Response to J. J. Lemasters et al.

In the February issue of the *Biophysical Journal* we ported a study on the modes of opening of the mitochondrial permeability transition pore (MTP) in hepatocytes and hepatoma cells (Petronilli et al., 1999). The method that we developed for monitoring MTP in intact cells was based on the decrease of the fluorescence emission of calcein localized in the mitochondrial matrix space. Indeed, we have unequivocally shown that calcein can be visualized inside mitochondria by three techniques: 1) permeabilization of the plasma membrane with digitonin, which revealed fluorescent mitochondria; 2) quenching of mitochondrial calcein fluorescence in intact cells by high concentrations of the potentiometric probe tetramethylrhodamine methyl ester (TMRM), a condition that resulted in the appearance of fluorescence voids corresponding to mitochondria by confocal microscopy; and 3) quenching of cytosolic calcein fluorescence by Co^{2+}, which resulted in the appearance of bright filamentous structures that costained with TMRM (see also Minamikawa et al., 1999).

Our findings differ from those of Lemasters and coworkers, who reported that mitochondria appear as fluorescence "voids" in confocal images of calcein-loaded hepatocytes (Nieminen et al., 1995). The voids were identified as mitochondria by coloading the cells with TMRM. In their letter, Lemasters et al. 1) suggest that the different calcein distribution may be explained by the loading temperature and the type of cells and 2) attribute the decrease of calcein emission induced by TMRM to an inner filter effect. An additional comment of Lemasters et al. is that negative imaging of mitochondria requires virtually perfect optical alignment of the confocal microscope.

1. In our hands, mitochondrial loading with calcein was obtained in all conditions tested, i.e., irrespective of loading temperature and cell type. In addition, since calcein emission is influenced by TMRM (Petronilli et al., 1999; Hüser et al., 1998), we suggested that in double-labeling experiments the occurrence of mitochondrial loading should always be assessed. Even results obtained after labeling with calcein alone must be considered with caution, however, because mitochondrially entrapped calcein can also undergo extensive self-quenching (Petronilli et al., 1998). Under these conditions the apperance of voids is due to an excessive loading of mitochondria with calcein rather than to its absence. In any case, the statement that "neutral AM ester of calcein cannot cross into mitochondria without first being hydrolyzed in the cytosol" should be validated experimentally.

2. Our argument that TMRM causes true quenching of calcein fluorescence is not based solely on the cuvette experiment cited in Lemasters' letter. Indeed, we have observed a decrease of calcein fluorescence after TMRM coloading of mitochondria at the confocal microscope. This finding is entirely consistent with the results of Hüser et al. in individually imaged isolated heart mitochondria, where the fluorescence of intramitochondrial calcein could be reversibly decreased by TMRM uptake (Hüser et al., 1998). These results demonstrate quite clearly that calcein fluorescence is quenched by TMRM, since inner filter effects are of minimal concern in the confocal microscope, as recognized by Lemasters et al. in their letter. Finally, we would like to point out that we do observe fluorescence voids in the calcein channel after TMRM coloading, whereas these voids cannot be detected after microinjection of the acid form of calcein alone (Petronilli et al., 1999). Thus the optical alignment of our confocal microscope appears to be good enough to see mitochondrial fluorescence voids when they are present.

The letter of Lemasters et al. closes with a brief comment on the potential side effects of Co^{2+}. We are aware of the potential hazards posed by this cation, although genotoxicity can hardly be one of them in experiments whose typical time course is in the range of a few minutes. We would like to point out, however, that Co^{2+} is complexed with calcein, so that its free concentration is presumably negligible; and that the lack of effects of Co^{2+} on the mitochondrial membrane potential and cell viability suggest that acute toxicity may not be a problem in these protocols.

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