Letters to the Editor

Negative Contrast Imaging of Mitochondria by Confocal Microscopy

The paper by Petronilli et al. in the February issue of the *Biophysical Journal* examined the distribution of calcein fluorescence in hepatocytes and hepatoma cells by confocal microscopy (Petronilli et al., 1999). Previously, we showed that warm incubation with calcein acetoxymethyl (AM) ester led to almost exclusive cytosolic distribution of calcein in both rat hepatocytes and rabbit cardiac myocytes (Nieminen et al., 1995; Ohata et al., 1998). Mitochondria excluded calcein and were visualized by confocal microscopy as round and oval voids about a micron in diameter in images of green calcein fluorescence. The identity of the dark voids as mitochondria was confirmed by colocalization with the red-fluorescing electrical potential-indicating dye, tetramethyrhodamine methyl ester (TMRM), which strongly labels mitochondria. A similar cytosolic distribution after warm ester loading occurred for the Ca^{2+}-indicating fluorophore, Fluo-3, in myocytes (Ohata et al., 1998) and for another Ca^{2+}-indicating fluorophore, Fura-2, and the pH-indicating fluorophore BCECF in hepatocytes (Nieminen et al., 1988; Gores et al., 1989). In contrast, after ester loading at 4°C, calcein and Fluo-3 entered both the cytosol and the mitochondria, and mitochondrial calcein voids were absent (Nieminen et al., 1995; Ohata et al., 1998).

In their paper, Petronilli et al. inferred that the voids we observed in images of calcein fluorescence were caused by quenching by TMRM. However, we observed an identical pattern of cytosolic calcein fluorescence in the absence of TMRM (Lemasters et al., 1998; Byrne et al., 1999). Subsequent TMRM addition did not alter the calcein image or enhance the mitochondrial voids (Lemasters et al., 1998). Moreover, after depolarization, mitochondrial voids did not disappear, provided that onset of the mitochondrial permeability transition (MPT) was prevented, although virtually all TMRM fluorescence was lost (Trost and Lemasters, 1997; Qian et al., 1997, 1999). Similarly, when mitochondria repolarized, TMRM was taken up into mitochondria without alteration of the mitochondrial voids (Qian et al., 1997). In contrast, when mitochondrial permeability to calcein increased at the onset of the MPT, the voids quickly filled with calcein fluorescence (Nieminen, 1995; Lemasters et al., 1998; Byrne et al., 1999; Trost and Lemasters, 1997; Qian et al., 1997, 1999).

Using conventional spectrofluorometry, Petronilli et al. showed that 50 μM TMRM strongly suppressed fluorescence from a 10-mm cuvette containing 50 μM calcein. This observation is a classic example of the inner filtereffect. In a spectrofluorometer, the excitation beam passes through the center of the cuvette, and fluorescence is measured at a 90° angle to the beam. Thus the average path length of emitted fluorescence out of the cuvette is $\sim$5 mm. TMRM has an extinction coefficient of 110 cm^{-1} mM^{-1} at its absorbance maximum of 554 nm. At 517 nm, the peak emission wavelength for calcein, TMRM absorbance is about half its peak value. At this wavelength, TMRM transmits only 4% of light through a 5-mm path length. The emission spectrum of calcein is asymmetrical with broadening to the red, and light absorbance by TMRM at longer wavelengths will be even greater. Moreover, light absorbed by TMRM will be reemitted as red fluorescence. Decreased green fluorescence and enhanced red fluorescence were exactly what Petronilli et al. observed when TMRM was added to calcein in a cuvette.

In confocal microscopy, the average path length of calcein fluorescence out of a TMRM-containing mitochondrion is not 5 mm but 0.5 μm (half the diameter of a mitochondrion). Indeed, the typical path length out of the entire cell is only $\sim$5 μm (distance from the confocal image plane to the coverslip surface). With such short path lengths, the inner filter effect becomes negligible. If one assumes that TMRM concentration rises to 1 mM in mitochondria and 0.25 mM for the cell as a whole, then only 0.6–1.6% of calcein fluorescence is absorbed by TMRM. Thus, although colorimetric quenching of calcein fluorescence by TMRM can be substantial in a cuvette, it becomes trivial at the microscopic scale.

Several factors may explain the failure of Petronilli et al. to observe dark mitochondrial voids after ester loading of calcein-AM. Ester loading is very temperature dependent. Loading at cool or ambient temperatures can cause mitochondrial loading and loss of voids (Nieminen, 1995; Ohata et al., 1998). Ester loading is also quite cell type specific. In fresh primary cultures of hepato-

Received for publication 29 April 1999 and in final form 10 June 1999.

0006-3495/99/09/1747/02 $2.00

cytes and myocytes, cytosolic esterase activity is apparently so high at 37°C that the neutral AM ester of calcein cannot cross into mitochondria without first being hydrolyzed in the cytosol to calcein free acid. At lower temperatures the esterase is inhibited sufficiently to allow neutral esters to cross the cytosol and enter mitochondria. However, in other cell types, such as transformed cell lines and 2–3-day cultured hepatocytes that have dedifferentiated in culture, cytosolic esterases may not be active enough to prevent calcein AM entry into mitochondria.

Cell lines also typically have mitochondria that are smaller than the mitochondria of hepatocytes and myocytes. Such mitochondria have average diameters of 0.3 μm and are impossible to visualize by negative contrast in confocal image slices, the *z* axis dimension of which is 0.7–0.8 μm at best. Indeed, negative imaging of heart and liver mitochondria with a diameter of 1 μm challenges the *z* axis resolving power of confocal microscopy. Thus the optical alignment of the confocal microscope must be virtually perfect to visualize the dark mitochondrial voids.

The technique introduced by Petronilli et al. of quenching cytosolic calcein fluorescence with cobalt is an important advance in visualizing small mitochondria. Loss of mitochondrial fluorescence after cobalt quenching may be used to monitor opening of the permeability transition pore. An alternative approach to labeling mitochondria selectively is to ester-load both the cytosol and the mitochondria at a low temperature. Subsequently, the cells are returned to the incubator. Over the next few hours, cytosolic fluorophores, such as calcein and Rhod-2, leak from the plasma membrane through an organic anion transporter. Mitochondrial fluorophore is retained, but cytosolic fluorophore is lost almost completely (Trollinger et al., 1997; Lemasters et al., 1999). The advantage of the cold loading/warm incubation technique is avoidance of Ca^{2+} channel blockade, dehydrogenase inhibition, genotoxicity, and other adverse effects of cobalt (Hughes and Barritt, 1989; Beyersmann and Hartwig, 1992; Seghizzi et al., 1994).

REFERENCES

Beyersmann, D., and A. Hartwig. 1992. The genetic toxicology of cobalt. *Toxicol. Appl. Pharmacol.* 115:137–145.

Byrne, A. M., J. J. Lemasters, and A.-L. Nieminen. 1999. Contribution of increased mitochondrial free Ca^{2+} to the mitochondrial permeability transition induced by *tert*-butylhydroperoxide in rat hepatocytes. *Hepatology.* 29:1523–1531.

Gores, G. J., A.-L. Nieminen, B. E. Wray, B. Herman, and J. J. Lemasters. 1989. Intracellular pH during "chemical hypoxia" in cultured rat hepatocytes: protection by intracellular acidosis against the onset of cell death. *J. Clin. Invest.* 83:386–396.

Hughes, B. P., and G. J. Barritt. 1989. Inhibition of the liver cell receptor-activated Ca^{2+} inflow system by metal ion inhibitors of voltage-operated Ca^{2+} channels but not by other inhibitors of Ca^{2+} inflow. *Biochim. Biophys. Acta.* 1013:197–205.

Lemasters, J. J., A.-L. Nieminen, T. Qian, L. C. Trost, S. P. Elmore, Y. Nishimura, R. A. Crowe, W. E. Cascio, C. A. Bradham, D. A. Brenner, and B. Herman. 1998. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim. Biophys. Acta.* 1366:177–196.

Lemasters, J. J., D. R. Trollinger, T. Qian, W. E. Cascio, and H. Ohata. 1999. Confocal imaging of Ca^{2+}, pH, electrical potential and membrane permeability in single living cells. *Methods Enzymol.* 302: 341–358.

Nieminen, A.-L., G. J. Gores, B. E. Wray, Y. Tanaka, B. Herman, and J. J. Lemasters. 1988. Calcium dependence of bleb formation and cell death in hepatocytes. *Cell Calcium.* 9:237–246.

Nieminen, A.-L., A. K. Saylor, S. A. Tesfai, B. Herman, and J. J. Lemasters. 1995. Contribution of the mitochondrial permeability transition to lethal injury after exposure of hepatocytes to *t*-butylhydroperoxide. *Biochem. J.* 307:99–106.

Ohata, H., E. Chacon, S. A. Tesfai, I. S. Harper, B. Herman, and J. J. Lemasters. 1998. Mitochondrial Ca^{2+} transients in cardiac myocytes during the excitation-contraction cycle: effects of pacing and hormonal stimulation. *J. Bioenerg. Biomembr.* 30:207–222.

Petronilli, V., G., Miotto, M. Canton, M. Brini, R. Colonna, P. Bernardi, and F. Di Lisa. 1999. 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.

Qian, T., B. Herman, and J. J. Lemasters. 1999. The mitochondrial permeability transition mediates both necrotic and apoptotic death of hepatocytes exposed to Br-A23187. *Toxicol. Appl. Pharmacol.* 154:117–125.

Qian, T., A.-L. Nieminen, B. Herman, and J. J. Lemasters. 1997. Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. *Am. J. Physiol.* 273:C1783–C1792.

Seghizzi, P., F. D'Adda, D. Borleri, F. Barbic, and G. Mosconi. 1994. Cobalt myocardiopathy. A critical review of literature. *Sci. Total Environ.* 150:105–109.

Trollinger, D. R., W. E. Cascio, and J. J. Lemasters. 1997. Selective loading of Rhod 2 into mitochondria shows mitochondrial Ca^{2+} transients during the contractile cycle in adult rabbit cardiac myocytes. *Biochem. Biophys. Res. Commun.* 236:738–742.

Trost, L. C., and J. J. Lemasters. 1997. Role of the mitochondrial permeability transition in salicylate toxicity to cultured rat hepatocytes: implications for the pathogenesis of Reye's syndrome. *Toxicol. Appl. Pharmacol.* 147:431–441.

John J. Lemasters
Ting Qian

University of North Carolina
Chapel Hill, North Carolina

Anna-Liisa Nieminen

Case Western Reserve University
Cleveland, Ohio