Monitoring of Relative Mitochondrial Membrane Potential in Living Cells by Fluorescence Microscopy

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ABSTRACT Permeant cationic fluorescent probes are shown to be selectively accumulated by the mitochondria of living cells. Mitochondria-specific interaction of such molecules is apparently dependent on the high trans-membrane potential (inside negative) maintained by functional mitochondria. Dissipation of the mitochondrial trans-membrane potential by ionophores or inhibitors of electron transport eliminates the selective mitochondrial association of these compounds. The application of such potential-dependent probes in conjunction with fluorescence microscopy allows the monitoring of mitochondrial membrane potential in individual living cells. Marked elevations in mitochondria-associated probe fluorescence have been observed in cells engaged in active movement. This approach to the analysis of mitochondrial membrane potential should be of value in future investigations of the control of energy metabolism and energy requirements of specific biological functions at the cellular level.

Fluorescent dyes have recently been employed as optical indicators of membrane potential differences in cells, isolated organelles, and lipid vesicles that are too small to make microelectrode measurements feasible (4, 5, 14, 18–20). The technique relies on a potential-dependent partitioning of charged lipophilic dye molecules across biological membranes. Changes in trans-membrane potential are reflected by concentration-dependent changes in dye fluorescence, termed "redistribution signals" (5). The fluorescence properties of these compounds allow their quantification by spectrofluorometric techniques and permit identification of their intracellular distribution by fluorescence microscopy (10).

By use of cationic cyanine dyes, it has been shown that erythrocyte-associated fluorescence varies in proportion to changes in membrane potential and is proportional to the potential measured by microelectrodes (8, 9, 15). Fluorescent probes of membrane potential have also been applied to axons, synaptosomes, tumor cells, lymphocytes, and platelets, *Paramecia*, *Neurospora*, bacteria, lipid bilayers, and liposomes (5). However, most studies of probe interaction with intact cells have not established the intracellular site of dye accumulation. Hladky and Rink (8) made the important observation that the proportion of cell-associated dye that is free inside erythrocytes and bound to the membrane is negligible compared to that associated with the cell contents (i.e., hemoglobin), and predicted that in cells containing organelles potential-dependent probes will partition not only across the plasma membrane but also across the membranes of these cytoplasmic constituents.

In fact, charged membrane potential probes have been extensively utilized in examinations of the electrochemical state of isolated mitochondria and submitochondrial particles. Such studies have further established the relationship between the equilibration of permeant ions across membranes and the magnitude of an associated trans-membrane potential. Bakeeva et al. (3) showed the energy-dependent uptake of cations in respiring intact mitochondria, while similar energy-dependent uptake of permeant anions was shown for sonicated submitochondrial particles (7). In both cases ion accumulation was energy requiring and linked to changes in proton distribution. Azzi et al. (2) noted that anionic 1-anilino-8-naphthalenesulfonate (ANS) was accumulated by submitochondrial particles but was lost from intact mitochondria in an energy-dependent manner, while the opposite behavior was observed for the cationic dye auramine O, confirming opposite membrane polarities in these two types of preparations.

More recent fluorometric investigations of mitochondrial membrane potential have employed permeant cationic dyes, such as safranine and the cyanine compounds. Studies by Laris and co-workers (12) showed changes in the fluorescence intensity of the dye 3,3'-dipropylthiodicarbocyanine associated with

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isolated hamster liver mitochondria, when a potassium diffusion potential was induced by valinomycin and when mitochondria became energized in the presence of succinate or ATP. Similar results have been described for the interaction of the dye safranine with isolated mitochondria (1, 6). However, conflicting evidence has been reported in which electrofluorometric dyes have reflected the lack of a metabolically dependent membrane potential in mitochondria (21).

In a previous study (10) it was demonstrated that the cationic compound rhodamine 123 could be utilized as a fluorescent stain for mitochondria in living cells. The studies described here make further use of potential-dependent fluorescent probes to monitor mitochondria in living cultured cells by fluorescence microscopy. Observed differences in the intensity of mitochondria-associated fluorescence may reflect differences in the functional state of mitochondria, more specifically, differences in the magnitude of mitochondrial trans-membrane potential.

MATERIALS AND METHODS

Fluorescent compounds tested for their ability to act as membrane potential probes in the mitochondria of living cells included: 5 μg/ml of rhodamines B, 3B, 6G, 19, 110, 116, 123, and tetramethylrhodamine, ANS, merocyanine 540, fluorescein (all from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.), safranine O (Fisher Scientific Co., Pittsburgh,), and 0.5 μM of the cyanine dyes 3,3'diethyloxacarbocyanine [$DiOC_2(3)$], 3,3'diethyloxadicarbocyanine [$DiOC_2(5)$], 3,3'-dibutyloxacarbocyanine [$DiOC_4(3)$], 3,3'-dipentyloxacarbocyanine [$DiOC_5(3)$], and 3,3'dihexyloxacarbocyanine [$DiOC_6(3)$] (supplied by Drs. H. M. Shapiro, Sidney Farber Cancer Institute, and A. S. Waggoner, Amherst College). These compounds were tested on a variety of cell lines including rat embryo fibroblasts (Rat-1), gerbil fibroma cells (IMR-33, ATCC CCL 146), mink lung fibroblasts (Mv 1 Lu, ATCC CCL 64), monkey kidney cells (CV-1, ATCC CCL 70), Chinese hamster lung fibroblasts (V-79), and mouse 3T3 fibroblasts. In addition, primary cultures of mouse bladder epithelium (17) and rat cardiac muscle cells (11) were also examined.

Cells grown on glass coverslips were exposed to fluorescent probes dissolved in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) for a period of 10 min at 37°C followed by 1-min rinsing and mounting in probe-free culture medium (7) or, in the case of cyanine dyes, under continuous exposure conditions in which cells were mounted and examined in the presence of the probe being tested. Cells were examined by epifluorescence microscopy at both 546 and 485 nm excitation and photographed as described previously (10).

Rhodamine 123 and the cyanine dye $DiOC_2(3)$ were selected for use in experiments analyzing the effects of inhibitors of mitochondrial function. Cells were incubated in culture medium containing valinomycin (5 ng/ml), nigericin (5 μg/ml), *p*-trifluoromethoxy-phenylhydrazone (FCCP, 10^{-6} M), 2,4-dinitrophenol (DNP, 10^{-3} M), potassium cyanide (10^{-4} M), sodium azide (10^{-2} M), antimycin A (10^{-5} M), rotenone (10^{-5} M), oligomycin (5 μg/ml), chloramphenicol (50 μg/ml), cycloheximide (10 μg/ml), or actinomycin D (10 μg/ml). The above compounds were obtained from Sigma Chemical Co. (St. Louis, Mo.), except for nigericin which was provided by Dr. W. E. Scott of Hoffmann-LaRoche, Inc. (Nutley, N. J.). The effects of such treatments on the mitochondrial accumulation of rhodamine 123 and $DiOC_2(3)$ were assessed after drug exposure periods of 1, 5, 10, 15, 30, and 60 min, and 3, 5, 8, 12, 16, and 24 h. The effects of such treatments on cells prestained with rhodamine 123 or $DiOC_2(3)$ were similarly tested.

RESULTS

Mitochondrial Accumulation of Cationic Probes in Living Cells

Examination of living cells by fluorescence microscopy after exposure to fluorescent probes reveals that cationic compounds such as rhodamines 3B, 6G, and 123, cyanine dyes, and safranine 0 are selectively accumulated by mitochondria (Fig. 1) in a manner similar to that previously described for rhodamine 123 (10). Conversely, neutral and anionic compounds (rhodamines B, 19, 110, and 116, tetramethylrhodamine, fluorescein, ANS, merocyanine 540) show no specific mitochondrial staining (Table I). Cells can be exposed to a probe for a period of 10 min, rinsed, and mounted for microscope examination in probe-free medium (10) or, in the case of cyanine dyes, mounted and examined in the continuous presence of the probe. It is possible to use the latter procedure for cyanine dyes because of low background fluorescence in the mounting medium in comparison with the intensely fluorescent mitochondria (Fig. 1). Examination of cells in the continuous presence of cyanine dye reduces the possibility of changes in probe distribution being induced by diffusion. However, indistinguishable results are obtained when cells are stained in the continuous presence of $DiOC_2(3)$ or by exposure to rhodamine 123 for 10 min followed by mounting in probe-free medium.

Effects of Inhibitors and Uncouplers of Mitochondrial Function

To confirm that the mitochondrial accumulation of cationic permanent probes in intact cells is in fact caused by the mitochondrial trans-membrane potential, a variety of agents that have been shown to disrupt the metabolism of isolated mitochondria were tested for their effects on probe-mitochondria interaction. These included inhibitors of electron transport (cyanide, azide, antimycin A, rotenone), and inhibitor of mitochondrial ATP synthetase (oligomycin), ionophores (valinomycin, FCCP, DNP, nigericin), and inhibitors of mitochondrial protein synthesis (chloramphenicol), cellular protein synthesis (cycloheximide), and RNA synthesis (actinomycin D) (Table II). The potassium ionophore valinomycin and the proton ionophores FCCP and DNP, all of which dissipate the mitochondrial trans-membrane potential, have the most marked effects on the retention of potential-dependent probes by mitochondria. Prestaining of cells with rhodamine 123 followed by exposure to any one of the above ionophores leads to the rapid release of the fluorescent dye from mitochondria and results in diffuse, low level cytoplasmic fluorescence with little to no mitochondrial fluorescence observable (Fig. 1). It appears that much of the fluorescent dye is discharged into the surrounding medium. This change takes place within 1–5 min of exposure to ionophore, occurs uniformly throughout the cell population, and is reversible as mitochondria can be restained upon the removal of ionophore. Conversely, pretreatment of cells with the ionophore nigericin, which induces an electrically neutral exchange of protons for potassium ions and results in the elimination of the pH gradient across the mitochondrial membrane and a compensating increase in membrane potential with continued respiration, enhances the accumulation of fluorescent probes by mitochondria (Table II, see also Fig. 4). Thus, the results obtained by the application of ionophores that both increase and decrease mitochondrial membrane potential suggest that permeant cationic probes can be used to reflect mitochondrial trans-membrane potential in living cells.

Prestaining of mitochondria in living cells with rhodamine 123 or cyanine dyes followed by mounting in medium containing inhibitors of electron transport such as azide, cyanide, antimycin A, or rotenone also leads to the release of fluorescent dye into the cytoplasm. The effect is similar to that observed in the presence of valinomycin, FCCP, or DNP except that the release of the fluorescent dye from mitochondria is slower (10–30 min), the residual fluorescence in mitochondria is higher, and much of the released dye remains in the cytoplasm. Rotenone has an interesting side effect in that the mitochon-

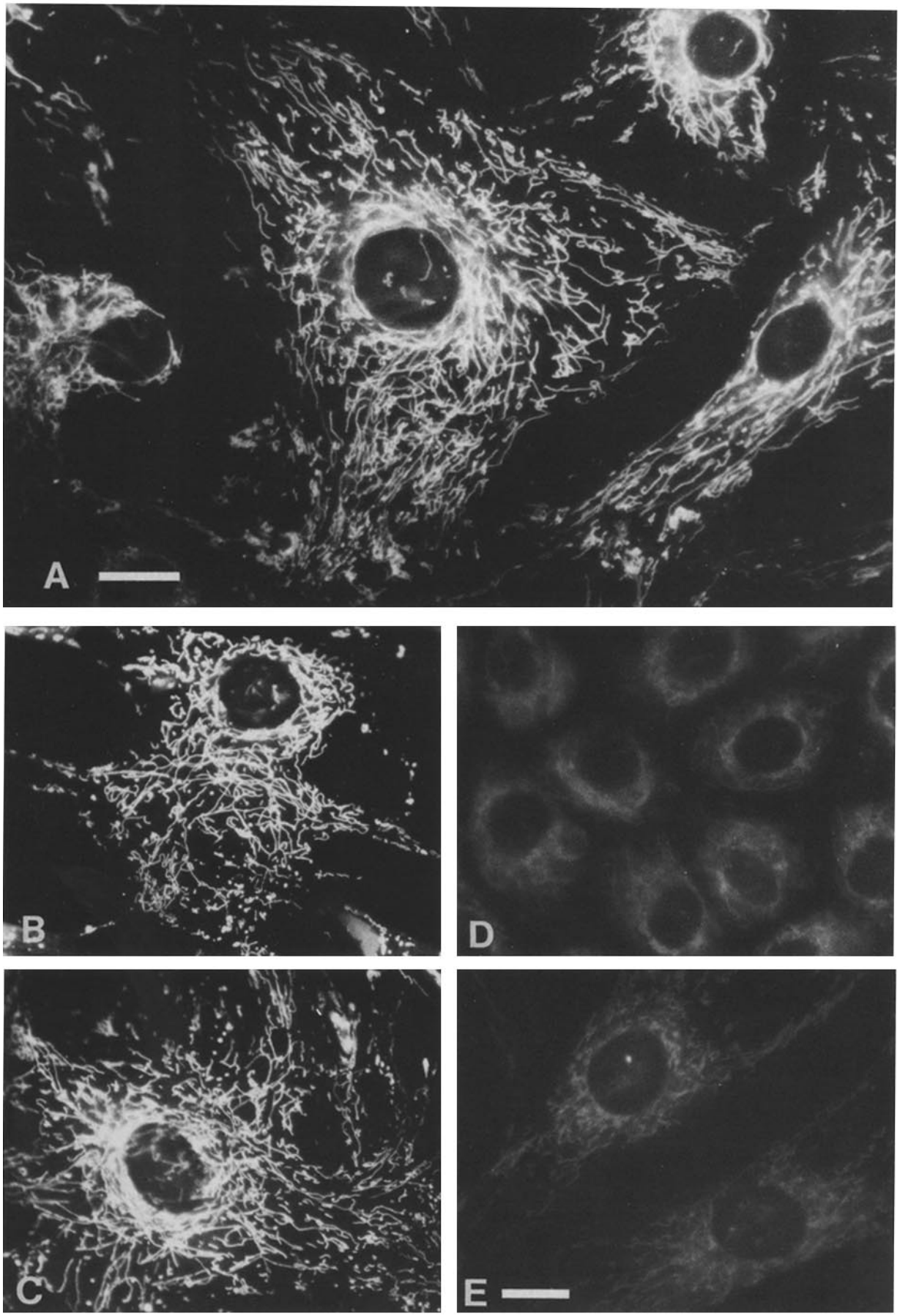

FIGURE 1 Mitochondria-specific staining with fluorescent probes in living cells. (*A*) CCL 146 gerbil fibroma cells stained with rhodamine 3B for 10 min and rinsed in dye-free medium for 15 min; (*B*) Human fibroblast FS-2 in the continuous presence of the cyanine dye, $DiOC_2(3)$, in the culture medium; (*C*) FS-2 stained with rhodamine 6G for 10 min and rinsed in dye-free medium for 15 min; (*D*) 3T3 cells treated with FCCP (10^{-6} M, 5 min) and (*E*) CCL 146 cells treated with valinomycin (5 ng/ml, 5 min) and then stained in the continuous presence of $DiOC_2(3)$ in the culture medium. Bars, 30 μm (*A*) and 20 μm (*B–E*).

drial shape and distribution are altered to a state similar to that induced by colchicine (10). In contrast, oligomycin, which inhibits mitochondrial ATP synthetase does not affect the fluorescent intensity of mitochondria prestained with rhodamine 123 or $DiOC_2(3)$. However, cells pretreated with oligomycin and then stained with rhodamine 123 or $DiOC_2(3)$ show a slight but detectable increase in fluorescence intensity. When cyanide is included with oligomycin, the mitochondrial staining is unaffected in prestained cells for 2 h. Chloramphenicol, cycloheximide, and actinomycin D are all without effect on either prestained cells or cells stained after drug treatment. Finally, mitochondria in cells grown under anaerobic conditions for 2 d are stained very weakly by rhodamine 123 in an anaerobic environment, but their staining increases to that normally observed within 10 min of a return to aerobic conditions.

Intercellular Variability in Mitochondrial Accumulation of Permeant Cationic Probes

Within a given cell, all mitochondria stain with a similar fluorescence intensity, suggesting that the functional state of mitochondria, as reflected by the magnitude of the trans-membrane potential, is maintained uniformly by all the mitochondria of an individual cell. However, in some cell populations marked cell-to-cell variations are observed in the intensity of mitochondria-associated fluorescence (Fig. 2). Even postmitotic daughter cells may sometimes show striking differences in the intensity of mitochondrial fluorescence (Fig. 3). Variation in dye accumulation within a population of cells is most marked in the IMR-33 (gerbil fibroma), CV-1 (monkey kidney epithelium), and Rat-1 (rat embryo fibroblast) cell lines.

To examine whether the observed differences in mitochondrial staining among different cells resulted from variability in dye permeability across the plasma membrane or even the mitochondrial membrane, we made use of the fact that with continued respiration nigericin allows the conversion of the pH gradient across the mitochondrial membrane to an increase in membrane potential and hence restores weakly stained cells to a normal level. CV-1 cells were treated with nigericin for 5 min, rinsed, and mounted in medium containing $DiOC_2(3)$ without nigericin. Fig. 4 shows that in the presence of nigericin every CV-1 cell is strongly stained, indicating that the fluorescent probe indeed reaches the mitochondria of every CV-1 cell. However, when nigericin is removed, the dye is rapidly released from those cells that had weakly stained mitochondria before nigericin treatment but not from cells already intensely stained before exposure to nigericin. Such an observation strongly suggests that the permeability of dye across plasma membrane or mitochondrial membrane is not the prime factor responsible for variability in mitochondrial staining within a population of cells. Rather, the heterogeneity in mitochondrial membrane potential within a population of cells is probably responsible for such differential staining.

TABLE I

Compounds Screened by Fluorescence Microscopy for Mitochondria-Specific Interaction in Living Cells

Compound	Charge at physiological pH	Mitochondria-specific fluorescence
Rhodamine 3B	Positive	Yes
Rhodamine 6G	Positive	Yes
Rhodamine 123	Positive	Yes
$DiOC_2(3)$	Positive	Yes
$DiOC_4(3)$	Positive	Yes
$DiOC_5(3)$	Positive	Yes
$DiOC_6(3)$	Positive	Yes
$DiOC_2(5)$	Positive	Yes
Safranine O	Positive	Yes
Rhodamine B	Neutral	No
Rhodamine 19	Neutral	No
Rhodamine 110	Neutral	No
Rhodamine 116	Neutral	No
Tetramethylrhodamine	Neutral	No
ANS	Negative	No
Merocyanine 540	Negative	No
Fluorescein	Negative	No

Possible Relationship between Mitochondrial Membrane Potential and Cell Motility

Two interesting examples in which intercellular variation in mitochondria-associated fluorescence may be related to cell motility have been noted in primary cultures of neonatal rat cardiac cells and mouse bladder epithelium. Primary cultures derived from neonatal rat hearts consist of morphologically distinguishable cardiac muscle cells and nonmuscle cells (epithelial and fibroblastic). The cardiac muscle cells are nondivid-

TABLE II

Effects of Various Treatments on the Mitochondrial Staining with Rhodamine 123

Treatment	Action	Effect on mitochondria-specific fluorescence
Valinomycin, *5 ng/ml, 5 min* *‡	K^+ Ionophore, dissipate electrochemical gradient	Diminished
FCCP, 10^{-6} *M, 5 min*‡	H^+ Ionophore, dissipate electrochemical gradient	Diminished
DNP, 10^{-3} *M, 5 min*‡	H^+ Ionophore, dissipate electrochemical gradient	Diminished
Nigericin, *5 μg/ml, 5 min*§	Na^+, H^+ Exchange ionophore, dissipate pH gradient but incease membrane potential	Increased
Oligomycin, *5 μg/ml, 24 hr*§	Inhibition of ATP synthetase	Partially increased
Sodium azide, 10^{-2} *M, 30 min*‡	Inhibition of electron transport	Diminished
Antimycin A, 10^{-5} *M, 30 min*‡	Inhibition of electron transport	Diminished
Rotenone, 10^{-5} *M, 30 min*‡	Inhibition of electron transport	Diminished
Chloramphenicol, *50 μg/ml, 24 h*§	Inhibition of mitochondrial protein synthesis	None
Cycloheximide, *10 μg/ml, 24 h*§	Inhibition of cellular protein synthesis	None
Actinomycin D, *10 μg/ml, 24 h*§	Inhibition of RNA synthesis	None
Anaerobic conditions, *24–48 h*§	Absence of terminal electron acceptor	Diminished

* Time in parentheses represents the minimum exposure period that reproducibly resulted in a marked decrease in mitochondria-associated fluorescence, or in treatments that did not have apparent effects, the maximum exposure period tested.
‡ CCL 146 gerbil fibroma cells were prestained with rhodamine 123 and mounted in medium containing antibiotics or drugs.
§ CCL 146 cells were pretreated with drugs or antibiotics for duration indicated and then stained for mitochondria with rhodamine 123.

ing and undergo rapid rhythmic contractions in culture (11). When exposed to potential-dependent probes, cardiac muscle cells show much higher levels of mitochondria-associated fluorescence than do the nonmuscle cells present in the same culture (Fig. 5).

Epithelial cells migrating outward in a continous monolayer from culture explants of mouse bladder (17) show regional variation in the accumulation of rhodamine 123 or the cyanine dye $DiOC_2(3)$. Cells at or near the periphery or leading edge of the outgrowth accumulate significantly more mitochondria-associated fluorescence than do cells deeper in the outgrowth (Fig. 6). Phase-contrast images of such epithelial outgrowths reveal extensive membrane ruffling at the leading edge while none is apparent on internal cells (Fig. 6). When a confluent culture of bladder-epithelium is "wounded" with a razor blade creating a gap in the epithelial cell sheet, the cells along the edge of the wound almost immediately (within 2 min) show increases in mitochondria-associated fluorescence and extensive membrane ruffling (Fig. 6). As with the peripheral cells of the migrating epithelial sheet, there does not appear to be stimulation of DNA synthesis at the wound edge as determined by autoradiography of [^{3}H]thymidine uptake (17). It appears

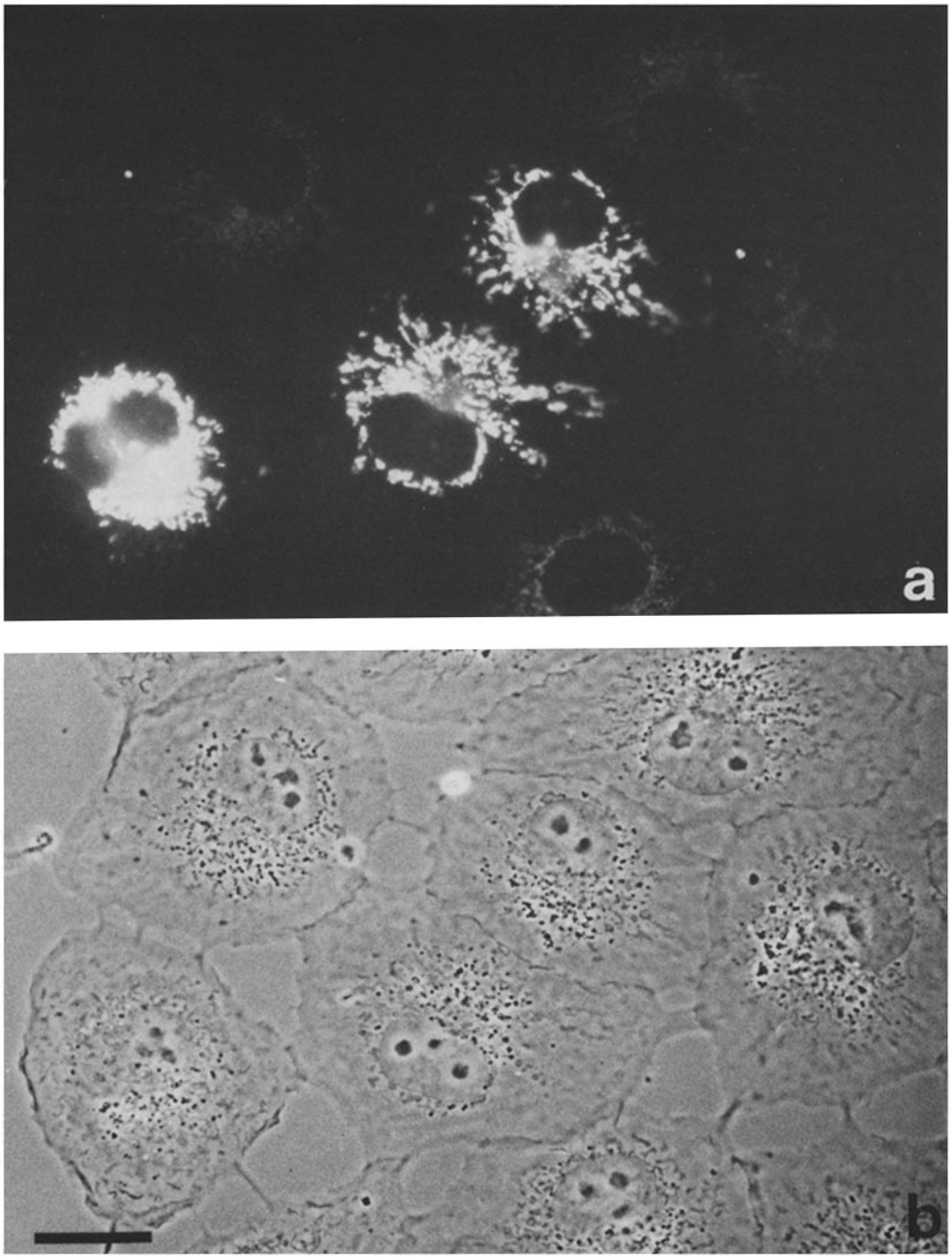

FIGURE 2 (*a*) Intercellular heterogeneity in the intensity of mitochondria-associated fluorescence in monkey kidney cells (CV-1) in the presence of $DiOC_2(3)$. (*b*) Phase contrast of the same field. Bar, 35 μm.

that wound healing in these preparations occurs by migration of cells across the wounded area until a continuous cellular sheet is reformed, at which time a cessation of ruffling and migrating activity is accompanied by a decrease in mitochondria-associated probe fluorescence in cells initially at the edge of the wound (Fig. 6).

DISCUSSION

In an earlier report it was demonstrated that the cationic, fluorescent compound rhodamine 123 is selectively accumulated by mitochondria in living cells (10). Of the additional fluorescent probes screened here, cationic molecules show selective accumulation by mitochondria while neutral and anionic molecules reveal no mitochondrial interaction. These results are likely to be caused by the relatively high electric potential (inside negative) that exists across the mitochondrial membrane. It has previously been established that changes in membrane potential can be reflected by such permeant fluorescent probes (4, 5, 18–20). Cationic probes used here appear to exhibit a potential-dependent interaction with the native mitochondria of living cells and may reflect intercellular variations in mitochondrial membrane potential based on variations in the intensity of mitochondria-associated fluorescence.

In support of this notion are the observations that treatment of cells with the ionophores valinomycin, DNP, and FCCP, which are known to dissipate the electrochemical gradient across the mitochondrial membrane (and, therefore, the transmembrane potential), results in a rapid loss of the specific association between cationic probes and mitochondria. Conversely, nigericin treatment, which induces an enhancement of mitochondrial membrane potential, leads to a significant increase in mitochondria-associated probe fluorescence. Furthermore, inhibitors of electron transport in the respiratory chain such as cyanide, azide, antimycin A, and rotenone all effectively release fluorescent probes from prestained mitochondria. Because electron transport is required for the maintenance of

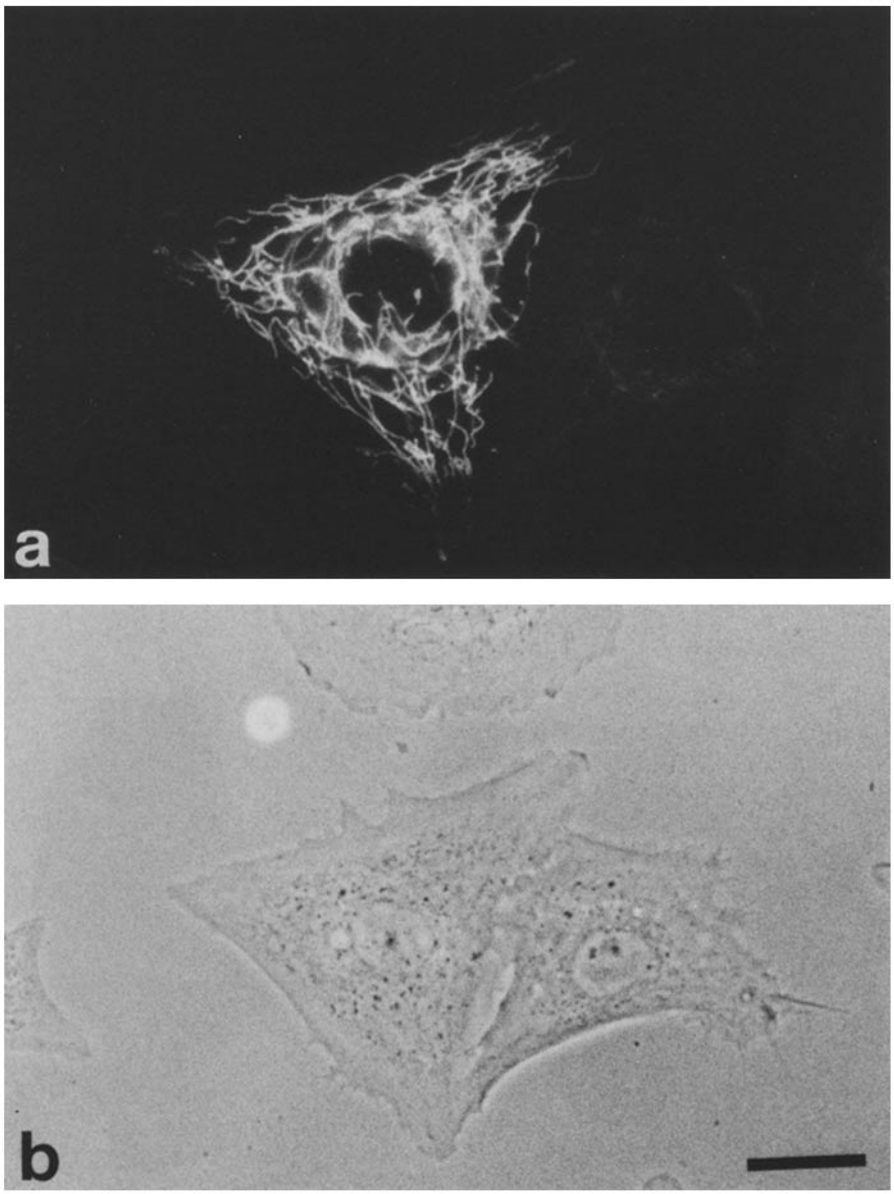

FIGURE 3 (*a*) Fluorescence micrograph of two CCL 146 gerbil fibroma daughter cells with a marked difference in the fluorescence intensity of mitochondria-associated rhodamine 123. (*b*) Phase-contrast micrograph of the same field. Bar, 40 μm.

the proton gradient across the mitochondrial membrane, these results are also consistent with the notion that the fluorescence intensity associated with mitochondria is likely to be a reflection of mitochondrial membrane potential. Reduction in mitochondrial staining under anaerobic conditions also supports this possibility. These results indicate that membrane potential probes can reflect fluctuations in mitochondrial trans-membrane potential and are consistent with those previously reported for isolated mitochondrial preparations (12). The observed effects are readily reversible and, therefore, are not likely to be the result of nonspecific effects on mitochondrial membranes.

The use of potential-dependent probes in conjunction with fluorescence microscopy has the advantage over conventional spectrofluorometric techniques of allowing the examination of individual cells. Thus, it is observed that all mitochondria within a given cell acquire a uniform level of fluorescence on exposure to such probes, suggesting that the entire mitochondrial population of a cell maintains a trans-membrane potential of equal magnitude. Such an occurrence could be explained by the existence of a continuous, interconnected mitochondrial network with the generated membrane potential distributed uniformly throughout. However, the frequent observation of individual mitochondria in addition to mitochondrial networks (10) would argue against this possibility. Alternatively, mitochondria may respond uniformly to factors influencing the rate of ATP synthesis and thus maintain similar trans-membrane potentials.

Another significant finding made possible by this approach to the study of membrane potential is the observation of marked intercellular variations in the mitochondrial accumulation of potential-dependent cationic probes. Some cell populations show extreme variations in the intensity of mitochondria-associated fluorescence, while other cell types are typically stained uniformly at a relatively high intensity. Such microscope analyses are, however, hampered by the lack of a strictly quantitative technique for the analysis of probe uptake by individual cells. Another factor that may be involved in variations in fluorescence intensity of mitochondria-associated probes is a change in the fluorescence properties of these molecules upon reaching the hydrophobic environment of the mitochondrial membrane. A number of fluorescent probes of hydrophobic binding sites in membranes and proteins have been shown to have increased quantum efficiency in hydrophobic solvents compared to that in aqueous media (13, 16). However, it will be reported elsewhere that the fluorescence intensity of mitochondria in fact correlates positively with the amount of mitochondria-associated dye (L. V. Johnson and L. B. Chen, manuscript in preparation).

In migrating sheets of epithelial cells, those at the periphery always show elevated levels of mitochondria-associated probe fluorescence compared to those located more internally. Likewise, cells at the edge of a wound in an epithelial sheet show increased mitochondrial fluorescence. In both cases, the cells that demonstrate increased mitochondrial accumulation of permeant cationic probes show extensive membrane ruffling activity, typical of actively migrating cells. It might be predicted that cells involved in such active movement would have increased ATP requirements, and the elevated mitochondrial membrane potential detected in these cells may reflect a requisite increase in the rate of mitochondrial ATP synthesis. Another example in which elevated levels of mitochondrial fluorescence have been observed to correlate with physical movement is in contracting cardiac muscle cells. The ATP requirements of such cells would be expected to be relatively high in comparison to noncontracting cells. Accordingly, mitochondrial accumulation of permeant cationic probes in cardiac muscle cells is sharply elevated in comparison with non-

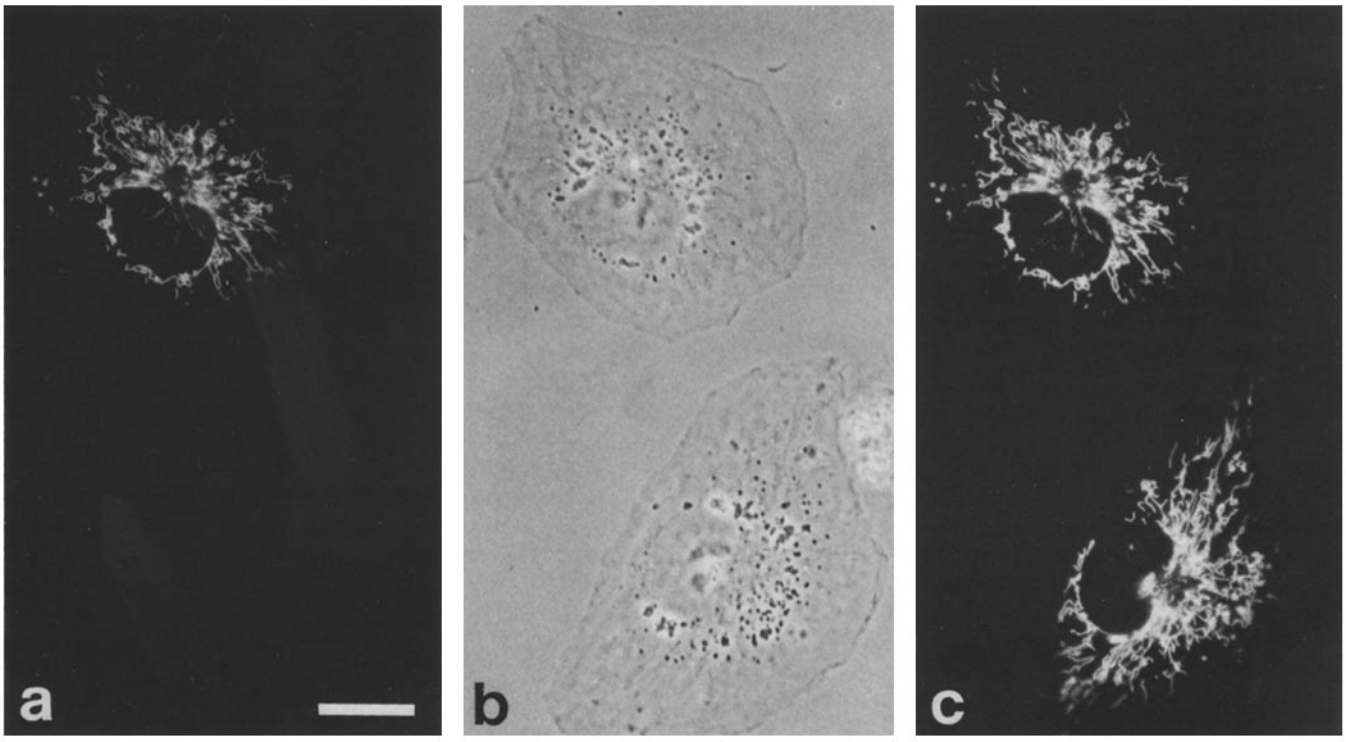

FIGURE 4 (a) Before nigericin, (b) phase contrast, and (c) 5 min after the addition of nigericin (5 μg/ml). Rat liver cells stained with continuous presence of cyanine dye, $DiOC_2(3)$. Bar, 30 μm.

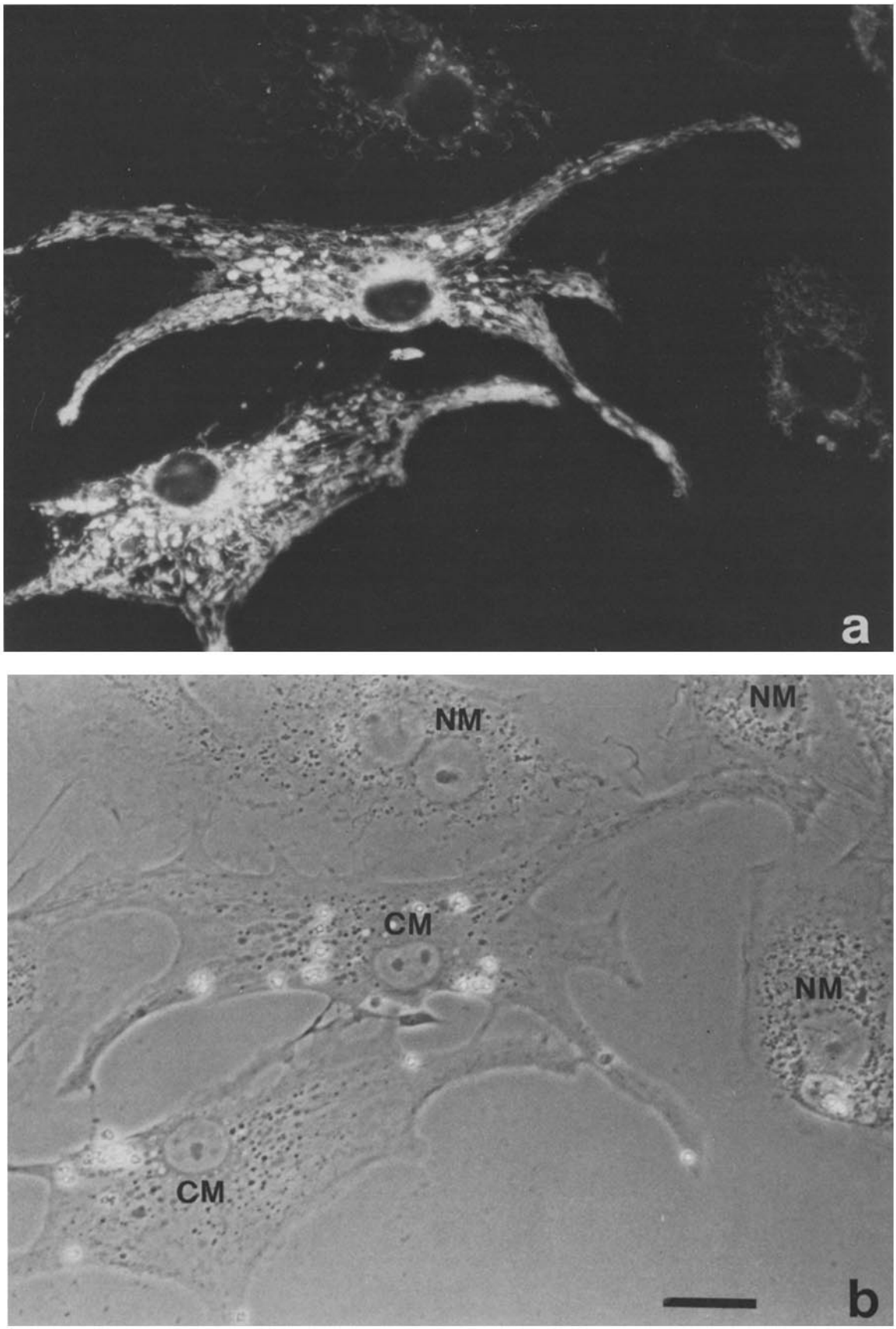

FIGURE 5 (*a*) Fluorescence micrograph showing the marked difference in the intensity of mitochondrial fluorescence between rat cardiac muscle (*CM*) and nonmuscle (*NM*) cells in the presence of $DiOC_2(3)$. (*b*) Phase-contrast micrograph of the same field. Bar, 20 μm.

muscle cells in the same culture, suggesting the existence of an elevated trans-membrane potential in mitochondria of the contracting muscle cells. The indication that cells that are using ATP faster have higher mitochondrial membrane potential suggests that the availability of substrates such as NADH is probably the rate-limiting factor rather than the availability of

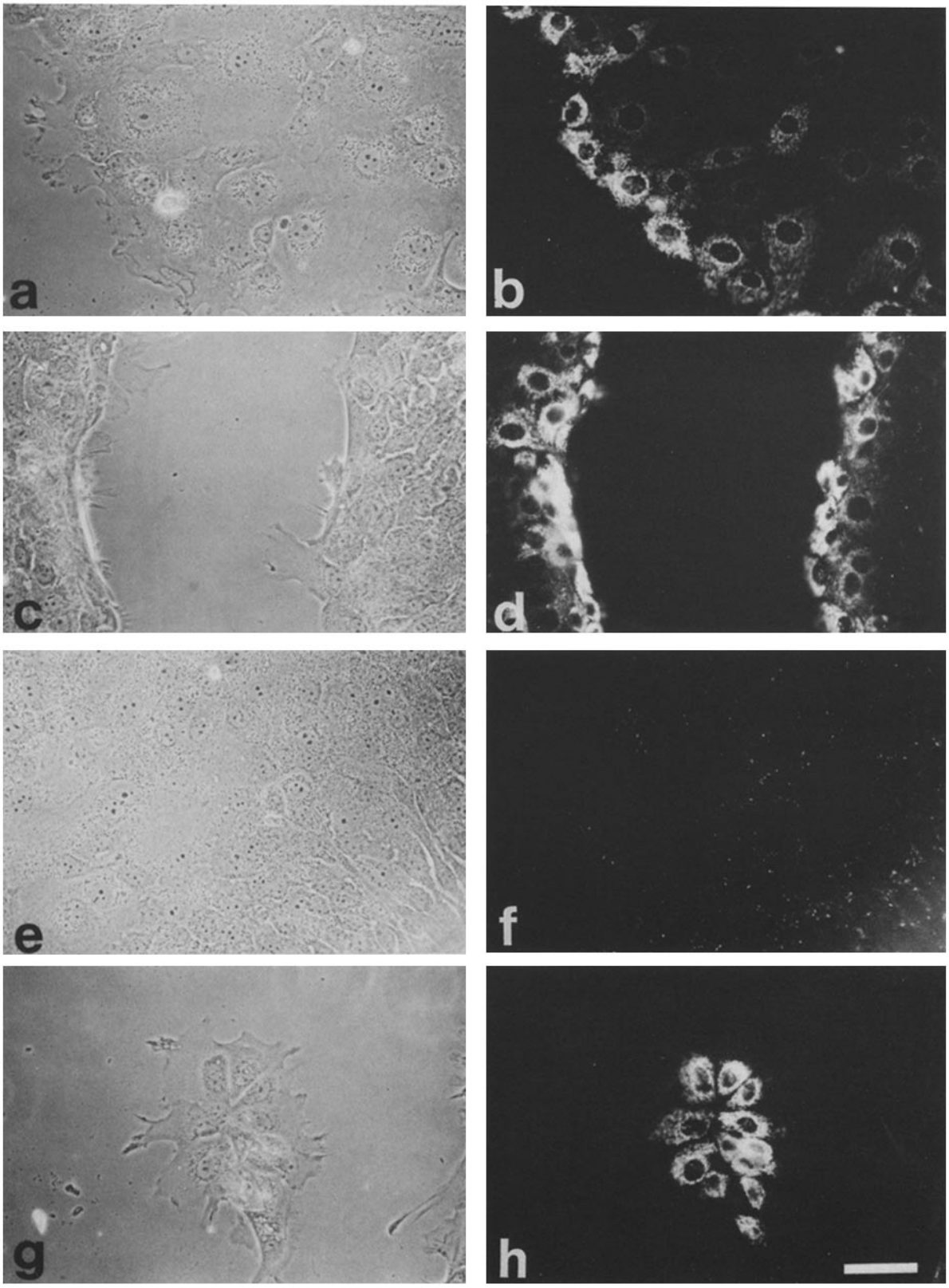

FIGURE 6 Primary cultures of mouse bladder epithelial cells at the leading edge of an outgrowth (*a*) phase contrast, (*b*) $DiOC_2(3)$ fluorescence; at the edge of a wound in the epithelial sheet 15 min after wounding (*c*) phase-contrast, (*d*) $DiOC_2(3)$ fluorescence; in a healed area of epithelial sheet 12 h after wounding (*e*) phase contrast, (*f*) $DiOC_2(3)$ fluorescence; in an island of epithelial cells (*g*) phase-contrast, (*h*) $DiOC_2(3)$ fluorescence. Bar, 100 μm.

ADP as commonly assumed. If ADP + P_i were rate limiting, cells with higher ADP concentration would be expected to have a lower membrane potential.

In summary, the results described here indicate that certain permeant cationic probes may reflect mitochondrial membrane potential in living cells. The binding of cationic probes by mitochondria should be considered in future spectrofluorometric investigations of membrane potential in intact, mitochondria-containing cells. In addition, it appears that intercellular variations in mitochondrial membrane potential occur and, in at least some cases, increases in mitochondrial accumulation of permeant cationic probes may reflect increased ATP requirements in cells engaged in active movement. The approach described here should be of value in future investigation of the control of energy metabolism and energy requirements of specific biological functions.

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