membrane depolarization (upper panel) and swelling (lower panel) in liver and brain mitochondria suspended in KCl assay medium supplemented with ATP and Mg²⁺. Mitochondria were incubated at 0.4 mg/ml (protein/ml) in KCl media (see Materials and Methods) supplemented with 4 mM MgCl₂, 3 mM ATP, 1 μ M rotenone, and 2 μ M TPP⁺. Arrows indicate timing of addition of mitochondria (Mito), succinate (S), signal peptide (P), and almetheticin (Alm). In all figures, membrane potential is designated by $\Delta\Psi$. Peptide concentration (μ M) is specified adjacent to each trace. Sixty micrograms of almetheticin/ml was used to obtain maximal swelling for both brain and liver mitochondria. Other details were as described under Experimental Procedures. *Note:* The response of the TPP⁺ electrode to peptide was negligible under these experimental conditions.

sequence from other signal peptides that have been previously tested. Initially the hypothesis that, like yeast COXIV₁₋₂₂, human COXIV₁₋₂₂ would increase the permeability of the inner mitochondrial membrane in a mannitol-sucrose-based assay reagent was confirmed. A decrease in light scattering and a decrease in accumulated TPP⁺ by liver mitochondria in response to hCOXIV₁₋₂₂ indicated an increase in mitochondrial volume and a decrease in mitochondrial membrane potential, respectively (data not shown). Figure 1 shows that unlike yeast COXIV₁₋₂₂, which produced little swelling of liver mitochondria at KCl concentrations above 40 mM (8), hCOXIV₁₋₂₂ was able to completely depolarize liver mitochondria and induce near-maximal swelling in KCl media supplemented with ATP and Mg²⁺. Although approximately twofold higher concentrations of peptide were necessary to induce a permeability increase in the presence of normal cytosolic components than were required in mannitol-sucrose assay media, permeability changes could nevertheless be observed. A KCl-based medium supplemented with ATP and

Mg^{2+} was therefore used throughout the remainder of the study to examine signal peptide effects. The depolarization and swelling induced by the nonselective pore-forming peptide alamethicin (33, 34) were considered maximal and used as a positive control.

Previously, signal peptide-induced permeability changes have primarily been examined in isolated liver mitochondria. It has been shown, however, that cytochrome *c* release induced by calcium occurs by different mechanisms in liver versus brain mitochondria (30). Specifically, although brain and liver mitochondria swelled to a similar extent and released all of their cytochrome *c* when treated with alamethicin, brain mitochondria, unlike liver mitochondria, released cytochrome *c* without swelling in response to calcium. Additionally, nearly threefold less cytochrome *c* was released from brain mitochondria than from liver mitochondria, further indicating a difference in the Ca^{2+} -induced permeabilization of the two types of mitochondria. The ability of hCOXIV₁₋₂₂ to affect mitochondrial membrane permeability in brain mitochondria was therefore examined. In the experiments depicted in Fig. 1, isolated rat brain mitochondria were treated with increasing concentrations of hCOXIV₁₋₂₂ and changes in mitochondrial volume and membrane potential were monitored. Similar to findings with liver mitochondria, swelling and membrane potential depolarization were observed in brain mitochondria treated with signal peptide. A concentration of hCOXIV₁₋₂₂ that was twice as great as that needed in liver mitochondria was necessary to induce swelling and a decrease in $\Delta\Psi$ in brain mitochondria. Furthermore, the maximum extent of swelling that could be observed in brain mitochondria as induced by alamethicin was less than half that which could be observed in liver mitochondria (see Discussion).

Cytochrome *c* release is an important step in the initiation of the apoptotic cascade (35, 36) and has been shown to occur following the opening of the classical, Ca^{2+} -triggered permeability transition pore (6, 37–39). It was therefore determined whether cytochrome *c* release accompanied the signal peptide-induced permeability transition and whether release differed between brain and liver mitochondria. Following treatment of isolated mitochondria with hCOXIV₁₋₂₂, the mitochondrial suspension was centrifuged and cytochrome *c* was assayed in the supernatant by immunoblotting. The data in Fig. 2 show that cytochrome *c* is released from both liver and brain mitochondria by signal peptide in a dose-dependent manner that was related to the extent of swelling and membrane potential depolarization. Additionally, the maximum amount of cytochrome *c* that could be released by hCOXIV₁₋₂₂ was comparable to that released by alamethicin.

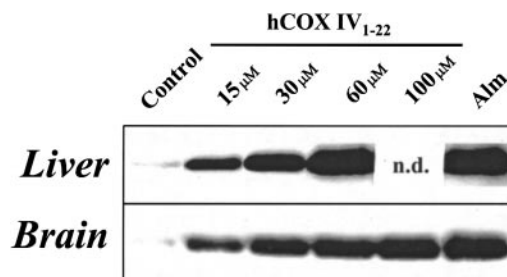


FIG. 2. Signal peptide induces the release of cytochrome *c* from isolated liver and brain mitochondria. Mitochondria (0.4 mg/ml) were incubated for 3.5 min in KCl media supplemented with 4 mM $MgCl_2$, 3 mM ATP, 1 μ M rotenone, and 2 μ M TPP⁺ and then energized with 5 mM succinate. Signal peptide, Alm (60 μ g Alm/ml), or vehicle control was added at 6.5 min. Mitochondrial suspension (1 ml) was centrifuged after 9.5 to 10.5 min total incubation time and cytochrome *c* was detected in the supernatant by immunoblot as described under Experimental Procedures. n.d., not determined.

Signal peptide-induced permeability changes and cytochrome c release are not permeability transition pore dependent. Previous measurements of signal peptide-induced permeability changes in liver mitochondria that were performed in mannitol–sucrose media do not appear consistent with a mechanism involving PTP opening (8, 20, 32). Inhibition by the cyclophilin-binding protein cyclosporin A and the requirement of Ca^{2+} for induction are the two characteristics typically used to define the classical permeability transition pore (40). The responses of brain mitochondria to hCOXIV₁₋₂₂ were therefore examined in the presence of EGTA and CsA to determine whether permeability changes and cytochrome *c* release could still be initiated under conditions in which permeability transition pore activity is suppressed. As shown in Fig. 3, a combination of CsA and Ca^{2+} chelation only slightly inhibited membrane depolarization and swelling and had no effect on the release of cytochrome *c*. Bongkreikic acid binds directly to the adenine nucleotide translocase and locks it in a conformation that is unfavorable to PTP opening. Like CsA, bongkreikic acid (5 μ M) did not prevent the signal peptide (60 μ M) from decreasing $\Delta\Psi$ or releasing cytochrome *c* (data not shown).

Signal peptide-induced permeability changes and cytochrome c release are largely prevented by inhibitors of protein import. It has been previously suggested that signal peptide-induced permeability changes could reflect an inherent pore-forming ability (41) or a capacity to nonspecifically disrupt lipid bilayers (17, 18). Strong evidence has since been provided linking the peptide-induced permeability increase to an interaction with the multiple conductance channel (9) which is thought to coincide with the protein import machinery of the inner mitochondrial membrane (12). Dissipation of the mitochondrial electrochemical gradient across the inner membrane by respiratory uncoupling has been re-

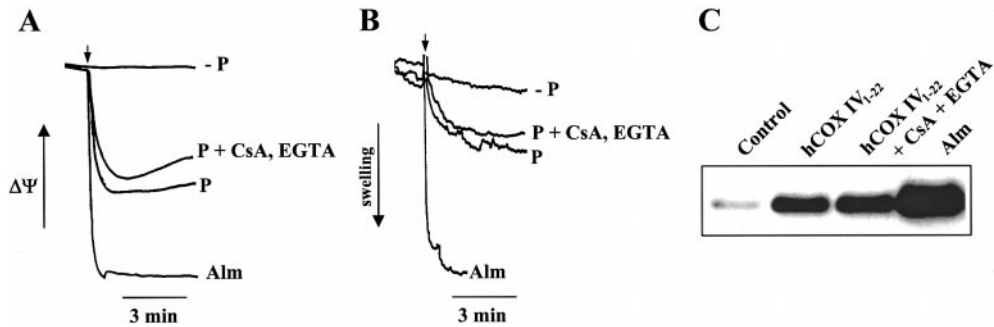


FIG. 3. PTP inhibitors do not block signal peptide-induced membrane potential depolarization, swelling, or cytochrome *c* release. Measurements of membrane potential are depicted in A, measurements of swelling are depicted in B, and measurements of cytochrome *c* release are depicted in C. Brain mitochondria were incubated as described in the legend to Fig. 1. EGTA (250 μ M) and 1 μ M cyclosporin A (CsA) were added prior to the addition of mitochondria. Signal peptide (60 μ M), alamethicin (60 μ g Alm/ml), or vehicle control was added at 6.5 min. Mitochondrial suspension (1 ml) was centrifuged after 10.5 min of total incubation time and cytochrome *c* was detected in the supernatant by immunoblot as described under Experimental Procedures. Here and in subsequent figures, -P denotes vehicle control. All other details are as described in the legend to Fig. 1.

ported to block mitochondrial protein import (17, 42, 43) and to also prevent swelling stimulated by yeast COXIV₁₋₂₂ treatment (8, 9). The data in Fig. 4 demonstrate that, in addition to completely preventing signal peptide-induced swelling in brain mitochondria, the protonophore uncoupler FCCP was also able to completely inhibit the release of cytochrome *c*. The requirement of $\Delta\Psi$ for hCOXIV₁₋₂₂-stimulated cytochrome *c* release argues against nonspecific membrane destabilization as the mechanism of signal peptide activity and implicates the mitochondrial protein import apparatus as a possible target for the peptide's action. Im-

portantly, SynB2, an artificial peptide of similar length and charge that is unable to mediate mitochondrial protein import (26) or swelling in liver mitochondria (9), was also unable to induce swelling of brain mitochondria or stimulate the efflux of cytochrome *c* (Fig. 4).

The amphiphilic cations dibucaine and propranolol have been shown to inhibit protein import (13), block the conductance through the multiple conductance channel (14), and block yeast COXIV₁₋₂₂-initiated swelling and dissipation of the transmembrane potential in liver mitochondria (8). To further investigate the involvement of mitochondrial import machinery in the novel signal peptide-induced permeability transition and cytochrome *c* release, the ability of these two protein import inhibitors to affect hCOXIV₁₋₂₂-mediated changes was examined in brain mitochondria. At concentrations that have been reported to completely inhibit protein import (13) but that do not cause uncoupling under the conditions employed in this study, dibucaine and propranolol largely inhibited signal peptide-stimulated swelling, membrane potential depolarization, and cytochrome *c* release (Fig. 5). No swelling or cytochrome *c* release was observed with the addition of dibucaine or propranolol in the absence of signal peptide (data not shown).

Mitochondrial swelling is a requirement for cytochrome c release by signal peptide. The release of cytochrome *c* following the classical permeability transition has been attributed to the rupturing of the relatively inflexible outer membrane subsequent to the expansion of the matrix space bordered by the highly invaginated inner membrane (6, 37–39). However, under some conditions and in certain cell types, both Ca²⁺ and the proapoptotic proteins Bax and tBid are capable of releasing cytochrome *c* in the absence of swelling (23,

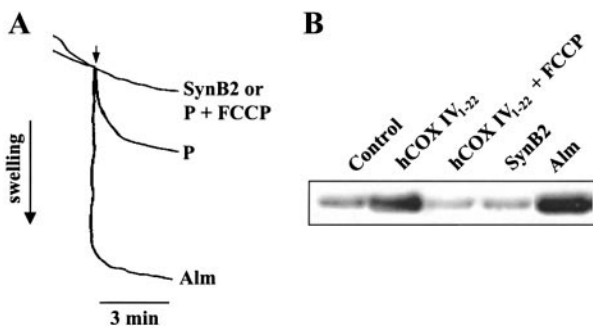


FIG. 4. Swelling and cytochrome *c* release are not observed in uncoupled mitochondria in the presence of signal peptide or in coupled mitochondria in the presence of an artificial peptide that cannot mediate protein import. Measurements of swelling are displayed in A and measurements of cytochrome *c* release are shown in B. Brain mitochondria (0.4 mg/ml) were incubated in KCl medium supplemented with 4 mM MgCl₂, 3 mM ATP, 250 μ M EGTA, 1 μ M rotenone, 5 mM succinate, and 2 μ M TPP⁺ for 3 min at which time 60 μ M signal peptide, 60 μ M SynB2, 80 μ g Alm/ml, or vehicle control was added. Where indicated, 1 μ M FCCP was added prior to the addition of mitochondria. Cytochrome *c* release was detected by immunoblot as described under Experimental Procedures. The arrow indicates the timing of signal peptide (P), SynB2, or alamethicin (Alm) addition.

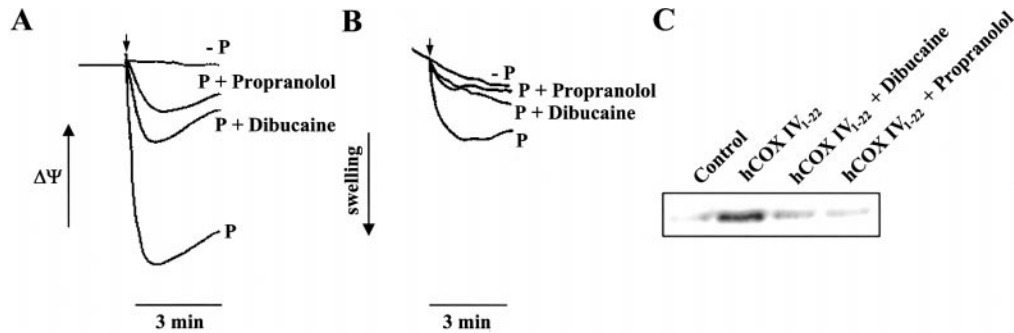


FIG. 5. Inhibitors of protein import reduce signal peptide-induced membrane potential depolarization (A), swelling (B), and cytochrome *c* release (C). Brain mitochondria were incubated under the conditions described in the legend to Fig. 4. In B and C, 5 μM safranin-O replaced 2 μM TPP⁺ in the incubation medium. Where indicated, 500 μM dibucaine or 500 μM propranolol was added prior to the addition of mitochondria. The arrow indicates the timing of 60 μM signal peptide (P) addition. Cytochrome *c* release was detected by immunoblot as described under Experimental Procedures.

44–47). Thus, it is possible that mitochondrial signal peptides can release cytochrome *c* by a mechanism independent of their ability to cause mitochondrial swelling. Low-molecular-weight dextran (MW 10,000) in the assay medium prevented both liver and brain mitochondria from swelling in the presence of signal peptide even though substantial collapse of $\Delta\Psi$ was still observed (Fig. 6). It was therefore possible to address the question of whether swelling is required for the hCOXIV₁₋₂₂-invoked release of cytochrome *c*. As shown in Fig. 6, the inhibition of swelling by dextran eliminated the release of cytochrome *c*, suggesting that swelling is essential to the mechanism of signal peptide-evoked cytochrome *c* efflux.

The release of cytochrome c by signal peptide is not selective. Adenylate kinase is a 25.2-kDa enzyme residing in the intermembrane space, the activity of which can be measured spectrophotometrically (31, 48). The release of adenylate kinase has commonly been used as a measure of outer membrane integrity (48–51). To determine whether hCOXIV₁₋₂₂ releases cytochrome *c* from the intermembrane space selectively, adenylate kinase activity was assayed in the supernatant and the pellet following centrifugation of liver and brain mitochondrial suspensions treated with signal peptide. The percentage of total adenylate kinase activity present in the supernatant following signal peptide treatment was significantly greater than that in the control for both liver and brain mitochondria (Table I, $P < 0.05$). By contrast, the large amount of adenylate kinase released by alamethicin was not significantly different from that released by hCOXIV₁₋₂₂. The observation that the elimination of swelling prevented the loss of mitochondrial cytochrome *c* and the finding that the much larger protein adenylate kinase was coreleased with cytochrome *c* together suggest that the release of cytochrome *c* by signal peptide occurred by swelling-induced outer membrane rupture.

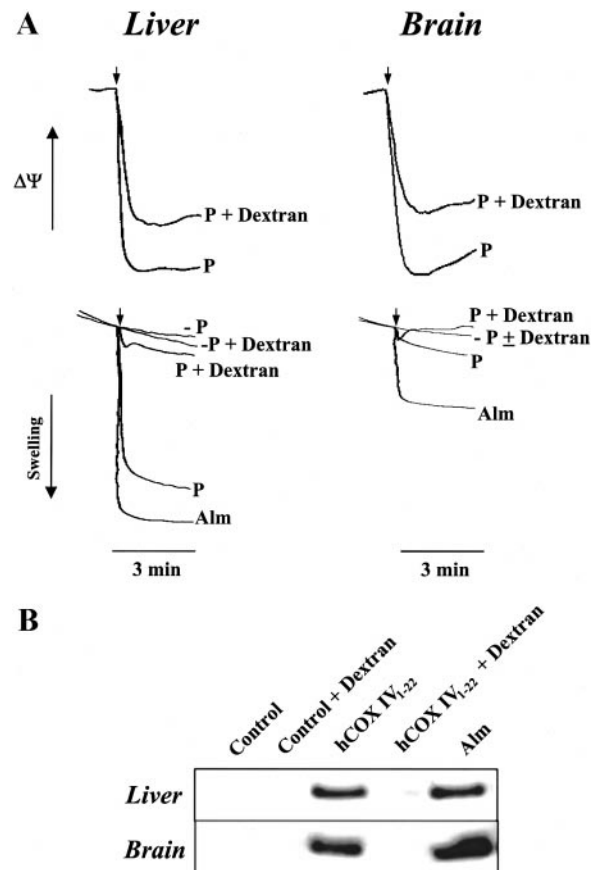


FIG. 6. Signal peptide-induced swelling and cytochrome *c* release in liver and brain mitochondria are suppressed by 10,000 MW dextran without an elimination of membrane potential or hCOXIV₁₋₂₂-induced depolarization. Measurements of membrane potential (upper panel) and swelling (lower panel) are shown in A and measurements of cytochrome *c* release are depicted in B. Mitochondria (0.4 mg/ml) were incubated as described in the legend to Fig. 4; arrows denote the addition of 56 μM signal peptide (P), 80 μg Alm/ml (Alm), or vehicle control (-P). Where indicated, 20% Dextran T-10 was also present (0.2 g/ml). Cytochrome *c* release was detected by immunoblot as described under Experimental Procedures.

TABLE I

Percentage Adenylate Kinase Release by hCOXIV₁₋₂₂^a

Treatment	Liver mitochondria	Brain mitochondria
Control	12.6 ± 5.2	25.3 ± 7.7
hCOXIV ₁₋₂₂	90.0 ± 0.5	63.3 ± 5.7
Alamethicin	86.2 ± 0.5	67.5 ± 4.0

^a Adenylate kinase release is expressed as a percentage of total activity. Results are displayed as means ± standard errors for three independent experiments. Mitochondria were treated with 60 μM hCOXIV₁₋₂₂, 80 μg alamethicin/ml, or vehicle control.

DISCUSSION

Evidence points to a central role for the mitochondrial permeability transition in necrotic and apoptotic cell death (1, 52), although the importance of this mechanism of mitochondrial dysfunction appears to vary between injury models and between cell types (30, 53, 54). Recent evidence also indicates that mitochondrial permeability transitions occur under physiological conditions in response to normal intracellular signal transduction (28, 55). Identification of the factors and conditions that can induce and regulate mitochondrial permeability transitions is therefore potentially quite important.

The current study expanded on previous observations using a yeast mitochondrial targeting peptide to induce a permeability transition in isolated liver mitochondria. The present results demonstrate that a cyclosporin A-insensitive permeability transition can occur in liver mitochondria in response to a human mitochondrial targeting peptide in the presence of physiological concentrations of K⁺, Mg²⁺, and ATP. In addition, the present study demonstrates that the mitochondrial swelling accompanying the permeability transition is sufficiently great to rupture the mitochondrial outer membrane and release both cytochrome *c* and adenylate kinase. Brain mitochondria have previously been shown to be relatively resistant to the classical Ca²⁺-induced permeability transition (22, 23, 30, 56, 57). This study shows for the first time that brain mitochondria are quite sensitive to the permeability transition induced by mitochondrial signal peptides, although the extent of swelling measured by light scattering is somewhat less than that observed with liver mitochondria at the same level of mitochondrial protein. The observation that swelling is responsible for release of intermembrane proteins like cytochrome *c* from brain as well as liver mitochondria in response to a targeting peptide is significant because Ca²⁺-induced cytochrome *c* release from brain mitochondria has been shown to be independent of swelling under the conditions used in the present study (23, 30).

Following the observation that a signal peptide releases cytochrome *c* in the presence of normal cytosolic

components, it was important to verify that the phenomenon was truly distinct from the classical permeability transition. The ability of CsA to inhibit the PTP has been shown to be dependent on its interaction with the matrix protein cyclophilin D (58, 59) and, under some conditions, CsA has been shown to be ineffective in preventing PTP opening (60). The adenine nucleotide translocase is believed to be a central component of the permeability transition pore (61–63). In this study, it was found that in addition to CsA and EGTA, bongkreic acid, a direct ligand of the adenine nucleotide translocase, was also unable to prevent hCOXIV₁₋₂₂-induced permeability transition and cytochrome *c* release. Previous work has indicated that matrix Ca²⁺, a requirement for PTP induction, is actually able to inhibit swelling induced by the yeast cytochrome oxidase VI targeting peptide (32). Altogether, these findings argue strongly against PTP induction as a mechanism to explain the signal peptide-mediated mitochondrial effects that have been observed.

In the past it has been argued that mitochondrial targeting peptides uncouple mitochondria and release adenylate kinase through direct and nonspecific outer and inner membrane disruption (18). Several lines of evidence presented here strongly suggest that the signal peptide-induced outer membrane permeabilization and the accompanying release of cytochrome *c* and adenylate kinase do not result from direct nonspecific lysis of the outer membrane. First, the artificial peptide SynB2, which resembles hCOXIV₁₋₂₂ in length and charge but cannot target proteins for mitochondrial import, failed to initiate mitochondrial swelling, membrane potential dissipation, or the release of cytochrome *c* (Fig. 4 and data not shown). Second, hCOXIV₁₋₂₂ was not able to induce a permeability transition or mediate cytochrome *c* release in uncoupled mitochondria (Fig. 4) or in the presence of protein import inhibitors (Fig. 5). FCCP dissipates the mitochondrial electrical potential across the inner membrane and dibucaine and propranolol have been proposed to block mitochondrial protein import at the level of the inner membrane. It is unlikely that these compounds that have been proposed to act on the inner membrane all inhibit hCOXIV₁₋₂₂-induced outer membrane permeabilization by preventing the direct disruption of the outer membrane bilayer by signal peptide. Third, it was found that the inhibition of swelling by 10,000 MW dextran blocked the release of cytochrome *c* even though inner membrane permeabilization, as evidenced by a drop in electrical potential, still occurred (Fig. 6). The requirement of swelling for the release of intermembrane proteins indicates that an interaction of signal peptides with mitochondrial membranes is in itself insufficient to permeabilize the outer membrane to these proteins. Notably, it has been found that yeast signal peptides are incapable of channel

formation in planar lipid bilayers (64), supporting the hypothesis that signal peptides increase the permeability of the inner membrane through activation of a preexisting conductance pathway rather than via a direct interaction with membrane lipids.

Several studies have suggested that a multisubunit inner membrane channel involved in protein import underlies the conductance pathway activated by mitochondrial signal peptides (8, 9, 20). Previously, it has been reported that a strong correlation exists between the ability of yeast signal peptides to induce swelling of rat liver mitochondria and their capacity to modulate the conductance of the MCC in mouse liver mitoplasts (9). Additionally, signal peptides were incapable of regulating the activity of the multiple conductance channel of yeast mutants with a defective Tim23p protein (12). Tim23p is a reported component of the inner membrane protein translocation complex (65). Pavlov and Glaser (13) have recently demonstrated that dibucaine, propranolol, and trifluoperazine are able to inhibit mitochondrial protein import at the level of the inner mitochondrial membrane. These compounds, as well as FCCP, have also been found to block the swelling of liver mitochondria in response to yeast signal peptides. Finally, propranolol and dibucaine block the conductance through the multiple conductance channel (14). Together, these findings raised the possibility that the ability of signal peptide to induce permeability changes and cytochrome *c* release is dependent on an interaction with the translocase of the inner membrane (TIM). The observation that maximal effects of the signal peptide on mitochondrial membrane potential, swelling, and cytochrome *c* release were obtained at 150–300 nmol mg⁻¹ protein presents the possibility that TIM may become “clogged” with peptides and forced to remain open in a state of high conductance.

The human COXIV₁₋₂₂-induced permeability transition, unlike yeast COXIV₁₋₂₂-induced permeability changes (8), was found to occur in the presence of normal cytosolic components. As the bioenergetic effects of the yeast signal peptide have been shown to be inhibited by FCCP, dibucaine, and propranolol, we tested their influence on the effects of the human signal peptide on mitochondria incubated in the presence of physiological concentrations of K⁺, Mg²⁺, and ATP. Both dibucaine and propranolol substantially inhibited signal peptide-induced mitochondrial depolarization and cytochrome *c* release (Fig. 5). Depolarization of mitochondria by the preaddition of the protonophore uncoupler FCCP similarly inhibited the swelling and the cytochrome *c* release in response to hCOXIV₁₋₂₂ (Fig. 4). The finding that hCOXIV₁₋₂₂ and previously characterized yeast signal peptides are able to initiate permeability changes with a similar pharmacological sensitivity suggests that the peptides act through a common pathway. Our evidence lends additional sup-

port to the hypothesis that signal peptides elicit mitochondrial membrane permeability changes through an interaction with the inner membrane protein import machinery.

Kroemer, Skulachev, and others have proposed that cytochrome *c* is released by free diffusion following matrix swelling and outer mitochondrial membrane rupture subsequent to PTP opening (5, 6, 37–39, 66). Recently, the hypothesis that cytochrome *c* release may occur via selective channels in the outer membrane has also emerged (23, 44–47, 67–69). The corelease of the much larger protein adenylate kinase accompanying cytochrome *c* efflux in response to various apoptotic stimuli has been used to promote the hypothesis favoring membrane disruption over the involvement of specific transport channels (70, 71).

Here it was demonstrated that hCOXIV₁₋₂₂ was able to stimulate adenylate kinase release from both liver and brain mitochondria (Table I), suggesting that outer mitochondrial membrane integrity had been compromised. However, one recent study has described cytochrome *c* and adenylate kinase release in the absence of swelling (48), raising the possibility that outer membrane breakage resulting from signal peptide-induced swelling was not, in fact, the mechanism of the cytochrome *c* and adenylate kinase efflux that was observed. Macromolecules such as dextran have been found to inhibit mitochondrial swelling by exerting oncotic pressure (72–74). Therefore, to determine whether swelling was truly crucial to the release of intermembrane proteins by hCOXIV₁₋₂₂, the ability of the signal peptide to invoke the release of cytochrome *c* in the absence of swelling was examined. Although liver and brain mitochondria incubated in the presence of dextran maintained membrane potential and mitochondrial cytochrome *c* localization, and respired normally under control conditions (Fig. 6 and unpublished observations), no swelling was exhibited following treatment with human signal peptide. Under these conditions, the release of cytochrome *c* was inhibited. Although alternative explanations are possible, such as direct effects of dextran on inner–outer membrane contact sites (see (75, 76)), the observation that adenylate kinase is released by hCOXIV₁₋₂₂ combined with the finding that release of cytochrome *c* does not occur in the absence of swelling suggests that the release of cytochrome *c* in this system is, in fact, due to swelling-induced outer membrane breakage. However, considering the relatively subtle changes in light scattering for brain mitochondria (Fig. 1) and previous observations that these mitochondria can release cytochrome *c* in response to Ca²⁺ by a swelling-independent mechanism (23, 30), a minor contribution of an outer membrane pore mechanism to signal peptide-induced cytochrome *c* release from brain mitochondria cannot be excluded.

In conclusion, the results of this study demonstrate that a mitochondrial precursor targeting peptide can induce a novel, cyclosporin A-insensitive permeability transition in the presence of physiological concentrations of K^+ , Mg^{2+} , and ATP. This transition occurs in both liver and brain mitochondria and the swelling associated with the transition is sufficient to cause disruption of the outer membrane and release of intermembrane proteins, including cytochrome *c*. Further investigations are necessary to determine the relevance of these phenomena observed with signal peptides to mechanisms of mitochondrial-cytosolic protein redistribution during apoptosis and to the mechanism of mitochondrial protein import. The finding that a human signal peptide can disrupt outer mitochondrial membrane integrity and release cytochrome *c* may also have important implications for studies using mitochondrial signal sequences for the targeting of molecules and drugs to mitochondria (77, 78).

ACKNOWLEDGMENTS

This work was supported by NIH Grant NS34152 to G.F. and GM57429 to K.W.K. The authors would like to thank Mr. S. Russell for expert technical assistance and Drs. A. Starkov and A. Andreyev for helpful discussions.

REFERENCES

- Lemasters, J. J., Qian, T., Bradham, C. A., Brenner, D. A., Cascio, W. E., Trost, L. C., Nishimura, Y., Nieminen, A. L., and Herman, B. (1999) *J. Bioenerg. Biomembr.* **31**, 305–319.
- Bernardi, P., Colonna, R., Costantini, P., Eriksson, O., Fontaine, E., Ichas, F., Massari, S., Nicolli, A., Petronilli, V., and Scorrano, L. (1998) *Biofactors* **8**, 273–281.
- Halestrap, A. P., Kerr, P. M., Javadov, S., and Woodfield, K. Y. (1998) *Biochim. Biophys. Acta* **1366**, 79–94.
- Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., and Kroemer, G. (1996) *J. Exp. Med.* **184**, 1331–1341.
- Skulachev, V. P. (1996) *FEBS Lett.* **397**, 7–10.
- Petit, P. X., Goubern, M., Dirolez, P., Susin, S. A., Zamzami, N., and Kroemer, G. (1998) *FEBS Lett.* **426**, 111–116.
- Petit, P. X., Susin, S. A., Zamzami, N., Mignotte, B., and Kroemer, G. (1996) *FEBS Lett.* **396**, 7–13.
- Sokolove, P. M., and Kinnally, K. W. (1996) *Arch. Biochem. Biophys.* **336**, 69–76.
- Kushnareva, Y. E., Campo, M. L., Kinnally, K. W., and Sokolove, P. M. (1999) *Arch. Biochem. Biophys.* **366**, 107–115.
- Pfeiffer, D. R., Guduz, T. I., Novgorodov, S. A., and Erdahl, W. L. (1995) *J. Biol. Chem.* **270**, 4923–4932.
- Kinnally, K. W., Lohret, T. A., Campo, M. L., and Mannella, C. A. (1996) *J. Bioenerg. Biomembr.* **28**, 115–123.
- Lohret, T. A., Jensen, R. E., and Kinnally, K. W. (1997) *J. Cell Biol.* **137**, 377–386.
- Pavlov, P. F., and Glaser, E. (1998) *Biochem. Biophys. Res. Commun.* **252**, 84–91.
- Antonenko, Y. N., Kinnally, K. W., Perini, S., and Tedeschi, H. (1991) *FEBS Lett.* **285**, 89–93.
- Lohret, T. A., and Kinnally, K. W. (1995) *J. Biol. Chem.* **270**, 15,950–15,953.
- Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., and Schatz, G. (1986) *EMBO J.* **5**, 1327–1334.
- Glaser, S. M., and Cumsky, M. G. (1990) *J. Biol. Chem.* **265**, 8808–8816.
- Nicolay, K., Laterveer, F. D., and van Heerde, W. L. (1994) *J. Bioenerg. Biomembr.* **26**, 327–334.
- Hugosson, M., Andreu, D., Boman, H. G., and Glaser, E. (1994) *Eur. J. Biochem.* **223**, 1027–1033.
- Lu, Y., and Beavis, A. D. (1997) *J. Biol. Chem.* **272**, 13,555–13,561.
- Friberg, H., Connern, C., Halestrap, A. P., and Wieloch, T. (1999) *J. Neurochem.* **72**, 2488–2497.
- Berman, S. B., Watkins, S. C., and Hastings, T. G. (2000) *Exp. Neurol.* **164**, 415–425.
- Andreyev, A. Y., Fahy, B., and Fiskum, G. (1998) *FEBS Lett.* **439**, 373–376.
- Rosenthal, R. E., Hamud, F., Fiskum, G., Varghese, P. J., and Sharpe, S. (1987) *J. Cereb. Blood Flow Metab.* **7**, 752–758.
- Lapidus, R. G., and Sokolove, P. M. (1993) *Arch. Biochem. Biophys.* **306**, 246–253.
- Allison, D. S., and Schatz, G. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9011–9015.
- Kamo, N., Muratsugu, M., Hongoh, R., and Kobatake, Y. (1979) *J. Membr. Biol.* **49**, 105–121.
- Kowaltowski, A. J., Vercesi, A. E., and Fiskum, G. (2000) *Cell Death Differ.* **7**, 207–214.
- Fiskum, G., Kowaltowski, A. J., Andreyev, A., Kushnareva, Y. E., and Starkov, A. A. (2000) *Methods Enzymol.* **322**, 222–234.
- Andreyev, A., and Fiskum, G. (1999) *Cell Death. Differ.* **6**, 825–832.
- Schmidt, B., Wachter, E., Sebald, W., and Neupert, W. (1984) *Eur. J. Biochem.* **144**, 581–588.
- Kushnareva, Y. E., Haley, L. M., and Sokolove, P. M. (1999) *Arch. Biochem. Biophys.* **363**, 155–162.
- Mathew, M. K., Nagaraj, R., and Balaran, P. (1981) *Biochem. Biophys. Res. Commun.* **98**, 548–555.
- Ritov, V. B., Tverdislova, I. L., Avakyan, T. Y., Menshikova, E. V., Leikin, Y., Bratkovskaya, L. B., and Shimon, R. G. (1992) *Gen. Physiol. Biophys.* **11**, 49–58.
- Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) *Cell* **90**, 405–413.
- Li, F., Srinivasan, A., Wang, Y., Armstrong, R. C., Tomaselli, K. J., and Fritz, L. C. (1997) *J. Biol. Chem.* **272**, 30,299–30,305.
- Kantrow, S. P., and Piantadosi, C. A. (1997) *Biochem. Biophys. Res. Commun.* **232**, 669–671.
- Scarlett, J. L., and Murphy, M. P. (1997) *FEBS Lett.* **418**, 282–286.
- Yang, J. C., and Cortopassi, G. A. (1998) *Free Radicals Biol. Med.* **24**, 624–631.
- Gunter, T. E., and Pfeiffer, D. R. (1990) *Am. J. Physiol.* **258**, C755–C786.
- Singer, S. J., Maher, P. A., and Yaffe, M. P. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1015–1019.
- Vallette, F. M., Juin, P., Pelleschi, M., and Henry, J. P. (1994) *J. Biol. Chem.* **269**, 13,367–13,374.
- Tokatlidis, K., and Schatz, G. (1999) *J. Biol. Chem.* **274**, 35,285–35,288.
- Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A., and Martinou, J. C. (1998) *J. Cell Biol.* **143**, 217–224.

45. Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J. C. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 4997–5002.
46. Finucane, D. M., Bossy-Wetzel, E., Waterhouse, N. J., Cotter, T. G., and Green, D. R. (1999) *J. Biol. Chem.* **274**, 2225–2233.
47. Shimizu, S., and Tsujimoto, Y. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 577–582.
48. Kluck, R. M., Esposti, M. D., Perkins, G., Renken, C., Kuwana, T., Bossy-Wetzel, E., Goldberg, M., Allen, T., Barber, M. J., Green, D. R., and Newmeyer, D. D. (1999) *J. Cell Biol.* **147**, 809–822.
49. Single, B., Leist, M., and Nicotera, P. (1998) *Cell Death. Differ.* **5**, 1001–1003.
50. Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P., and Orrenius, S. (1999) *EMBO J.* **18**, 2040–2048.
51. Stefanelli, C., Stanic, I., Zini, M., Bonavita, F., Flamigni, F., Zambonin, L., Landi, L., Pignatti, C., Guarneri, C., and Caldarera, C. M. (2000) *Biochem. J.* **347**, 875–880.
52. Hirsch, T., Susin, S. A., Marzo, I., Marchetti, P., Zamzami, N., and Kroemer, G. (1998) *Cell Biol. Toxicol.* **14**, 141–145.
53. Martinou, I., Desagher, S., Eskes, R., Antonsson, B., Andre, E., Fakan, S., and Martinou, J. C. (1999) *J. Cell Biol.* **144**, 883–889.
54. Ushmorov, A., Ratter, F., Lehmann, V., Droge, W., Schirrmacher, V., and Umansky, V. (1999) *Blood* **93**, 2342–2352.
55. Smaili, S. S., and Russell, J. T. (1999) *Cell Calcium* **26**, 121–130.
56. Brustovetsky, N., and Dubinsky, J. M. (2000) *J. Neurosci.* **20**, 103–113.
57. Kristian, T., Gertsch, J., Bates, T. E., and Siesjo, B. K. (2000) *J. Neurochem.* **74**, 1999–2009.
58. Scorrano, L., Nicolli, A., Basso, E., Petronilli, V., and Bernardi, P. (1997) *Mol. Cell. Biochem.* **174**, 181–184.
59. Halestrap, A. P., Connern, C. P., Griffiths, E. J., and Kerr, P. M. (1997) *Mol. Cell. Biochem.* **174**, 167–172.
60. Nieminen, A. L., Saylor, A. K., Tesfai, S. A., Herman, B., and Lemasters, J. J. (1995) *Biochem. J.* **307**, 99–106.
61. Halestrap, A. P., and Davidson, A. M. (1990) *Biochem. J.* **268**, 153–160.
62. Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C., and Kroemer, G. (1998) *Science* **281**, 2027–2031.
63. Bauer, M. K., Schubert, A., Rocks, O., and Grimm, S. (1999) *J. Cell Biol.* **147**, 1493–1502.
64. Henry, J. P., Chich, J. F., Goldschmidt, D., and Thieffry, M. (1989) *J. Membr. Biol.* **112**, 139–147.
65. Emtage, J. L., and Jensen, R. E. (1993) *J. Cell Biol.* **122**, 1003–1012.
66. Scorrano, L., Petronilli, V., Di Lisa, F., and Bernardi, P. (1999) *J. Biol. Chem.* **274**, 22581–22585.
67. Shimizu, S., Narita, M., and Tsujimoto, Y. (1999) *Nature* **399**, 483–487.
68. Shimizu, S., Ide, T., Yanagida, T., and Tsujimoto, Y. (2000) *J. Biol. Chem.* **275**, 12321–12325.
69. Saito, M., Korsmeyer, S. J., and Schlesinger, P. H. (2000) *Nat. Cell Biol.* **2**, 553–555.
70. Pastorino, J. G., Tafani, M., Rothman, R. J., Marcineviciute, A., Hoek, J. B., and Farber, J. L. (1999) *J. Biol. Chem.* **274**, 31,734–31,739.
71. Kohler, C., Gahm, A., Noma, T., Nakazawa, A., Orrenius, S., and Zhivotovsky, B. (1999) *FEBS Lett.* **447**, 10–12.
72. Gellerich, F. N., Laterveer, F. D., Korzeniewski, B., Zierz, S., and Nicolay, K. (1998) *Eur. J. Biochem.* **254**, 172–180.
73. Laterveer, F. D., Gellerich, F. N., and Nicolay, K. (1995) *Eur. J. Biochem.* **232**, 569–577.
74. Laterveer, F. D., Nicolay, K., and Gellerich, F. N. (1996) *FEBS Lett.* **386**, 255–259.
75. Wicker, U., Bucheler, K., Gellerich, F. N., Wagner, M., Kapischke, M., and Brdiczka, D. (1993) *Biochim. Biophys. Acta* **1142**, 228–239.
76. Doran, E., and Halestrap, A. P. (2000) *Biochem. J.* **348**, 343–350.
77. Murphy, M. P. (1997) *Trends Biotechnol.* **15**, 326–330.
78. Murphy, M. P., and Smith, R. A. (2000) *Adv. Drug Deliv. Rev.* **41**, 235–250.