

# Elevation of resting mitochondrial membrane potential of neural cells by cyclosporin A, BAPTA-AM, and Bcl-2

ALICIA J. KOWALTOWSKI,<sup>1,2,\*</sup> SORAYA S. SMAILI,<sup>3,4,\*</sup>  
JAMES T. RUSSELL,<sup>3</sup> AND GARY FISKUM<sup>1</sup>

<sup>1</sup>Department of Anesthesiology, The University of Maryland Baltimore, Baltimore, Maryland 21201;

<sup>2</sup>Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brazil;

<sup>3</sup>Section on Neuronal Secretory Systems, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; and

<sup>4</sup>Departamento de Farmacologia, Universidade Federal de São Paulo, UNIFESP, São Paulo, Brazil

Received 20 May 1999; accepted in final form 30 March 2000

**Kowaltowski, Alicia J., Soraya S. Smaili, James T. Russell, and Gary Fiskum.** Elevation of resting mitochondrial membrane potential of neural cells by cyclosporin A, BAPTA-AM, and Bcl-2. *Am J Physiol Cell Physiol* 279: C852–C859, 2000.—This study tested the hypothesis that the activity of the mitochondrial membrane permeability transition pore (PTP) affects the resting mitochondrial membrane potential ( $\Delta\Psi$ ) of normal, healthy cells and that the anti-apoptotic gene product Bcl-2 inhibits the basal activity of the PTP.  $\Delta\Psi$  was measured by both fluorometric and nonfluorometric methods with SY5Y human neuroblastoma cells and with GT1–7 hypothalamic cells and PC12 pheochromocytoma cells in the absence and presence of Bcl-2 gene overexpression. The resting  $\Delta\Psi$  of Bcl-2 nonexpressing PC12 and wild-type SY5Y cells was increased significantly by the presence of the PTP inhibitor cyclosporin A (CsA) or by intracellular  $\text{Ca}^{2+}$  chelation through exposure to the acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM). The  $\Delta\Psi$  of Bcl-2-overexpressing PC12 cells was larger than that of Bcl-2-negative cells and not significantly increased by CsA or by  $\text{Ca}^{2+}$  chelation. CsA did not present a significant effect on the  $\Delta\Psi$  monitored in unstressed GT1–7 cells but did inhibit the decrease in  $\Delta\Psi$  elicited by the addition of *t*-butyl hydroperoxide, an oxidative inducer of the mitochondrial permeability transition. These results support the hypothesis that an endogenous PTP activity can contribute to lowering the basal  $\Delta\Psi$  of some cells and that Bcl-2 can regulate the endogenous activity of the mitochondrial PTP.

calcium; mitochondrial permeability transition; energy metabolism

---

EXPOSURE OF ISOLATED MITOCHONDRIA to  $\text{Ca}^{2+}$  ions can cause a nonselective permeabilization of the inner mitochondrial membrane due to the opening of the mitochondrial permeability transition pore (PTP) (30, 53). The PTP promotes a drop in mitochondrial membrane potential ( $\Delta\Psi$ ) and a loss of accumulated  $\text{Ca}^{2+}$  and

even induces large amplitude swelling of mitochondria (30, 53). These phenomena are stimulated by the presence of inorganic phosphate, oxidative stress, or dithiol reagents and are typically inhibited by cyclosporin A (CsA) (25, 27, 30, 53).

Although the PTP has been studied extensively using isolated mitochondria or permeabilized cells, these experiments have rarely been conducted under physiologically relevant conditions (2). In some cells and tissues, the PTP has been implicated as an early event in both apoptotic and necrotic cell death (17, 29, 30). In addition, the anti-apoptotic protein Bcl-2 inhibits the PTP and prevents mitochondrial release of cytochrome *c*, a trigger for apoptosis (26, 28, 52). However, few studies have detected the activity of the PTP in intact cells in the absence of potentially lethal stressful conditions (11, 16, 19, 46), e.g., in the presence of greatly elevated intracellular  $\text{Ca}^{2+}$  or toxic hydroperoxides.

Classically, PTP opening has been associated with generalized mitochondrial dysfunction, which is consistent with a role of the PTP in cell death but would be incompatible with a physiological role for this pore. Some studies suggest that, under certain conditions, the PTP mediates a limited transport of small ions, which could allow for the maintenance of viable mitochondrial energy-transducing activities (13, 49). This activity state of the PTP has been referred to as the “low-conductance state” (19, 20, 37) but can also be interpreted as a transient opening of the PTP that, unlike a relatively high-conductance state, is insufficient to cause high-amplitude swelling and irreversible mitochondrial destruction (38).

Recent elucidation of the multiple roles that mitochondria play in normal cellular  $\text{Ca}^{2+}$  homeostasis has provided additional evidence for a physiological PTP activity. Upon mobilization of  $\text{Ca}^{2+}$  from the endoplasmic reticulum by the second messenger inositol 1,4,5-

---

\*A. J. Kowaltowski and S. S. Smaili contributed equally to this work.

Address for reprint requests and other correspondence: G. Fiskum, Univ. of Maryland, Baltimore, Dept. of Anesthesiology, 685 W. Baltimore St., Baltimore, MD, 21201 (E-mail: gfishum@anesthlab.ummc.umaryland.edu).

---

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

trisphosphate (IP<sub>3</sub>), mitochondria adjacent to the Ca<sup>2+</sup> release sites play an important role in clearance of cytosolic Ca<sup>2+</sup> (16, 39, 43). Under these circumstances, activation of mitochondrial Ca<sup>2+</sup> influx can modulate IP<sub>3</sub> receptors and cytosolic Ca<sup>2+</sup> signaling (43). Moreover, it has been shown that mitochondrial Ca<sup>2+</sup> uptake triggers mitochondrial Ca<sup>2+</sup> release, which, in turn, leads to an amplification of the cytosolic Ca<sup>2+</sup> signals (19). A low-conductance PTP would present a tendency to flicker between the opened and closed states as Ca<sup>2+</sup> is taken up and released (Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release), generating and conveying Ca<sup>2+</sup> signals (20, 44). Thus the low-conductance PTP may be responsible for the mitochondrial participation in modulating and shaping Ca<sup>2+</sup> transients during Ca<sup>2+</sup> signaling.

In this report, we investigated the contribution of PTP activity to mitochondrial  $\Delta\Psi$  in healthy, unstressed neural cells. We found that the PTP contributes significantly toward the reduction in mitochondrial  $\Delta\Psi$  in two of three different cell lines. The anti-apoptotic gene product Bcl-2, which has been shown to inhibit the PTP in stressed cells, was also found to minimize the contribution of the PTP to the resting  $\Delta\Psi$  of unstressed cells.

## MATERIALS AND METHODS

**Cell cultures.** Immortalized PC12 adrenal pheochromocytoma cells, GT1-7 hypothalamic tumor cells, and SY5Y human neuroblastoma cells were maintained as described previously (34, 3). PC12 and GT1-7 cells were transfected with the human *bcl-2* gene (Bcl-2<sup>+</sup>) or with a control retroviral construct (Bcl-2<sup>-</sup>) (24). Experiments were performed either with cells plated on coverslips or with cells that were grown normally, trypsinized, and suspended in the incubation medium. All cells presented >98% viability at the time they were used, as assayed by trypan blue staining.

**Standard incubation conditions.** All assays were conducted at 37°C, in medium containing 130 mM NaCl, 5.6 mM KCl, 0.8 mM MgSO<sub>4</sub>, 1 mM Na<sub>2</sub>PO<sub>4</sub>, 25 mM glucose, 20 mM HEPES (pH 7.3), 1.5 mM CaCl<sub>2</sub>, 2.5 mM NaHCO<sub>3</sub>, 1.5 mg/ml BSA, and 1 mM ascorbic acid. Cells in suspension were continuously stirred while cells on coverslips were continuously superfused with medium. All additions during experiments were made to the suspension or superfusion medium and did not involve a change in media.

**Determination of mitochondrial  $\Delta\Psi$  using TMRE.** Cells were maintained during the experimental assays in media containing tetramethylrhodamine ethyl ester (TMRE, 50 nM), a cationic dye that is rapidly and reversibly accumulated by mitochondria, due to their  $\Delta\Psi$  (12, 31). As TMRE-based measurements of  $\Delta\Psi$  may underestimate the absolute value of the membrane potential, these determinations were used to compare relative levels rather than assigning specific values (41). Moreover, because TMRE can, under some conditions, produce superoxide radicals and even induce the permeability transition when photodynamically excited (18), all incubations were conducted in the dark, and light exposure was kept to the minimum necessary for accurate measurements.

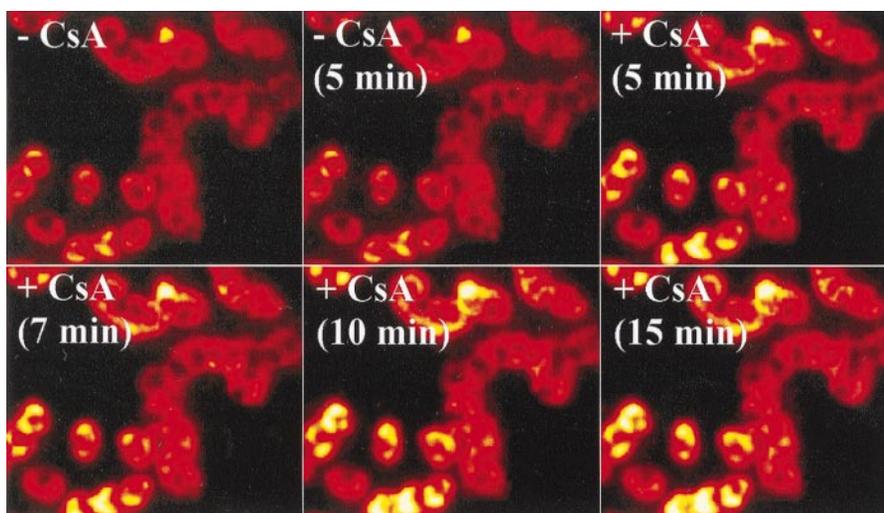
TMRE fluorescence of cells in suspension was measured with a Perkin-Elmer LS-3 fluorescence spectrophotometer equipped with continuous stirring, operating at excitation and emission wavelengths of 546 and 573 nm, respectively. Measurements of  $\Delta\Psi$  are expressed as the difference in

fluorescence of the suspension before and after the addition of the protonophore uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 10  $\mu$ M). In the presence of a respiration-driven mitochondrial  $\Delta\Psi$ , the fluorescence of TMRE in mitochondrial or cellular suspensions is quenched as a result of TMRE accumulation within mitochondria, an effect which is rapidly reversed by the addition of FCCP. TMRE measurements of  $\Delta\Psi$  are generally conducted with high-resolution fluorescent microscopy at the single cell level, where mitochondrial images can be discerned (11, 12, 18, 19, 31, 46). To assess the possible contribution of changes in TMRE fluorescence due to changes in plasma membrane potential, we tested the effects of FCCP in the absence and presence of the combination of the respiratory inhibitor antimycin A and the mitochondrial ATP hydrolase inhibitor oligomycin. The change in fluorescence observed after the addition of FCCP in the presence of these mitochondria-specific poisons was ~4% of that observed in their absence, indicating that TMRE fluorescence changes observed under our experimental conditions were due almost exclusively to changes in mitochondrial  $\Delta\Psi$ , rather than fluctuations in the plasma membrane electrical potential. Although we cannot exclude the possibility that the effects of drugs used in our experiments, e.g., CsA, are at least partially due to their influence on plasma membrane potential, we believe this to be unlikely, because no effects of these compounds on cellular membrane potentials have been reported. Results are expressed as the average  $\pm$  SE of 3–5 individual determinations. Comparisons between cell types (e.g.,  $\pm$ Bcl-2) and between experimental conditions (e.g.,  $\pm$ CsA) were made using a Tukey's test multiple pairwise comparison procedure, run by Sigmapstat.

In addition to TMRE measurements of  $\Delta\Psi$  with cells in suspension, TMRE fluorescence was recorded using images of individual cells acquired with a charge-coupled device (CCD) camera equipped with an intensifier (51). In these experiments, cells were plated on coverslips that were then placed in a perfusion chamber at 37°C and positioned on the stage of an inverted microscope. In contrast to the decrease in fluorescence of TMRE that occurs with an increased  $\Delta\Psi$  of cells in suspension, TMRE fluorescence of individually imaged cell bodies under the microscope is proportional to the  $\Delta\Psi$ , due to the concentration of TMRE into the cells in response to the respiration-dependent  $\Delta\Psi$ . Cells on coverslips were superfused with TMRE-containing media at a constant rate. Added drugs were diluted in the perfusion medium and applied by switching the reservoirs of the perfusion system. Therefore, cells were under the same perfusion conditions during the experiment, and additions did not represent any change in the equilibrium of TMRE uptake. Cells were exposed to perfusion medium containing TMRE (50 nM) 10 min before the initial recordings and during all data acquisition. Fluorescence images were acquired every 20 s for 30 min at 525-nm excitation and 610-nm emission wavelengths. Data were extracted using Synapse image processor (Synergy Research Systems, Silver Spring, MD), and results were plotted as arbitrary units or normalized fluorescence ( $\Delta F/F$ ) for comparison. Fluorescence intensities in the nonzero pixels within each slice were averaged ( $F$ ) and plotted as normalized fluorescence against time.  $\Delta F$  is calculated as the difference between the mean value of the first 20 data points before stimulation of the cell and  $F$ . TMRE fluorescence signals were not calibrated to membrane potential and represent relative values (46). All calculations were performed using Synapse.

**Determination of  $\Delta\Psi$  using TPP<sup>+</sup>.** Cells were incubated in standard media supplemented with 0.5  $\mu$ M tetraphenylphos-

Fig. 1. Fluorescence images of mitochondrial membrane potential ( $\Delta\Psi$ ) in PC12 Bcl-2<sup>-</sup> cells before (-) and after (+) exposure to cyclosporin A (CsA). Cells were stained with 50 nM tetramethylrhodamine ethyl ester (TMRE) for mitochondrial  $\Delta\Psi$  visualization using a charge-coupled device (CCD) camera. After 5 min, 1  $\mu$ M CsA was added, and fluorescence was monitored for an additional 15 min. Images are representative of 3 experiments.



phonium (TPP<sup>+</sup>), and the concentration of TPP<sup>+</sup> was continuously monitored in the extracellular medium using a TPP<sup>+</sup>-selective electrode constructed according to Kamo et al. (23). TPP<sup>+</sup> uptake by cells treated with antimycin A plus oligomycin was <1% of that in respiring cells, again indicating that TPP<sup>+</sup> measurements reflect the mitochondrial  $\Delta\Psi$  rather than the plasma membrane potential.

**Materials.** *Tert*-butyl hydroperoxide (*t*-bOOH), FCCP, antimycin A, oligomycin, and TPP<sup>+</sup> were purchased from Sigma Chemical. The acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM) was purchased from Calbiochem, CsA was from Alexis, and TMRE was obtained from Molecular Probes. FK-506 was a gift from Fujisawa, Japan. CsA and BAPTA were diluted in ethanol or DMSO. The final concentration of these vehicles was 0.001%, which was determined to have no effect on TMRE fluorescence intensity or the response of the TPP<sup>+</sup> electrode.

## RESULTS

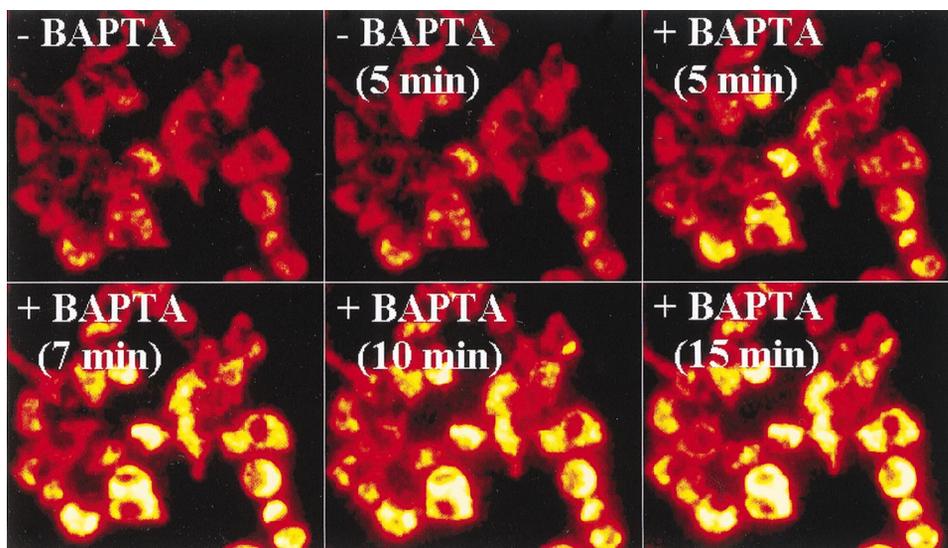
The mitochondrial  $\Delta\Psi$  in normal, intact cells was initially evaluated by fluorescence microscopy using TMRE, a fluorescent probe of  $\Delta\Psi$ . Although the base-

line TMRE fluorescence of Bcl-2<sup>-</sup> PC12 cells remained constant over the first 5 min of measurements (Fig. 1) and for at least 15 min thereafter (not shown), cells treated with the PTP inhibitor CsA exhibited a substantial increase in TMRE fluorescence that appeared to reach a plateau 10–15 min after the addition of CsA (Fig. 1).

To ascertain that the increase in TMRE response observed could be attributed to the PTP, we treated the cells with BAPTA-AM (Fig. 2), which chelates intracellular Ca<sup>2+</sup>, a necessary trigger for PTP opening (53). We observed that PC12 cells treated with BAPTA-AM also presented an increase in TMRE response over time, similar to that observed with CsA. Thus both intracellular Ca<sup>2+</sup> chelation, which prevents PTP opening, and CsA, which inhibits the PTP, increased the resting mitochondrial  $\Delta\Psi$  in PC12 cells.

The anti-apoptotic protein Bcl-2, which inhibits PTP opening induced by Ca<sup>2+</sup> and pro-oxidants, has been reported to elevate the resting  $\Delta\Psi$  of isolated mitochondria (42, 28). As a test of the hypothesis that Bcl-2 can

Fig. 2. Fluorescence images of mitochondrial  $\Delta\Psi$  in PC12 Bcl-2<sup>-</sup> cells before (-) and after (+) exposure to the acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM). Cells were stained with 50 nM TMRE for mitochondrial  $\Delta\Psi$  visualization using a CCD camera. Images were acquired for 5 min before and 15 min after the addition of 10  $\mu$ M BAPTA-AM. Images are representative of 3 experiments.



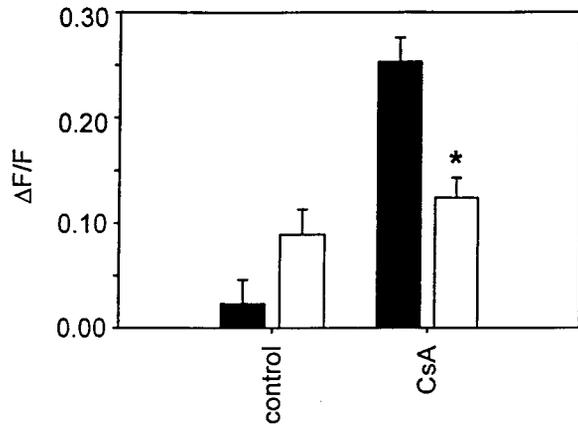


Fig. 3. Comparison between the effect of CsA on the mitochondrial  $\Delta\Psi$  imaged within Bcl-2<sup>-</sup> and Bcl-2<sup>+</sup> PC12 cells. Fluorescence data were obtained from images acquired with the CCD camera as described in Fig. 1. Fluorescence intensities (arbitrary units) within each cell were extracted and normalized for comparison (see MATERIALS AND METHODS). PC12 Bcl-2<sup>-</sup> (solid bars) and Bcl-2<sup>+</sup> cells (open bars) were stained with TMRE, and fluorescence was measured over 20 min in the absence (control) and presence of exposure to 1  $\mu\text{M}$  CsA for 15 min. Values are means  $\pm$  SE for 74–116 cells from 3 different experiments. \*Difference between Bcl-2<sup>-</sup> and Bcl-2<sup>+</sup> is significant at  $P < 0.05$ .

elevate the resting mitochondrial  $\Delta\Psi$  within intact, unstressed cells, TMRE fluorescence measurements were also performed with transfected PC12 cells that overexpress the human *bcl-2* gene. Quantification of changes in fluorescence over a 20-min period during which data were obtained is provided in Fig. 3. In the absence of CsA, TMRE fluorescence for both Bcl-2<sup>-</sup> and Bcl-2<sup>+</sup> cells increased slightly, with a trend toward a greater increase in the Bcl-2<sup>+</sup> cells (Fig. 3). The fluorescence increase that occurred in the presence of 1  $\mu\text{M}$  CsA was significantly greater for Bcl-2<sup>-</sup> than for Bcl-2<sup>+</sup> cells ( $P < 0.05$ ). By analyzing the changes in fluorescence of individual cells with time, we also found that CsA evoked an increase in  $\Delta\Psi$  for  $82.7 \pm 6.0\%$  of the Bcl-2<sup>-</sup> cells vs.  $62.4 \pm 4.2\%$  of the Bcl-2<sup>+</sup> cells ( $n = 3$ ;  $P < 0.05$ ).

Results obtained with fluorescence microscopy and cells grown on coverslips were expanded upon using spectrofluorometric measurements of PC12 cells suspended in medium containing TMRE (Fig. 4A). These

measurements of  $\Delta\Psi$  are expressed as the difference in TMRE fluorescence measured before and after the addition of the uncoupler FCCP to quantify the response to PTP inhibitors and to exclude potential artifacts. We observed that the difference in TMRE fluorescence in the Bcl-2<sup>-</sup> PC12 cells was significantly increased ( $P < 0.05$ ) by a 20-min exposure to either CsA or BAPTA-AM. The concomitant presence of both inhibitors did not present an additive effect. Also, FK-506, an immunosuppressant similar to CsA, which also inhibits protein phosphatase activity but does not inhibit the PTP (15), did not significantly alter the FCCP-sensitive fluorescence observed under these conditions.

TMRE, as well as other cell-permeant fluorescent probes of  $\Delta\Psi$ , can under some conditions significantly alter mitochondrial function (41). Indeed, it has been demonstrated that TMRE-loaded mitochondria undergo PTP opening in a manner dependent on the concentration of the probe and light exposure, due to photodynamically induced free radical generation by TMRE (18). To minimize these possible effects of TMRE, a relatively very low concentration of dye was used (50 nM), and light exposure was kept to a minimum (see MATERIALS AND METHODS). Further validation of our measurements employed the use of an alternative, nonfluorometric method for monitoring  $\Delta\Psi$ . In Fig. 4B, mitochondrial  $\Delta\Psi$  in intact PC12 Bcl-2<sup>-</sup> cells was monitored by continuously measuring the extracellular concentration of TPP<sup>+</sup> (see MATERIALS AND METHODS). TPP<sup>+</sup> is a lipophilic cation that is actively accumulated into mitochondria due to the high inside-negative potential ( $>180$  mV) that exists across the mitochondrial inner membrane. Consistent with the results observed using TMRE (Fig. 4A), we observed that the cellular TPP<sup>+</sup> uptake was greater in the presence of CsA or BAPTA-AM (Fig. 4B). Furthermore, preincubation of the cells for 40 min in media containing 5 mM EGTA to reduce intracellular Ca<sup>2+</sup> content resulted in a TPP<sup>+</sup> uptake similar to that observed in the presence of CsA or BAPTA-AM (not shown). As with the TMRE measurements, FK-506 did not present a significant effect on the  $\Delta\Psi$  measured by TPP<sup>+</sup> uptake. These results are fully consistent with the results obtained with the fluorescent  $\Delta\Psi$  probe TMRE and

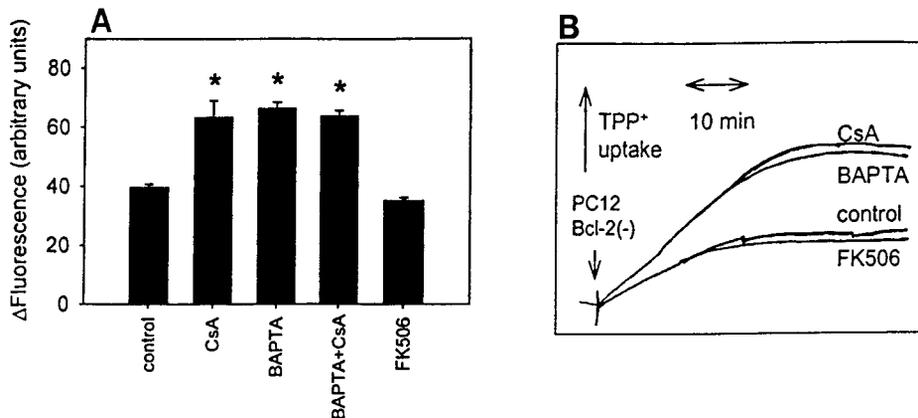
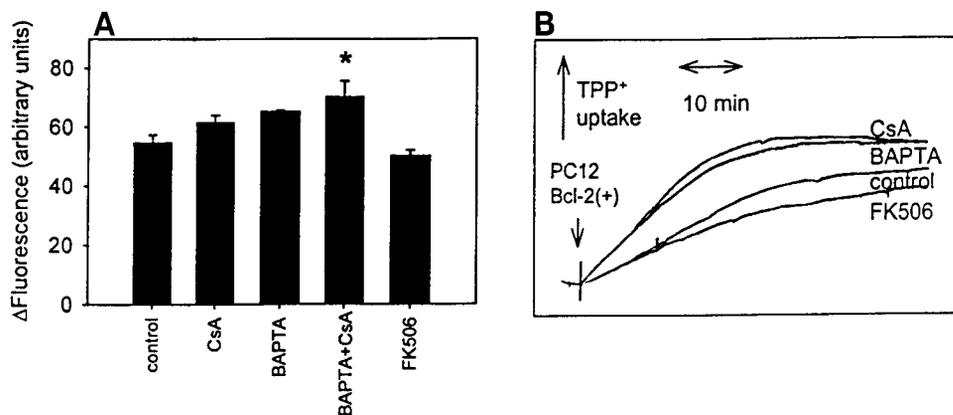


Fig. 4. Fluorescence and tetraphenylphosphonium (TPP<sup>+</sup>) electrode measurements of mitochondrial  $\Delta\Psi$  in suspended PC12 Bcl-2<sup>-</sup> cells. A: suspended cells ( $2 \times 10^7$  cells/ml) were incubated in standard media containing 50 nM TMRE. After 5 min, 1  $\mu\text{M}$  CsA, 10  $\mu\text{M}$  BAPTA-AM, or 1  $\mu\text{M}$  FK-506 was added, as indicated. After 20 min, TMRE fluorescence was quantified as described in MATERIALS AND METHODS. B: suspended cells ( $4 \times 10^7$  cells/ml) were incubated in standard media containing 0.5  $\mu\text{M}$  TPP<sup>+</sup> and 1  $\mu\text{M}$  CsA, 10  $\mu\text{M}$  BAPTA-AM, or 1  $\mu\text{M}$  FK-506, as indicated. TPP<sup>+</sup> uptake was monitored continuously with a TPP<sup>+</sup> electrode, as described in MATERIALS AND METHODS. \* $P < 0.05$ .

Fig. 5. Fluorescence and TPP<sup>+</sup> electrode measurements of mitochondrial  $\Delta\Psi$  in suspended PC12 Bcl-2<sup>+</sup> cells. **A:** cells ( $2 \times 10^7$  cells/ml) were incubated in standard media containing 50 nM TMRE. After 5 min, 1  $\mu$ M CsA, 10  $\mu$ M BAPTA-AM, or 1  $\mu$ M FK-506 was added, as indicated. After 20 min, TMRE fluorescence was quantified as described in MATERIALS AND METHODS. **B:** cells ( $4 \times 10^7$  cells/ml) were incubated in standard media containing 0.5  $\mu$ M TPP<sup>+</sup> and 1  $\mu$ M CsA, 10  $\mu$ M BAPTA-AM, or 1  $\mu$ M FK-506, as indicated. TPP<sup>+</sup> uptake was monitored continuously as described in MATERIALS AND METHODS. \* $P < 0.05$ .



support the conclusion that PTP opening lowers the resting  $\Delta\Psi$  in this cell line.

Using TMRE fluorescence measurements with cells in suspension, it was also possible to verify that the resting  $\Delta\Psi$  of PC12 Bcl-2<sup>+</sup> cells was significantly higher ( $P < 0.05$ ) than that of the control cells (compare Figs. 5A and 4A). We have previously established that *bcl-2* overexpression in transformed neural cells does not affect the content of mitochondria present within the cells, as indicated by measurements of mitochondrial O<sub>2</sub> consumption and DNA levels (33, 34). Therefore, the present results expand upon previous reports that Bcl-2 increases the  $\Delta\Psi$  of isolated mitochondria (42) and mitochondria present within permeabilized cells (28) to indicate that the same effect occurs within unstressed, intact cells. Neither CsA or BAPTA-AM added alone had any significant effect on the TMRE fluorescence of Bcl-2-overexpressing PC12 cells. Although the combined presence of these agents did result in a significant increase in  $\Delta F$ , this increase (<30%) was substantially lower than that observed with Bcl-2<sup>-</sup> cells (>60%). This finding suggests that the resting PTP activity in Bcl-2<sup>+</sup> cells is lower than in control cells. Indeed, because the final levels of  $\Delta\Psi$  after the addition of CsA or BAPTA to Bcl-2<sup>-</sup> vs. Bcl-2<sup>+</sup>

cells were not statistically different, our results suggest that the difference in resting  $\Delta\Psi$  of Bcl-2<sup>-</sup> and Bcl-2<sup>+</sup> cells is due to inhibition of the resting PTP activity by Bcl-2. Alternative measurements of  $\Delta\Psi$  by monitoring the cellular accumulation of TPP<sup>+</sup> in the suspending medium were qualitatively similar to the results obtained from fluorescent TMRE measurements (Fig. 5B). Although either CsA or BAPTA-AM slightly increased TPP<sup>+</sup> uptake, their effects on Bcl-2<sup>+</sup> cells appeared less than those observed with Bcl-2<sup>-</sup> cells (compare with Fig. 4B). FK-506 exhibited a slight depression of TPP<sup>+</sup> uptake, which is also consistent with the effects it had on TMRE-based measurements of  $\Delta\Psi$ .

In addition to the difference in the extent of the contribution of PTP opening to the resting  $\Delta\Psi$  of Bcl-2<sup>-</sup> and Bcl-2<sup>+</sup> cells, we also detected variability in the apparent endogenous PTP activity among different cell lines. Figure 6A describes TMRE fluorescence responses obtained with suspensions of GT1-7 cells, a hypothalamic transformed neural cell line. Unlike that of PC12 cells, the TMRE fluorescence of GT1-7 cells was not significantly affected by the intracellular Ca<sup>2+</sup> chelator BAPTA-AM or the PTP inhibitor CsA, even when the concentration of CsA was increased to 5  $\mu$ M.

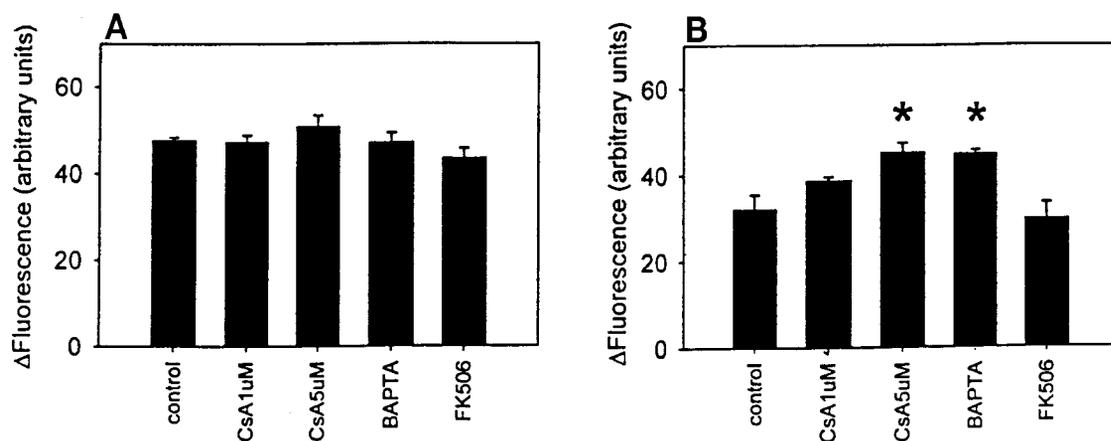


Fig. 6. Fluorescence measurements of mitochondrial  $\Delta\Psi$  in suspended GT1-7 cells. **A:** cells ( $2 \times 10^7$  cells/ml) were incubated in standard media containing 50 nM TMRE. After 5 min, 1  $\mu$ M CsA, 10  $\mu$ M BAPTA-AM, or 1  $\mu$ M FK-506 was added, as indicated. **B:** at 10 min, 200  $\mu$ M *tert*-butyl hydroperoxide (*t*-BOOH) was added to all samples, and TMRE fluorescence was quantified at 20 min, as described in MATERIALS AND METHODS. \* $P < 0.05$ .

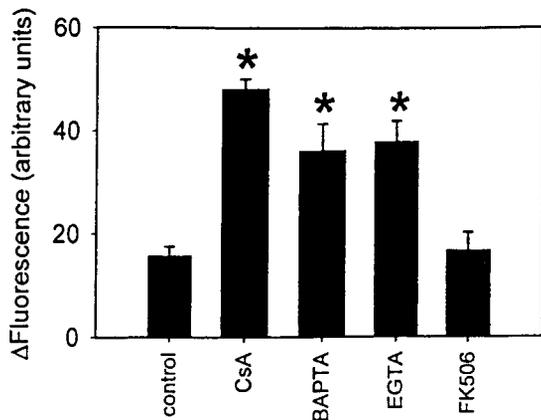


Fig. 7. Fluorescence measurements of mitochondrial  $\Delta\Psi$  in suspended SY5Y cells. Cells ( $2 \times 10^7$  cells/ml) were incubated in standard media containing 50 nM TMRE. After 5 min, 1  $\mu$ M CsA, 10  $\mu$ M BAPTA-AM, or 1  $\mu$ M FK-506 was added, as indicated. After 20 min, TMRE fluorescence was quantified as described in MATERIALS AND METHODS. EGTA-treated cells were preincubated for 40 min in reaction media containing 5 mM EGTA, before the addition of 50 nM TMRE. \* $P < 0.05$ .

The  $\Delta\Psi$  of unstressed GT1-7 cells was also not affected by exposure to FK-506. However, if these cells were treated with *t*-BOOH, a compound capable of enhancing  $\text{Ca}^{2+}$ -induced PTP in both isolated mitochondria and cells by oxidizing mitochondrial pyridine nucleotides (4, 6, 14, 21, 28, 36), then an average value for  $\Delta F$  was obtained ( $32 \pm 4$ ; Fig. 6B) that is significantly lower than that obtained in the absence of *t*-BOOH ( $47 \pm 1$ ; Fig. 6A). The fluorescence values obtained in the presence of *t*-BOOH were significantly increased by exposure of cells to CsA (5  $\mu$ M) or BAPTA but not FK-506. Thus GT1-7 cells do not present a detectable resting PTP activity but do exhibit a drop in  $\Delta\Psi$  consistent with PTP activity when exposed to the oxidant *t*-BOOH.

In contrast to the insensitivity of GT1-7 cells to alterations in resting  $\Delta\Psi$  caused by CsA or BAPTA-AM, but in agreement with the sensitivity of PC12 cells, the resting  $\Delta\Psi$  of human SY5Y neuroblastoma cells was significantly elevated by the addition of the MPT inhibitor CsA or by exposure to the intracellular  $\text{Ca}^{2+}$  chelator BAPTA-AM (Fig. 7). In addition, we found that exposure of these cells to the extracellular  $\text{Ca}^{2+}$  chelator EGTA for 40 min resulted in an increase in TMRE fluorescence. Thus, with SY5Y cells, we observed an increase in the normal  $\Delta\Psi$  after treatment with three different conditions that can inhibit PTP activity by two different mechanisms. As with the other cell lines, the  $\Delta\Psi$  of SY5Y cells was not elevated by exposure to FK-506.

## DISCUSSION

Taken together, our results strongly suggest that the PTP is active and contributes toward a decrease in mitochondrial  $\Delta\Psi$  in resting PC12 and SY5Y cells but not in unstressed GT1-7 cells. We have determined that the PTP influences the resting  $\Delta\Psi$  in PC12 and SY5Y cells by demonstrating that  $\Delta\Psi$  can be elevated

by the presence of CsA or  $\text{Ca}^{2+}$  chelators but not by the immune suppressor FK-506, which does not inhibit PTP activity in isolated mitochondria (15). In addition, fluorescence microscopy measurements on individual cells indicate that the increase in  $\Delta\Psi$  induced by CsA or BAPTA-AM in PC12 cells is a common phenomenon and occurs in the majority of cells that are analyzed. This finding argues against the possibility that the observed responses are due to a fraction of cells that are undergoing PTP as part of a cell death process. The GT1-7 cell line that did not exhibit sensitivity of resting  $\Delta\Psi$  to PTP inhibitors nevertheless did demonstrate a CsA- and BAPTA-AM-sensitive fraction of  $\Delta\Psi$  in the presence of the PTP inducer *t*-BOOH. The finding that *t*-BOOH does not completely eliminate the  $\Delta\Psi$  of GT1-7 cells, as it does in hepatocytes (4, 21, 36), may relate to the reason why GT1-7 cells do not express a detectable endogenous PTP activity. For example, variability of endogenous and induced PTP activity could be due to variability in cellular redox state or sensitivity of mitochondrial pyridine nucleotides to oxidation (14).

The PTP has been studied most extensively with isolated mitochondria with the use of conditions in vitro that bear little resemblance to those that exist within intact cells. Within the last few years, however, evidence obtained with models of cell and tissue injury has supported the involvement of the PTP in necrotic and apoptotic cell death (8, 17, 29, 30). Moreover, it has been proposed that a  $\text{Ca}^{2+}$ - and proton-selective, low-conductivity state of the PTP may be active in normal Ehrlich tumor cells, generating and conveying electrical and  $\text{Ca}^{2+}$  signals (19). In the study reported by Ichas et al. (19), the PTP is activated upon  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  mobilization from the endoplasmic reticulum, whereupon PTP-mediated mitochondrial  $\text{Ca}^{2+}$  efflux contributes to the amplification of cytosolic  $\text{Ca}^{2+}$  signals. These findings are supported by studies showing that binding of  $\text{IP}_3$  to its receptors results in discrete areas of elevated intracellular  $\text{Ca}^{2+}$  that are sensed by neighboring mitochondria (39). The ensuing increase in intramitochondrial  $\text{Ca}^{2+}$  can lead to an activation of mitochondrial dehydrogenases and, therefore, ATP production (40). However, the rapid stimulation of mitochondrial  $\text{Ca}^{2+}$  uptake by focal spikes in extramitochondrial  $\text{Ca}^{2+}$  concentrations could result in transient reductions in mitochondrial  $\Delta\Psi$  (9, 47), which would promote activation of the PTP. Opening of the PTP would be expected to prolong the period of mitochondrial depolarization and induce the release of at least some fraction of the accumulated  $\text{Ca}^{2+}$ . Additional support for this scenario comes from observations that CsA increases mitochondrial  $\text{Ca}^{2+}$  accumulation in normal cardiomyocytes (1) and decreases  $\text{Ca}^{2+}$ -induced mitochondrial  $\text{Ca}^{2+}$  release in Ehrlich ascites tumor cells and endothelial cells (10, 50). In addition, blockade of MPT inhibits agonist-evoked  $\text{Ca}^{2+}$  oscillations in glial cells, reinforcing the hypothesis that, during physiological stimulation, transient PTP openings support  $\text{Ca}^{2+}$  signaling (46). Further evidence for the baseline activity of a low-conductance state of the PTP has come from  $\text{TPP}^+$  uptake measurements (5), fluorescent

flow cytometry and microscopic imaging of  $\Delta\Psi$  in SY5Y neuroblastoma cells (11), and rat oligodendrocyte progenitors (46).

The activity of the MPT under physiological conditions would qualify it as an endogenous uncoupler of oxidative phosphorylation. However, the degree of uncoupling and energy expenditure by the movement of ions through the PTP would, by necessity, need to be very limited so that metabolic homeostasis could be preserved. Like the activity of well-characterized tissue-specific uncoupling proteins (22), the resting state PTP activity may act similarly to increase energy consumption without obstructing ATP synthesis. Studies are in progress to determine the extent to which the endogenous PTP contributes to the basal rate of respiration by the PC12 and SY5Y cells used in our experiments. Increased energy utilization associated with the cycling of protons,  $\text{Ca}^{2+}$ , and other ions mediated by PTP activity should be manifested as heat generation. Indeed, CsA has been demonstrated to decrease the heat output of normal lymphocytes (25). In addition to the influence PTP activity can exert on intracellular  $\text{Ca}^{2+}$  signaling, oxygen utilization, and heat production, the associated reduction in  $\Delta\Psi$  would reduce the formation of superoxide due to "leakage" of electrons from the ubiquinone region of the electron transport (45). It is therefore possible that a controlled endogenous PTP activity could actually protect mitochondria against self-inflicted oxidative stress (45).

Bcl-2 overexpression has previously been shown to increase  $\Delta\Psi$  in isolated mitochondria (42) and permeabilized cells (28). Our findings further indicate that Bcl-2 can elevate the resting  $\Delta\Psi$  of intact, unstressed cells by inhibiting the endogenous activity of the PTP, as previously suggested by JC-1 fluorescence probe measurements of  $\Delta\Psi$  in another strain of PC12 cells (7). It is possible that the differences in uncoupler-sensitive TMRE fluorescence and  $\text{TPP}^+$  uptake between Bcl-2<sup>+</sup> and Bcl-2<sup>-</sup> cells are due to differences in mitochondrial volume, even though maximal rates of respiration and mitochondrial DNA contents are equivalent. However, the observation that overexpression of Bcl-2 minimizes the effects of PTP inhibitors on resting  $\Delta\Psi$  constitutes evidence that Bcl-2 actually increases  $\Delta\Psi$  possibly via inhibition of endogenous PTP activity. Bcl-2 has been proposed to act as a  $\text{H}^+$  channel that contributes to rather than detracts from the mitochondrial electrochemical gradient of protons (42). However, considering the known ability of Bcl-2 to inhibit the stress-evoked PTP opening in isolated mitochondria and permeabilized cells (28, 32, 42, 48) and the ability of Bcl-2 to inhibit endogenous PTP activity in our experiments, its endowment for MPT inhibition may be its primary mechanism of action. Although the physiological role for Bcl-2 is generally thought to be one of protection against cytotoxicity (24, 26, 32, 34, 35, 48, 52), the present results suggest that Bcl-2 may also serve as an enhancer of the efficiency of mitochondrial energy coupling by decreasing the endogenous PTP activity.

We thank S. J. Russell for the excellent technical assistance.

Transfected cells were kindly provided by Dr. Dale Bredesen (Burnham Research Institute, La Jolla, CA), and FK-506 was provided by Fujisawa, Japan.

This work was supported by the FAPESP, by National Institutes of Health Grant NS-34152, and by the Bayer Corporation.

## REFERENCES

1. Altschuld RA, Hohl CM, Castillo LC, Garleb AA, Starling RC, and Brierley GP. Cyclosporin inhibits mitochondrial calcium efflux in isolated adult rat ventricular cardiomyocytes. *Am J Physiol Heart Circ Physiol* 262: H1699–H1704, 1992.
2. Andreyev A and Fiskum G. Calcium induced release of mitochondrial cytochrome c by different mechanisms selective for brain versus liver. *Cell Death Differ* 6: 825–832, 1999.
3. Baumgold J, Paek R, and Fiskum G. Calcium independence of phosphoinositide hydrolysis-induced increase in cyclic AMP accumulation in SK-N-SH human neuroblastoma cells. *J Neurochem* 58: 1754–1759, 1992.
4. Byrne AM, Lemasters JJ, and Nieminen AL. Contribution of increased mitochondrial free  $\text{Ca}^{2+}$  to the mitochondrial permeability transition induced by *tert*-butylhydroperoxide in rat hepatocytes. *Hepatology* 29: 1523–1531, 1999.
5. Cassarino DS, Swerdlow RH, Parks JK, Parker WDJ, and Bennett JPJ. Cyclosporin A increases resting mitochondrial membrane potential in SY5Y cells and reverses the depressed mitochondrial membrane potential of Alzheimer's disease cybrids. *Biochem Biophys Res Commun* 248: 168–173, 1998.
6. Castilho RF, Kowaltowski AJ, Meinicke AR, Bechara EJ, and Vercesi AE. Permeabilization of the inner mitochondrial membrane by  $\text{Ca}^{2+}$  ions is stimulated by *t*-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria. *Free Radic Biol Med* 18: 479–486, 1995.
7. Dispersyn G, Nuydens R, Connors R, Borgers M, and Geerts H. Bcl-2 protects against FCCP-induced apoptosis and mitochondrial membrane potential depolarization in PC12 cells. *Biochim Biophys Acta* 1428: 357–371, 1999.
8. Dubinsky JM and Levi Y. Calcium-induced activation of the mitochondrial permeability transition in hippocampal neurons. *J Neurosci Res* 53: 728–741, 1998.
9. Duchen MR, Leyssens A, and Crompton M. Transient mitochondrial depolarizations reflect focal sarcoplasmic reticular calcium release in single rat cardiomyocytes. *J Cell Biol* 142: 975–988, 1998.
10. Evtodienko Y, Teplova V, Khawaja J, and Saris NE. The  $\text{Ca}^{2+}$ -induced permeability transition pore is involved in  $\text{Ca}^{2+}$ -induced mitochondrial oscillations. A study on permeabilised Ehrlich ascites tumour cells. *Cell Calcium* 15: 143–152, 1994.
11. Fall CP and Bennett JPJ. Visualization of cyclosporin A and  $\text{Ca}^{2+}$ -sensitive cyclical mitochondrial depolarizations in cell culture. *Biochim Biophys Acta* 1410: 77–84, 1999.
12. Farkas DL, Wei MD, Febrriello P, Carson JH, and Loew LM. Simultaneous imaging of cell and mitochondrial membrane potentials. *Biophys J* 56: 1053–1069, 1989. [published erratum appears in *Biophys J* 1990 Mar 57(3): following p. 684]
13. Fiskum G and Lehninger AL. Regulated release of  $\text{Ca}^{2+}$  from respiring mitochondria by  $\text{Ca}^{2+}/2\text{H}^+$  antiport. *J Biol Chem* 254: 6236–6239, 1979.
14. Fiskum G and Pease A. Hydroperoxide-stimulated release of calcium from rat liver and AS-30D hepatoma mitochondria. *Cancer Res* 46: 3459–3463, 1986.
15. Friberg H, Ferrand-Drake M, Bengtsson F, Halestrap AP, and Wieloch T. Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. *J Neurosci* 18: 5151–5159, 1998.
16. Hajnoczky G, Robb-Gaspers LD, Seitz MB, and Thomas AP. Decoding of cytosolic calcium oscillations in the mitochondria. *Cell* 82: 415–424, 1995.
17. Halestrap AP, Kerr PM, Javadov S, and Woodfield KY. Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochim Biophys Acta* 1366: 79–94, 1998.

18. **Huser J, Rechenmacher CE, and Blatter LA.** Imaging the permeability pore transition in single mitochondria. *Biophys J* 74: 2129–2137, 1998.
19. **Ichas F, Jouaville LS, and Mazat JP.** Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. *Cell* 89: 1145–1153, 1997.
20. **Ichas F and Mazat JP.** From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. *Biochim Biophys Acta* 1366: 33–50, 1998.
21. **Imberti R, Nieminen AL, Herman B, and Lemasters JJ.** Mitochondrial and glycolytic dysfunction in lethal injury to hepatocytes by *t*-butylhydroperoxide: protection by fructose, cyclosporin A and trifluoperazine. *J Pharmacol Exp Ther* 265: 392–400, 1993.
22. **Jezeq P, Engstova H, Zackova M, Vercesi AE, Costa AD, Arruda P, and Garlid KD.** Fatty acid cycling mechanism and mitochondrial uncoupling proteins. *Biochim Biophys Acta* 1365: 319–327, 1998.
23. **Kamo N, Muratsugu M, Hongoh R, and Kobatake Y.** Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J Membr Biol* 49: 105–121, 1979.
24. **Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, and Bredesen DE.** Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* 262: 1274–1277, 1993.
25. **Karlsson H, DePierre JW, and Nassberger L.** Energy levels in resting and mitogen-stimulated human lymphocytes during treatment with FK506 or cyclosporin A in vitro. *Biochim Biophys Acta* 1319: 301–310, 1997.
26. **Kluck RM, Bossy-Wetzell E, Green DR, and Newmeyer DD.** The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275: 1132–1136, 1997.
27. **Kowaltowski AJ and Vercesi AE.** Mitochondrial damage induced by conditions of oxidative stress. *Free Radic Biol Med* 26: 463–471, 1999.
28. **Kowaltowski AJ, Vercesi AE, and Fiskum G.** Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides. *Cell Death Different.* In press.
29. **Kroemer G, Dallaporta B, and Resche-Rigon M.** The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* 60:619–42: 619–642, 1998.
30. **Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, and Herman B.** The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta* 1366: 177–196, 1998.
31. **Loew LM, Carrington W, Tuft RA, and Fay FS.** Physiological cytosolic Ca<sup>2+</sup> transients evoke concurrent mitochondrial depolarizations. *Proc Natl Acad Sci USA* 91: 12579–12583, 1994.
32. **Marzo I, Brenner C, Zamzami N, Susin SA, Beutner G, Brdiczka D, Remy R, Xie ZH, Reed JC, and Kroemer G.** The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins. *J Exp Med* 187: 1261–1271, 1998.
33. **Murphy AN, Bredesen DE, Cortopassi G, Wang E, and Fiskum G.** Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. *Proc Natl Acad Sci USA* 93: 9893–9898, 1996.
34. **Myers KM, Fiskum G, Liu Y, Simmens SJ, Bredesen DE, and Murphy AN.** Bcl-2 protects neural cells from cyanide/anglycemia-induced lipid oxidation, mitochondrial injury, and loss of viability. *J Neurochem* 65: 2432–2440, 1995.
35. **Newmeyer DD and Green DR.** Surviving the cytochrome seas. *Neuron* 21: 653–655, 1998.
36. **Nieminen AL, Byrne AM, Herman B, and Lemasters JJ.** Mitochondrial permeability transition in hepatocytes induced by *t*-BuOOH: NAD(P)H and reactive oxygen species. *Am J Physiol Cell Physiol* 272: C1286–C1294, 1997.
37. **Novgorodov SA and Gudz TI.** Permeability transition pore of the inner mitochondrial membrane can operate in two open states with different selectivities. *J Bioenerg Biomembr* 28: 139–146, 1996.
38. **Petronilli V, Miotto G, Canton M, Brini M, Colonna R, Bernardi P, and Di Lisa F.** Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys J* 76: 725–734, 1999.
39. **Rizzuto R, Brini M, Murgia M, and Pozzan T.** Microdomains with high Ca<sup>2+</sup> close to IP<sub>3</sub>-sensitive channels that are sensed by neighboring mitochondria. *Science* 262: 744–747, 1993.
40. **Robb-Gaspers LD, Burnett P, Rutter GA, Denton RM, Rizzuto R, and Thomas AP.** Integrating cytosolic calcium signals into mitochondrial metabolic responses. *EMBO J* 17: 4987–5000, 1998.
41. **Scaduto RCJ and Grotjohann LW.** Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys J* 76: 469–477, 1999.
42. **Shimizu S, Eguchi Y, Kamiike W, Funahashi Y, Mignon A, Lacronique V, Matsuda H, and Tsujimoto Y.** Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. *Proc Natl Acad Sci USA* 95: 1455–1459, 1998.
43. **Simpson PB and Russell JT.** Mitochondria support inositol 1,4,5-trisphosphate-mediated Ca<sup>2+</sup> waves in cultured oligodendrocytes. *J Biol Chem* 271: 33493–33501, 1996.
44. **Simpson PB and Russell JT.** Role of mitochondrial Ca<sup>2+</sup> regulation in neuronal and glial cell signalling. *Brain Res Brain Res Rev* 26: 72–81, 1998.
45. **Skulachev VP.** Membrane-linked systems preventing superoxide formation. *Biosci Rep* 17: 347–366, 1997.
46. **Smaili SS and Russell JT.** Permeability transition pore regulates both mitochondrial membrane potential and agonist-evoked Ca<sup>2+</sup> signals in oligodendrocyte progenitors. *Cell Calcium* 26: 121–130, 1999.
47. **Sparagna GC, Gunter KK, Sheu SS, and Gunter TE.** Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. *J Biol Chem* 270: 27510–27515, 1995.
48. **Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M, and Kroemer G.** Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med* 184: 1331–1341, 1996.
49. **Vercesi AE.** Stimulation of mitochondrial Ca<sup>2+</sup> efflux by NADP<sup>+</sup> with maintenance of respiratory control. *An Acad Bras Cienc* 57: 369–375, 1985.
50. **Wood PG and Gillespie JL.** Evidence for mitochondrial Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release in permeabilised endothelial cells. *Biochem Biophys Res Commun* 246: 543–548, 1998.
51. **Yagodin SV, Holtzclaw L, Sheppard CA, and Russell JT.** Nonlinear propagation of agonist-induced cytoplasmic calcium waves in single astrocytes. *J Neurobiol* 25: 265–280, 1994.
52. **Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, and Wang X.** Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 275: 1129–1132, 1997.
53. **Zoratti M and Szabo I.** The mitochondrial permeability transition. *Biochim Biophys Acta* 1241: 139–176, 1995.