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Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1

(fluorescence microscopy/vital dye/respiration/5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide)

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ABSTRACT **By using a potential-dependent J-aggregate-forming delocalized lipophilic cation, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1), we find that membrane potentials across mitochondria in a living cell can be heterogeneous. Remarkably, even within a long contiguous mitochondrion, regional heterogeneity in membrane potentials appears to be possible.**

Delocalized lipophilic cations such as rhodamine-123 have been used to monitor mitochondrial membrane potential in a single living cell (1–4). However, two long-standing issues have not been resolved. Do all mitochondria within one cell necessarily adopt the same membrane potentials? Is membrane potential maintained uniformly throughout a long single mitochondrion?

It has been known for more than a half-century that some dyes form J-aggregates in certain environments (5, 6). The formation of J-aggregates is accompanied by dramatic shifts in both the absorption and fluorescence maxima of the dye. A unique feature of these dyes potentially useful for cell biological studies is that J-aggregates may form rapidly at favorable sites and display "resonance fluorescence" (7). Their large size would cause them to diffuse slowly so they may be useful as reporter molecules for localized biochemical events. Surprisingly, this remarkable phenomenon has not been purposefully exploited by biologists. Here we report that J-aggregates may serve as reporter molecules for heterogeneity in mitochondrial membrane potentials in living cells.

MATERIALS AND METHODS

Cell Culture. 3T3, NRK, BHK, CCL22, CCL64, CCL146 (ATCC), FS-2 (R. Sager, Dana–Farber Cancer Institute), CX-1 (S. Bernal, Boston University), and MCF-7 (Michigan Cancer Foundation) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum (iron-supplemented, HyClone) at 37°C, 5% CO_2/95% air, and 100% humidity.

Spectroscopic Analysis. Uptake of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (Polaroid) (JC-1) by CX-1 cells was examined by a Kontron SFM25 fluorescent spectrophotometer. Cells were washed with (5 ml) and incubated in (1 ml) low-K^+ buffer containing 137 mM NaCl, 3.6 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 4 mM Hepes, dextrose (1 mg/ml), and 1× mixed amino acids of modified Eagle's medium (GIBCO) at pH 7.2 (8) and then incubated in 1 ml of JC-1 in the same buffer at concentrations specified for 10 min. Cells were then washed with three 2-ml washes and left in 1 ml of trypsin (1×, Whittaker Bioproducts) in low-K^+ buffer for 5 min. About 0.8 ml of cell suspension was mixed with 1.2 ml of low-K^+ buffer in a 1-cm quartz cuvette for 5 min. Fluorescence scans were made by synchronously varying both the emission and excitation wavelengths with a constant differential of 15 nm. Slit widths were 10 nm. Recordings of spectra from 550 to 620 nm were repeated at a higher detector sensitivity.

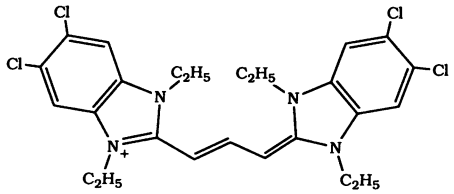

FIG. 1. Chemical structure of JC-1.

Fluorescence Microscopy. All cells were grown on 12-mm square glass coverslips (Bradford Scientific, Epping, NH). A stock solution of JC-1 was made at 1 mg/ml in dimethyl sulfoxide. Fresh staining solution (10 μg/ml) was prepared by diluting the stock solution in warm (37°C) culture medium supplemented with 10% calf serum. Fifty microliters was immediately applied to a coverslip, which was then kept in a cell culture incubator for 10 min (or as specified). (Staining solution older than 3 min should be discarded.) Cells were rinsed in warm dye-free culture medium and mounted in a living-cell chamber made of 0.7-mm-thick silicon rubber (N.A. Reiss, Belle Mead, NJ) as described (9). To dissipate plasma membrane potentials, cells were stained and maintained in high-K^+ buffer (same as low K^+ buffer except 137 mM KCl and 3.6 mM NaCl), instead of culture medium. A Zeiss Axiophot microscope or a Zeiss Photomicroscope III equipped with epifluorescence optics and a 100-W mercury lamp was used. Objective lenses used included Plan Apo 40× [numerical aperture (n.a.) 1.3], Plan Apo or Neofluor 100× (n.a. 1.2). For visualizing the green fluorescence of JC-1 monomer, any filter combinations (such as Zeiss' barrier 515–565 nm, dichroic mirror FT 510 nm, and exciter 450–490 nm) used for fluorescein dye are adequate. Likewise, any rhodamine filter sets (such as Zeiss' barrier LP 590 nm, dichroic mirror FT 580 nm, and exciter BP 546/12 nm) are suitable for detecting the red fluorescence of J-aggregates. However, to visualize green and red fluorescence simultaneously, a long-pass filter system (such as Zeiss' barrier LP 520 nm, dichroic mirror FT 510 nm, and exciter 450–490 nm) is desirable. For Figs. 2, 4, 6, and 7*A*, fluorescent images were recorded on Kodak Professional Ektamatic P800/1600 positive films at exposure index (E.I.) 1600 for green and yellow fluorescence and at E.I. 6400 for red fluorescence (Kodak E-6 processing at Push 1) and printed on Ilford

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Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

Cibachrome A-II papers. Fig. 7*B* was recorded on Kodak Ektar 1000 negative films at E.I. 1600 (Kodak C-41 processing) and printed on Fuji color paper with a +4 neutral-density filter.

Uptake of JC-1 by Mitochondria *in Vitro*. Rat-liver mitochondria were isolated as described by Johnson and Lardy (10) and Reers *et al.* (11). Briefly, the initial homogenization was carried out at 4°C in buffer I (210 mM sucrose/70 mM mannitol/3 mM Mops·KOH, pH 7.4) supplemented with 0.2 mM EGTA·KOH and 2% (wt/vol) bovine serum albumin. The final mitochondrial fraction was suspended at a concentration higher than 60 mg/ml in buffer I. The respiration control ratios with glutamate/malate as substrate were above 18. Mitochondria (0.25 mg/ml) were suspended in a cuvette a 3 ml of buffer II (200 mM sucrose/20 mM mannitol/1 mM EDTA·NaOH/20 mM Mops·NaOH, pH 7.5) at 37°C. After additions of rotenone (1 mM), oligomycin (2.5 mM), JC-1 (1.7 mM), and valinomycin (20 nM), subsequent additions of a 1 M KCl solution were applied to change the membrane potential across the inner mitochondrial membrane. The cuvette was placed in a sample compartment of a Spex Fluorolog (Edison, NJ) equipped with a magnetic stirrer and a temperature controlled cuvette holder. JC-1 was excited at 575 ± 1.8 nm. The J-aggregate fluorescence was monitored at

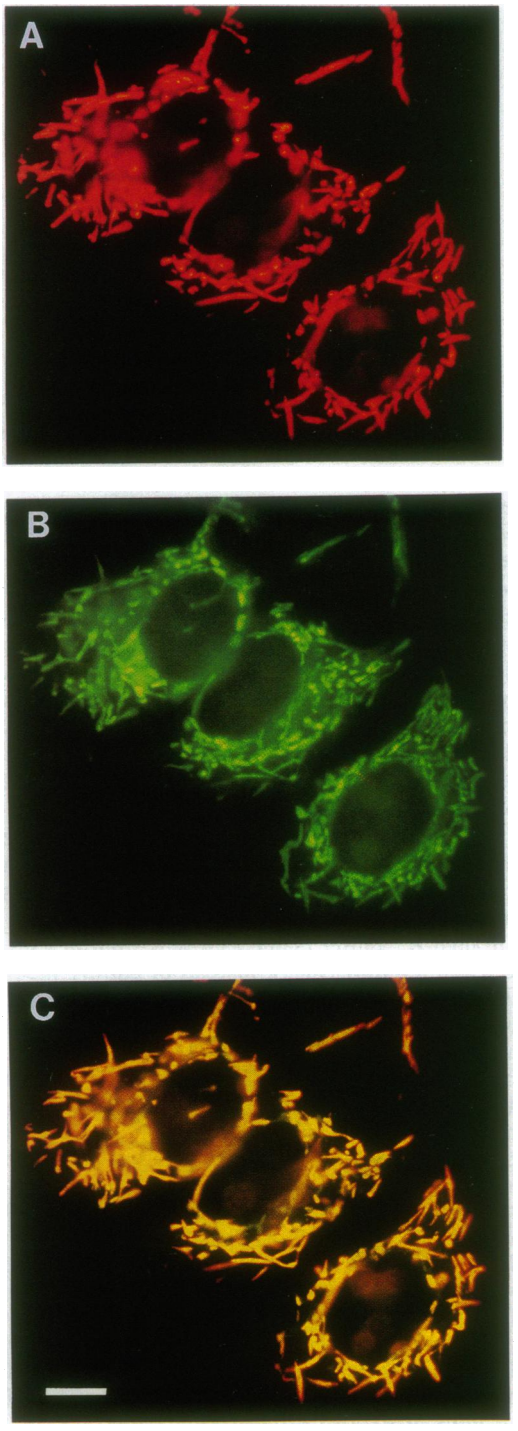

FIG. 2. Localization of JC-1 in MCF-7 cells by epifluorescence microscopy. (*A*) Red fluorescence under green excitation corresponding to J-aggregates fluorescence. (*B*) Green fluorescence under blue excitation corresponding to monomer fluorescence. (*C*) Yellow fluorescence under blue excitation and a long-pass filter. (Bar = 10 μm.)

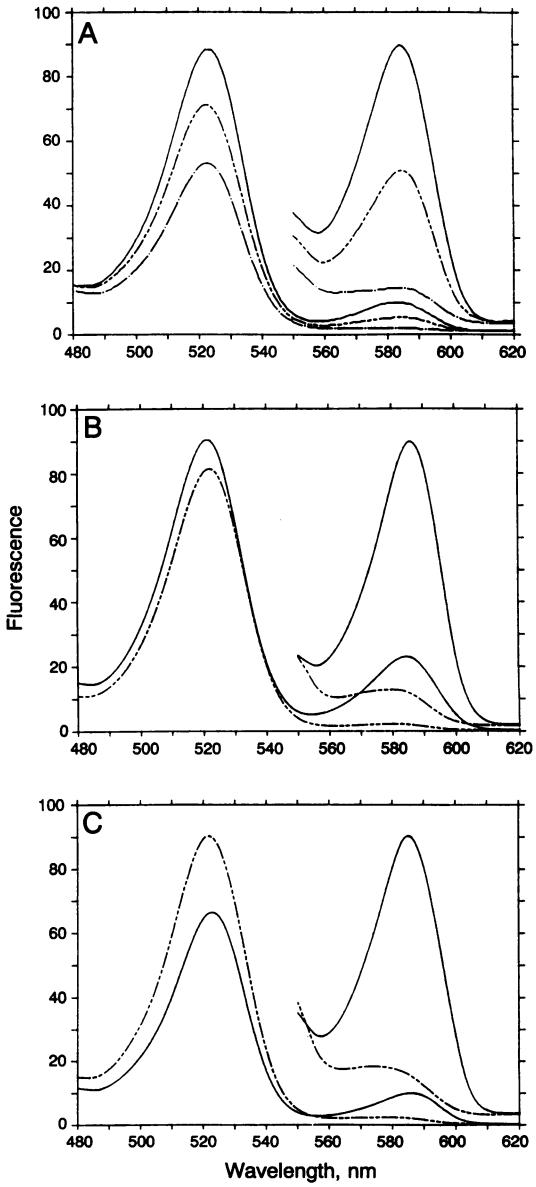

FIG. 3. Fluorescent spectra of JC-1 in CX-1 cells under various conditions. Recordings of spectra from 550 to 620 nm were also repeated at a higher detector sensitivity. (*A*) Uptake of JC-1 in low-K^+ buffer by CX-1 cells in 5 μg/ml (solid line), 2.5 μg/ml (dashed line), or 1.25 μg/ml (dotted line). (*B*) CX-1 cells were incubated with JC-1 (10 μg/ml) in the presence of 5 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 0.5% ethanol (dashed line) or 0.5% ethanol (solid line). (*C*) CX-1 cells were first incubated with JC-1 (10 μg/ml) and then treated with FCCP (5 μM with 0.5% ethanol, dashed line) or 0.5% ethanol (solid line).

590 ± 3.6 nm in the front face mode. The membrane potential was calculated by using the Nernst equation for the potassium diffusion potential (negative inside). The intramitochondrial potassium concentration was assumed to be 120 mM (12). The fluorescence of JC-1 at each potassium diffusion potential was normalized and plotted as a function of the calculated membrane potential.

RESULTS AND DISCUSSION

The aggregation of cyanine molecules is accompanied by large shifts in their absorption spectra, violating Beer's law (7). The formation of H-aggregates (for hypsochromic) is accompanied by a shift to a shorter wavelength and quenching its fluorescence. This feature has been previously exploited for the quantitation of membrane potentials (4). In contrast, J-aggregates formation is associated with a large shift of its absorption and fluorescence maxima to a longer wavelength. Because of the lack of a Stokes' shift, J-aggregates exhibit intense "resonance fluorescence." Among the numerous J-aggregate-forming dyes we screened, JC-1 (Fig. 1) appears to be most suitable for probing mitochondria in living cells. It has an absorption maximum of 510 nm and a fluorescence maximum of 520 nm for monomers and an absorption maximum of 585 nm and a fluorescence maximum of 585 nm for the J-aggregates (13). It has relatively low toxicity, reasonable solubility, and appropriate pK_a and fluorescence characteristics convenient for detection by filter systems commonly used in epifluorescence microscopy.

When cultured cells were stained with JC-1 at 10 μg/ml for 10 min at 37°C and examined by epifluorescence microscopy, either green fluorescence (under blue excitation) or red fluorescence (under green excitation) was detected. However, by use of blue excitation and a long-pass filter system, yellow fluorescence may be detected in regions where both red and green fluorescences coexisted. Fig. 2 illustrates how these three colors may be detected in JC-1-stained human breast carcinoma MCF-7 cells, known to have high mitochondrial membrane potentials (3).

To establish that in living cells the green fluorescence represents the monomer and the red fluorescence represents the J-aggregates, human colon carcinoma CX-1 cells, also known to have high mitochondrial membrane potentials (3), were incubated with JC-1 at various concentrations treated with trypsin, transferred to cuvettes, and analyzed by fluorescence spectrophotometry. The results are shown in Fig. 3*A*. The two peaks at 520 nm and 585 nm correspond to the fluorescence of monomers and the J-aggregate, respectively (13). Thus, the green fluorescence in living cells represents the monomer and the red fluorescence represents the J-aggregate.

The uptake of JC-1 and formation of J-aggregates were monitored at 1 min intervals by epifluorescence microscopy. CX-1 cells were mounted in a live cell chamber containing JC-1 (10 μg/ml) in the culture medium and maintained at 37°C on a microscope stage with an air curtain. After 3 min, green fluorescence and a few speckles of orange fluorescence was detected in mitochondria. At 5 min, the intensity of green fluorescence significantly increased and rod-like structures with orange fluorescence were detected. At 7 min, almost every mitochondrion fluoresced orange. After 10 min, all mitochondria were intensely illuminated with orange fluo-

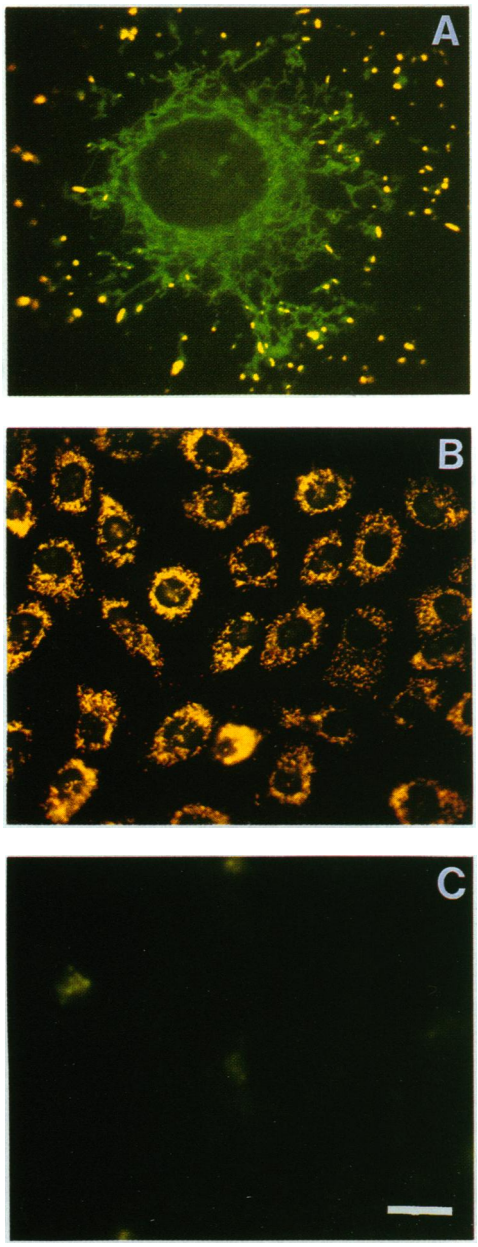

FIG. 4. Epifluorescence localization of JC-1 in drug-treated CCL22 bovine kidney cells. (*A*) Untreated control of CCL22 bovine kidney cells in high-K^+ buffer. (*B*) Treated with nigericin (5 μg/ml) plus ouabain (5 μg/ml) for 30 min in high-K^+ buffer. (*C*) Treated with FCCP (5 μM for 30 min) in high-K^+ buffer. (Bar: for *A*, 12 μm; for *B* and *C*, 55 μm.)

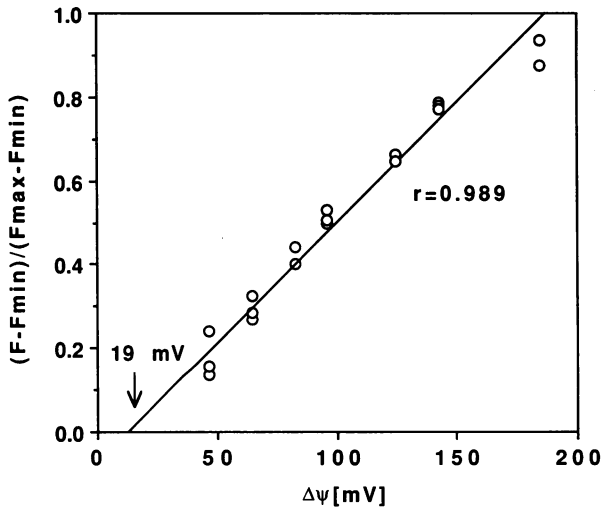

FIG. 5. Relationship between the J-aggregate fluorescence of JC-1 and the membrane potential (ψ) in isolated rat liver mitochondria *in vitro*. F, fluorescence.

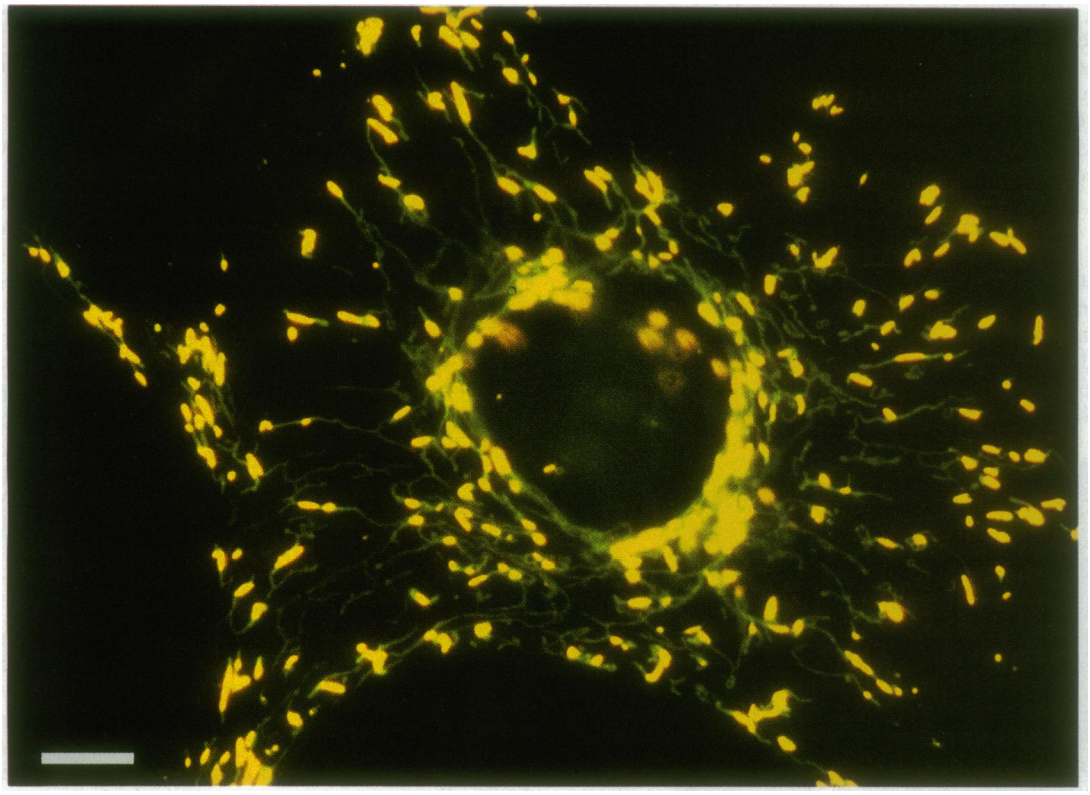

FIG. 6. Epifluorescence localization of JC-1 in a normal mink fibroblast CCL64. As seen, there are two types of mitochondria, one with green fluorescence and the other with yellow. (Bar = 8 μm.)

resncence. No J-aggregate was detected when CX-1 cells were incubated at 4°C with JC-1 (10 μg/ml) in culture medium for 5 hr. A small amount was formed after 1 hr at 25°C.

In the course of our dye screenings, none of the neutral or anionic dyes with a structure similar to JC-1 were taken up by mitochondria. This suggests that the uptake of cationic JC-1 by mitochondria may be related to the presence of a negative sink inside mitochondria created by proton pumps. To confirm that the uptake of JC-1 and subsequent formation of J-aggregates in living cells is caused principally by the mitochondrial membrane potential, a variety of agents known to inhibit normal mitochondrial functions were tested. Figs.

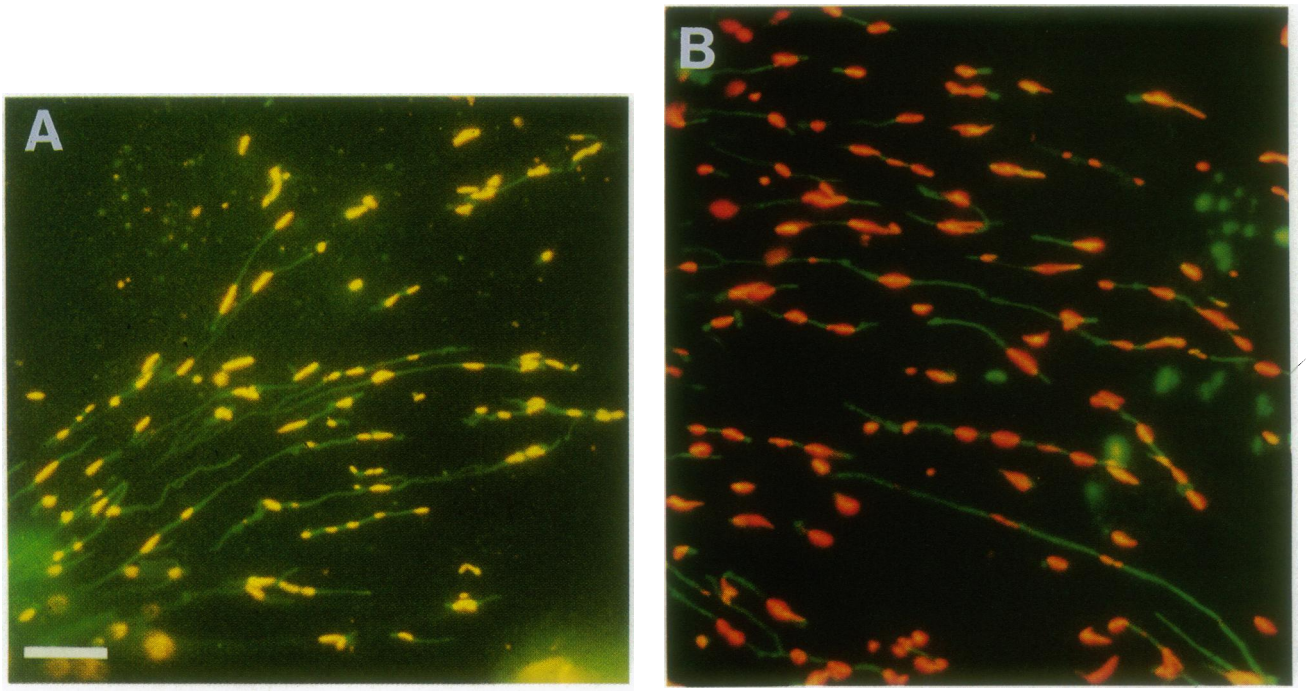

FIG. 7. Epifluorescence micrographs of human fibroblast FS-2 stained with JC-1. (*A*) Recorded with a positive film that gives yellow when green and red colocalize. (*B*) Recorded with a negative film that gives reddish orange when green and red colocalize. Regional heterogeneity in color is detected within long mitochondria. (Bars: *A*, 7 μm; *B*, 5 μm.)

3*B* and 4*C* show that in the presence of FCCP, a proton ionophore that abolishes the electrochemical gradient, a very small amount of J-aggregates was detected. This suggests that the formation of J-aggregates within mitochondria is dependent upon the presence of an electrochemical gradient. When cells were allowed to form intramitochondrial J-aggregates and then placed in medium containing FCCP, the J-aggregates fluorescence rapidly disappeared (Fig. 3*C*). Thus, the maintenance of J-aggregates within mitochondria is also dependent upon the electrochemical gradient. Other agents known to abolish the mitochondrial electrochemical gradient [including carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol, azide plus oligomycin, antimycin A plus oligomycin, and rotenone plus oligomycin] also inhibited the formation of J-aggregates and dissociated preformed J-aggregates (data not shown).

To identify which components of the electrochemical gradient are responsible for the formation and maintenance of J-aggregates, the effects of two other ionophores were investigated: valinomycin, a K^+ ionophore that dissipates the membrane potential but not the pH gradient, and nigericin, a K^+/H^+ ionophore that abolishes the pH gradient while inducing a compensatory increase in the membrane potential with continued respiration. Fig. 4*B* shows that nigericin (5 μg/ml for 30 min) in the presence of ouabain (5 μg/ml; to inhibit hyperpolarization of the plasma membrane) dramatically increased the formation of J-aggregates in kidney epithelial cells. On the other hand, valinomycin drastically reduced the uptake of JC-1 and J-aggregate formation. Moreover, when cells were prestained with JC-1 and placed in valinomycin or nigericin in the absence of dye, the former abolished the J-aggregate fluorescence and the latter had no observable effect. Thus, the pH gradient is not required either for the uptake of JC-1 and subsequent formation of J-aggregates or for the maintenance of preformed J-aggregates. These results suggest that the component of the electrochemical gradient responsible for the formation and maintenance of J-aggregates in mitochondria is likely to be the membrane potential.

In living cells, mitochondria are surrounded by the plasma membrane whose membrane potential has been shown to have a pre-concentrating effect on the accumulation of lipophilic cations by mitochondria (8). If J-aggregate formation is largely membrane potential dependent, a reduction in the plasma membrane potential should also lead to a reduction in J-aggregate formation. Indeed, by incubating CX-1 cells in a high K^+ buffer that dissipates the plasma membrane potential (8), the amount of J-aggregate formation is reduced (data not shown).

To further elucidate the relationship between the membrane potential and the J-aggregate formation, isolated rat liver mitochondria were used. As shown in Fig. 5, the formation of J-aggregates and their fluorescence indeed responds linearly to an increase in membrane potential.

Various cell types and cell lines including FS-2, 3T3, NRK, BHK, CCL64, CCL146, and CCL22 were stained with JC-1 at 10 μg/ml for 10 min. In contrast to MCF-7 and CX-1 cells described above, these cells displayed mostly two populations of mitochondria: one with only green fluorescence and the other with additional J-aggregate fluorescence. Fig. 6 shows such an example in a single mink fibroblast (CCL64). Remarkably, within a single long mitochondrion of some cell types/lines, multiple regions with yellow or orange fluorescence separated by green fluorescence were detected. Fig. 7 shows such mitochondria in normal human fibroblasts FS-2.

It appears that all mitochondria in the same cell do not necessarily maintain the same membrane potential. Considering the increasing certainty that mitochondria are descendents of bacteria and that all mitochondria within one metazoan cell are not interconnected as is the endoplasmic reticulum (14), it is conceivable that some degree of autonomy and individuality may be maintained by mitochondria within a single cell. For short mitochondria, the color of JC-1 fluorescence is mostly uniform (i.e., either green or yellow). For long mitochondria such as those in fibroblast, the fluorescence color is often not uniform (i.e., interdigited between green and yellow). This raises an intriguing possibility that in long mitochondria there may be a regional heterogeneity in mitochondrial membrane potentials.

Note Added in Proof. Quantitative aspects of the use of JC-1 are presented elsewhere (15).

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