Biophysical Journal Volume 65 December 1993 2269–2272

New and Notable

Imaging Mitochondrial Membrane Potentials

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While the chemiosmotic theory (Mitchell, 1979) provides a conceptual framework for mitochondrial energy transduction, most of the details of this process remain to be elucidated. The elusiveness of this goal can be attributed, at least in part, to the absence of structural information about the mitochondrion itself at several levels of complexity. It is obvious, for example, that obtaining atomic structures of the respiratory chain protein complexes by x-ray or electron crystallography would greatly aid the interpretation of existing data and spur new biophysical and molecular genetic approaches into oxidative phosphorylation. This process has already begun in the field of photosynthesis with the solution of the crystal structure of bacterial photosynthetic reaction centers (Deisenhofer, 1984). On a different structural level, there is still considerable uncertainty about the characteristics (spatial and temporal) of the gradients and fields that lie at the heart of the chemiosmotic theory. Information is lacking about the internal compartmentation of the mitochondrion, i.e., the three-dimensional shape of the inner membrane, its physical associations with the outer membrane, and the distribution of transport proteins on these membranes. These structural details, which are needed to map the pathways of ion and metabolite diffusion within the mitochondrion, may soon be provided by electron microscopic techniques like tomography (Mannella et al., 1994). However, electron microscopy cannot provide direct information about transmembrane electrical potentials. The article by Loew et al. in this issue of *Biophysical Journal* describes an elegant new technique to get quantitative, time-dependent information about the spatial distribution of mitochondrial potentials at the resolution limit of the light microscope.

The use of fluorescence light microscopy to study mitochondrial potentials began to attract serious attention during the late 1970s. (See review by Chen (1989).) A variety of lipophilic cationic fluorescent dyes were found which are selectively taken up by mitochondria and which are nontoxic, at least with short exposure, to mitochondrial function. The general consensus is that these organic cations accumulate in mitochondria as a function of membrane potential. Thus, not only do the dyes make dramatic vital stains, they can (depending on the choice of dye and the skills of the microscopist) be used to "image" the membrane potentials of individual mitochondria.

Several reports have hinted at the utility of fluorescence microscopy for the spatial and kinetic analysis of mitochondrial membrane potentials. In one of the earlier studies of this type (Siemens et al., 1982), a focussed laser beam was used to excite subregions of rhodamine-loaded mitochondria within myocardial cells. Local variations in the patterns of fluorescence emission (detected with a sensitive photomultiplier tube) could be mapped at a lateral resolution of about 0.5 μm across individual mitochondria. Fluorescence patterns from the myocardial mitochondria displayed strong intensity fluctuations with periods of several seconds. The implication was that these fluorescence oscillations might reflect local events (alterations in respiratory activity and/or membrane permeability) that are missed in solution studies which average over large numbers of mitochondria.

Despite the tremendous promise of fluorescence microscopy (widefield and confocal), quantitation of single-mitochondrion membrane potentials has been hampered by technical obstacles. These have included identifying dyes that distribute across biological membranes in a strictly Nernstian fashion (i.e., no aggregation or binding to cell components), and measuring rapidly and precisely the fluorescence from intra- and extramitochondrial compartments. In the paper by Loew et al., great strides have been made at overcoming these obstacles. This laboratory had previously developed a class of nontoxic fluorescent dyes which follow the Nernst relation closely, requiring only minor corrections for nonspecific binding. In the current report, these researchers measure the fluorescence from neurites treated with these dyes using a "fast 3D microscope." The key feature of this instrument is a 512 $\times$ 512 cooled CCD array used to rapidly acquire through-focus image sets from a conventional epifluorescence microscope. Subsequent application of a deconvolution algorithm and correction for residual vertical distortion leads to a three-dimensional map of the intracellular fluorescence intensity. Plugging the corrected intensities from subvolumes inside and outside individual mitochondria into the Nernst equation then yields a value for each organelle's membrane potential.

Despite the limited spatial resolution inherent in light microscopy, the increasingly sophisticated fluorescence microscopic techniques being developed in several laboratories should yield important new insights into the nature of mitochondrial potentials. An example is the finding by Loew et al. that a few mitochondria in the neurites display drops in potential lasting several seconds, reminiscent of the fluctuations observed in the above-mentioned cardiac cell study. The advantage of the newer technique is that the magnitude

Received for publication 1 October 1993 and in final form 4 October 1993.

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0006-3495/93/12/2269/03 $2.00

of local potential changes can be calculated and correlated with changes in other mitochondrial activities. An obvious example is respiratory activity, another is membrane permeability. Large-conductance, voltage-gated ion channels have been detected in the inner mitochondrial membrane by patch-clamping (e.g., Sorgato and Moran (1993)). While available evidence suggests that these channels are usually closed, might transient openings correlate with membrane potential fluctuations? These questions and others relating to the distribution and regulation of mitochondrial membrane potentials should begin to be answered as 3D fluorescence microscopy continues to advance.

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$[Ca^{2+}]_i$ Waves in Heart Cells: More Than a Passing Fancy

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Images of calcium ion concentration ($[Ca^{2+}]_i$) in living cells take a starting number of forms. In heart cells, such images reveal that regions of elevated $[Ca^{2+}]_i$ can propagate as "waves" (at constant velocity) or, as in the elegant images from Lipp and Niggli (1993) in this issue, as waves that spiral about subcellular cores. These author's use of high spatial and temporal resolution confocal microscopy enabled them to observe details of $[Ca^{2+}]_i$ waves in three dimensions that had escaped notice previously with lower resolution techniques. They saw for the first time that spiral waves may initiate "linear" waves and that when such waves collide, they annihilate each other. Their results indicate unequivocally that $[Ca^{2+}]_i$ waves in heart cells are produced by a process with high positive feedback. As strongly suspected already, that process is certainly Ca^{2+}-induced release of Ca^{2+} from internal stores (sarcoplasmic reticulum or SR). The new idea compelled by their observations is that (in their own words) there is a "variability of positive feedback even on the subcellular level" and that "this notion implies the existence of functionally separate SR elements exhibiting differeces in gain." As I explain below, this fits in well in a larger picture of control of $[Ca^{2+}]_i$ in heart cells, in which certain paradoxes concerning $[Ca^{2+}]$-induced release of Ca^{2+} and its role in both $[Ca^{2+}]_i$ waves and in normal excitation-contraction (E-C) coupling may finally be resolved.

The $[Ca^{2+}]_i$ transients (waves) observed by Lipp and Niggli (1993) and others appear to be "uncontrolled" in the sense that they may develop spontaneously and may propagate without change until an obstacle (nucleus), another wave, or the end of the cell is reached. Under other circumstances however, $[Ca^{2+}]_i$ transients appear (in the available images, at least) to be uniform throughout the cell, and to be controlled, in the sense that transmembrane Ca^{2+} current is required to initiate them, and that stopping the Ca^{2+} current stops the $[Ca^{2+}]_i$ transient. Under yet other circumstances, imaging reveals that $[Ca^{2+}]_i$ transients may be localized in subcellular regions, and that the elevated $[Ca^{2+}]_i$ may fail to propagate out of such a region (Valdeolmillos et al., 1989). At first, all this might seem to indicate that the control of $[Ca^{2+}]_i$ is highly diverse; surely such a diversity of types of changes in $[Ca^{2+}]_i$ implies a diversity of control mechanisms. In particular, it has always been difficult for researchers in this area to understand how the autocatalytic process of Ca^{2+}-induced release of Ca^{2+} from SR, which can easily be imagined to underlie spontaneous regenerative, propagating $[Ca^{2+}]_i$ waves, might be "harnessed", under other circumstances, to produce controlled changes in $[Ca^{2+}]_i$. Indeed, Fabiato (1985) postulated, in his original description, that "spontaneous cyclic Ca^{2+} release and Ca^{2+}-induced release of Ca^{2+} do not occur through the same mechanism." Nevertheless, the evidence, from many types of experiments, is that all these types of changes in $[Ca^{2+}]_i$ involve exactly the same cellular structures and molecules (e.g., ryanodine receptors or SR Ca^{2+} release channels). There is little doubt now that, however it might work, Ca^{2+}-induced release of Ca^{2+} from SR underlies both $[Ca^{2+}]_i$ waves and the $[Ca^{2+}]_i$ transients of normal E-C coupling. Thus, the major challenge to those attempting to understand the roles of $[Ca^{2+}]_i$ in heart cells is to unify these diverse, even seemingly contradictory phenomena, all of which seem to involve Ca^{2+}-induced release of Ca^{2+}. As noted by Lipp and Niggli (1993), the explanation of spiral $[Ca^{2+}]_i$ waves may lie in the fact that Ca^{2+}-activated SR Ca^{2+}-release channels are not homogeneously arranged throughout the cell but are arranged, functionally, at least, in "clusters." The notion of functional clusters of SR release channels was introduced in a seminal article by Stern in recently *Biophysical Journal* (Stern, 1992), and this notion has since been invoked to explain both controlled and uncontrolled changes in $[Ca^{2+}]_i$ in heart cells. Indeed the "new and notable" idea is that Ca^{2+}-induced release of Ca^{2+} at functional clusters of SR channels may be able to explain how

Received for publication 12 October 1993 and in final form 11 October 1993.

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0006-3495/93/12/2270/03 $2.00