Evaluation of fluorescent dyes for the detection of mitochondrial membrane potential changes in cultured cardiomyocytes

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Abstract

\textbf{Objective:} Maintenance of the mitochondrial membrane potential ($\Delta \psi_m$) is fundamental for the normal performance and survival of cells such as cardiomyocytes, that have a high energy requirement. Measurement of $\Delta \psi_m$ is therefore essential in order to develop an understanding of the molecular mechanisms controlling cardiomyocyte function. Here we have evaluated various potentiometric dyes for their ability to detect alterations of $\Delta \psi_m$, using flow cytometry and confocal microscopy. \textbf{Methods:} Primary cultures of cardiomyocytes from neonate rats were treated with mitochondrial uncouplers before or after loading with Rho123, DiOC\textsubscript{3}, CMXRos or JC-1, and then analysed by flow cytometry. Apoptotic cells were identified by light scatter and Annexin V staining. \textbf{Results:} The four potentiometric dyes tested were able to discriminate between viable and apoptotic cells. However, only JC-1 was able to detect the collapse of $\Delta \psi_m$ induced by uncouplers of mitochondrial respiration. Confocal microscopic analysis confirmed that JC-1 stained mitochondria in a potential-dependent manner. In contrast, CMXRos stained cardiomyocytes irrespective of alterations in $\Delta \psi_m$. \textbf{Conclusions:} We conclude that JC-1 is the optimal dye to use when measuring $\Delta \psi_m$ in cardiomyocytes.

Keywords: Apoptosis; Cardiomyopathy; Membrane potential; Mitochondria; Myocytes

\textit{This article is referred to in the Editorial by E.J. Griffiths (pages 24–27) in this issue.}

1. Introduction

The supply of energy by the mitochondrion depends on the maintenance of the chemiosmotic gradient across its inner membrane [1]. This gradient, also known as the proton motive force (PMF), is generated by three respiratory enzyme complexes which use the free energy released during electron transport to translocate protons from the mitochondrial matrix into the intermembrane space [2].

The energy stored in PMF drives the phosphorylation of ADP by ATP synthase and also supports other mitochondrial activities such as the uptake of calcium, the importation of mitochondrial proteins and the transport of metabolites across the inner membrane. PMF has two components: the membrane potential ($\Delta \psi_m$) which arises from the net movement of positive charge across the inner membrane, and the pH gradient [3]. Of these two components $\Delta \psi_m$ contributes most of the energy stored in the gradient, typically $\sim$150 mV. Hence, for practical purposes, $\Delta \psi_m$ may be used on its own as an indicator of the energization state of the mitochondrion.

Maintenance of $\Delta \psi_m$ is fundamental for the normal performance and survival of cells that have a high-energy requirement, such as the beating cardiomyocyte. Hence,
cardiomyopathies appear as one of the prominent clinical manifestations of mitochondrial diseases in which $\Delta \psi_m$ is compromised [4]. Furthermore, the collapse of $\Delta \psi_m$ due to the opening of a high conductance pore in the inner mitochondrial membrane, has been implicated in the molecular mechanism associated with reperfusion injury to the heart [5]. Therefore, the development of reliable methods to evaluate $\Delta \psi_m$ in cardiomyocytes is of considerable relevance for biomedical research.

The Nernst equation predicts that cationic lipophilic compounds would become more concentrated in the relatively more electronegative mitochondria than in the cytoplasm [3,6]. Based on this principle lipophilic fluorescent cations, including rhodamines [6–9], rosamines [10,11], and carbocyanine derivatives [5,7,10,12–18], have been widely used in combination with fluorometric [12–14], microscopic [5,6,9,11,13,14,16,18], or flow cytometric methods [7,8,10,11,15,17], to monitor relative changes of $\Delta \psi_m$. Flow cytometry has been previously used to study certain aspects of cardiomyocyte physiology [19–21], but not to evaluate $\Delta \psi_m$ in these cells. Here, we have studied intact cultured cardiomyocytes by flow cytometry and confocal microscopy, and have compared a number of commonly used mitochondrial dyes for their ability to detect the collapse of $\Delta \psi_m$ induced by mitochondrial uncouplers.

2. Methods

2.1. Materials

Rhodamine 123 (Rho123), 3,3′-dihexyloxacarbocyanine iodide (DiOC$_3$(3)), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracyclolimidazolocarbocyanine iodide (JC-1) and chloromethyl-X-rosamine (CMXRos) were purchased from Molecular Probes (Eugene, OR). Propidium iodide (PI), carbonyl cyanide $m$-chlorophenylhydrazone (mCCCP) and carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone (FCCP) were from Sigma (Dorset, UK). Tissue culture media and additives were from Gibco Life Technologies (Paisley, UK). Other reagents were from standard suppliers or as listed in the text. Stock solutions of DiOC$_3$(3) (40 $\mu$M), JC-1 (2.5 mM) and CMXRos (1 mM) were made in dimethylsulfoxide. Rho123 (1.25 mM) was dissolved in dimethylformamide. FCCP and mCCCP (25 mM) were dissolved in ethanol. Stock solutions were stored in small aliquots at $-20^\circ$C and diluted in experimental medium immediately before adding to the cells.

2.2. Cardiomyocytes

Primary cultures of neonatal rat cardiomyocytes were prepared by a modification of a previously described method [22]. Briefly, hearts were removed from 1-day-old Sprague–Dawley rat pups and digested with 0.03% collagenase (Worthington Biochemicals, Lakewood, NJ)/0.6% pancreatin (Gibco, Life Technologies) dissolved in (mM) 116 NaCl, 0.8 NaH$_2$PO$_4$, 5.5 glucose, 5.4 KCl, 0.4 MgSO$_4$ and 20 HEPES, pH 7.4. The dissociated cells were pre-plated onto 100-mm tissue culture dishes (Falcon) in 68% DMEM, 17% Medium 199, 10% horse serum, 5% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin, and incubated for 1 h at 37°C under 5% CO$_2$ to remove the non-myocytic cells. The non-adherent cardiomyocytes were then harvested and seeded onto six-well tissue culture plates (Falcon) at a density of 20,000 cells/cm$^2$. Twenty-four hours later the cultures were switched to maintenance medium which consisted of 80% DMEM, 19% Medium 199, 1% FBS and antibiotics as before. Experiments were performed 4–7 days after the initial seeding at which time more than 70% of the cells were beating spontaneously.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Immunocytochemistry

Cell monolayers were fixed for 10 min in cold methanol ($-20^\circ$C) and then air dried. After rinsing in PBS/0.1% bovine serum albumin (BSA; fraction V, Sigma) the cells were pre-incubated with PBS/1% BSA for 15 min to block non-specific binding sites and then stained for 30 min at room temperature with an anti-sarcomeric $\alpha$-actin antibody (clone 5C5, Sigma) at 1:500 dilution. Parallel incubations were carried out using an equivalent amount of an isotype-matched control antibody (clone DAK-G08, DAKO). Dishes were then rinsed three times in PBS/0.1% BSA, incubated for 30 min with a 1:20 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (1:20, DAKO) and rinsed again. The antibody-alkaline phosphatase complex was detected using the new fuchsin chromogenic substrate system (DAKO). Samples were viewed under phase contrast with an Axiovert 25 CFL inverted microscope using a 20× objective (Carl Zeiss, Germany). Randomly selected fields were photographed using Kodak Ektachrome 64 T film.

2.4. Flow cytometry

To measure $\Delta \psi_m$ the fluorescent probes Rho123, DiOC$_3$(3), JC-1 and CMXRos were used. Cell monolayers were washed with experimental medium (DMEM containing 20 mM HEPES, pH 7.4 and 0.1% BSA) and then incubated in this medium with the fluorescent probes at 37°C under 5% CO$_2$ for the indicated lengths of time. Samples were treated in parallel with mCCCP or an equivalent amount of vehicle (control), which were added to the monolayers either before or after loading the probes, as indicated. At the end of the incubations cells were
washed twice with cold PBS, and resuspended by trypsinization at room temperature with 0.5 ml trypsin/EDTA (Biowhittaker, Wokingham) per well. After adding 0.5 ml trypsin neutralizing solution (Biowhittaker) the cell suspension was stored in the dark at 4°C until the time of analysis (usually within 30 min). PI (2.5 μg/ml) was added to cell suspensions stained with Rho123 or DiOC₆(3) to identify dead cells. In some experiments cells were first trypsinized, resuspended in experimental medium and then incubated with fluorescent probes prior to flow cytometric analysis (i.e. without washing away the free dye). Flow cytometry was performed on a FACScan instrument (Becton Dickinson, Oxford, UK) connected to an Apple G3 Computer. Data were acquired and analysed using CellQuest software (Becton Dickinson). The analyser threshold was adjusted on the FSC channel to exclude noise and most of the subcellular debris. Photomultiplier settings were adjusted to detect Rho123 and DiOC₆(3) fluorescence on the FL1 detector, CMXRos and PI fluorescence on the FL3 detector, and the JC-1 monomer and aggregate fluorescence signals on the FL1 and FL2 detectors, respectively. In each case the photomultiplier voltage was set so that the signal peak from non-stained cells (mostly due to autofluorescence), fell within the first decade of the logarithmic amplifier. Owing to the dual wavelength emission of JC-1, electronic compensation was used in this case to correct for spillage of the green (monomer) and orange/red (aggregate) fluorescence signals into the FL2 and FL1 channels, respectively. The linearity of the logarithmic amplifiers was checked routinely using standardized fluorescent beads (Immunobrite, Coulter). Light scatter parameters were used to establish size gates and to detect apoptotic cells [23].

Apoptotic cells were also identified by staining with fluorescein isothiocyanate-conjugated (FITC)-Annexin V and PI using a commercial kit (Annexin-V-FLUOS, Boehringer Mannheim). Briefly, after trypsinization, ~10⁵ cells were collected by centrifugation (5 min, 300 ×g), washed once with PBS and resuspended in 0.1 ml binding buffer containing FITC-Annexin V and PI. After 10-min incubation at room temperature in the dark, 0.7 ml of binding buffer was added and the cell suspension was analysed by flow cytometry as described above.

2.5. Confocal microscopy

Cardiomyocytes were plated into six-well plates containing methanol-sterilized glass cover slips and grown for 4–7 days as described above. Cells were treated with uncouplers and stained with fluorescent dyes in the same way as for flow cytometry. For JC-1, following staining the coverslips were washed with PBS at 4°C, mounted onto silicon-coated glass microscope slides and examined immediately. For CMXRos, prior to mounting, cells were fixed for 30 min in 2% paraformaldehyde at 4°C. Samples were viewed at room temperature in a darkened room with a Leica TCSNT laser scanning confocal imaging system coupled to a Leica DMRBE microscope, using a Leica 100 ×/1.4 NA fluorar objective. Probes were excited with the 488 or 568-nm lines of a 25-mW multi-line argon–krypton laser. The laser was set to the lowest power that was able to produce a fluorescent signal. JC-1 green fluorescence was visualized with a RSP580 and BF530/30 emission filter combination. Red fluorescence from JC-1 aggregates or from CMXRos was detected using a LP590 emission filter. Photomultiplier settings were the same for all acquisitions with a given dye. Images were acquired from randomly selected fields. For each field eight optical slices were taken along the optical axis at ~1-μm intervals. Optical sections close to the middle of the cells were chosen for visual evaluation of experimental treatments. Images were downloaded onto Adobe Photoshop and printed using a Kodak thermal printer.

2.6. Statistical analysis

Experiments were performed at least three times and unless otherwise indicated, results from one representative experiment are shown. Where indicated, levels of statistical significance were determined using the Student’s t-test. A P value below 0.05 was considered significant.

3. Results

3.1. Evaluation of the fluorescent dyes Rho123, DiOC₆(3) and CMXRos by flow cytometry

The traditional mitochondrial probe Rho123 has been widely used to evaluate Δψm in a variety of cell types [7,8,17,24]. Flow cytometric analysis of cardiomyocytes stained with this probe and PI revealed the presence of three distinct cell subpopulations (Fig. 1A). One subpopulation, which accounted for less than 5% of all the cells, was labelled with both PI and Rho123, suggesting that these are necrotic cells bearing leaky plasma membranes. The other two subpopulations consisted of cells that were impermeable to PI, giving respectively dim or bright Rho123 green fluorescence. Light scatter analysis (Fig. 1B) revealed that the cells within the Rho123dim subpopulation produced a distinct low FSC signal (Fig. 1B, purple dots), while those within the Rho123bright subpopulation produced a relatively high FSC signal, though with a broader distribution (Fig. 1B, red dots). Pre-treatment with the protonophore mCICCP at a concentration which normally should disrupt the Δψm of cardiomyocytes, did not affect significantly the Rho123 fluorescence distribution (Fig. 1C). Failure to reveal the collapse of Δψm was also observed when these experiments were repeated under various other experimental conditions, including the use of FCCP instead of mCICCP, adding the uncoupler after the dye had been already loaded, extending the period of dye
loading, using a range of Rho123 concentrations (1–25 μM), and/or loading the dye at 4°C followed by washing and further incubation at 37°C (data not shown).

Staining of cardiomyocytes with either 40 nM DiOC₃(3) or 25 nM CMXRos, two alternative putative Δψₘ-sensitive fluorescent probes [7,10,11], revealed in each case two cellular subsets displaying different levels of fluorescence (Fig. 1D and G) which corresponded, respectively, to cells with low and high FSC signals (Fig. 1E and H). However, like Rho123, these probes also failed to detect the collapse of Δψₘ induced by mCCCP (Fig. 1F and I). Furthermore, in the case of DiOC₃(3), in contrast to recent findings in lymphocytes [25], its lack of sensitivity to the effect of mCCCP was also observed at dye concentrations below 1
Fig. 2. Fluorescence histograms of cardiomyocytes stained with DioC(3): lack of effect of dye concentration on the response to mClCCP. Cardiomyocyte monolayers were incubated with the indicated concentrations of DioC(3). Thirty minutes after the initiation of dye loading mClCCP (5 µM, open tracings) or an equivalent amount of vehicle (filled tracings) was added and the incubation continued for another 30 min. Then cells were detached and analyzed by flow cytometry. Histograms depict all the acquired events. The dotted tracings correspond to a non-stained sample.

3.2. Phenotypic characterization of cardiomyocyte cultures

The apparent heterogeneity of the light scatter signals (Fig. 1), together with the observation that a significant fraction of cells (~30%) did not appear to beat, raised the possibility that our cell cultures contained, in addition to cardiomyocytes, a significant proportion of cells of one or more different phenotypic types. However, immunocytochemical analysis using an antibody against sarcomeric α-actin revealed that more than 90% of the cells stained positive for this antigen, suggesting that the majority were indeed cardiomyocytes (Fig. 3A). Alternatively, the heterogeneous nature of the light scatter patterns, and in particular the coincidence of the low FSC events with the low fluorescence signals of the mitochondrial dyes, could be attributed to the presence of a significant number of apoptotic cells. Consistent with this possibility, we found that maintenance of cardiomyocytes in suspension at 37°C in a glucose-containing balanced salt solution following trypsinization, resulted in a time-dependent increase in the proportion of Rho123<sub>high</sub>/FSC<sub>low</sub> cells (data not shown). Confirmation that the subpopulation with low FSC comprised mostly apoptotic cells was obtained by Annexin V staining, as shown in Fig. 3B and C. Taken together, the above series of experiments indicated that although staining with Rho123, DiOC<sub>6</sub>(3) or CMXRos enabled discrimination between viable and apoptotic cardiomyocyte subpopulations, this discrimination was probably not based on an ability of the dye to sense differences in Δψm.

3.3. Evaluation of the fluorescent dye JC-1 by flow cytometry

The carbocyanine dye JC-1 has been generally considered a more reliable and sensitive fluorescent probe for detecting differences in Δψm due to its dual emission characteristics [12–14]. At low concentrations JC-1 exists mainly in a monomeric form which emits green fluorescence. At high concentrations this molecule forms aggregates, known as J-aggregates, which emit orange-red fluorescence. Thus, since the amount of cationic dye taken up by the mitochondrion depends on its transmembrane potential, at low Δψm the fluorescence emission will be mostly green, whereas at high Δψm it will shift to orange-red. To evaluate this prediction preliminary experiments were performed in which cardiomyocyte monolayers were stained with JC-1 for 30 min in the absence or presence of mClCCP. Results of a typical experiment performed under these conditions are shown in Fig. 4. A two-parameter fluorescence display of untreated cultures, reveals that apoptotic cells (region R1, Fig. 4A) emitted mostly green fluorescence (Fig. 4B), whereas viable cells (region R2, Fig. 4A) emitted relatively high levels of both green and orange-red fluorescence (Fig. 4C). JC-1 fluorescence histograms of all the cells in the culture show that pre-treatment with mClCCP did not cause a substantial change in the green fluorescence pattern (Fig. 4D), but in contrast resulted in a striking decrease in the orange-red fluores-
Fig. 3. Phenotypic characterization of cardiomyocyte cultures. (A) Photomicrograph of cells stained for sarcomeric α-actin. In this culture 6.6±1.2% (mean of seven fields±S.D.) of the cells were negative for this marker; the arrow points to a negative cell. (B, C) Flow cytometric identification of apoptotic cells. Annexin V fluorescence histograms in C correspond to the events gated within regions R1 and R2 in B. PI positive events (4% of total) were excluded before analysis.

Fluorescence emission. These results suggested that prior disruption of Δψm inhibited the formation of JC-1 aggregates in mitochondria (Fig. 4D and E) and indicated that JC-1 could be an effective probe to monitor differences in Δψm using flow cytometry.

Incubation of cardiomyocytes with different doses of JC-1 revealed that the percentage of cells emitting bright orange-red fluorescence was strongly influenced by the concentration of dye used (Fig. 5). This study also indicated that under this set of experimental conditions the minimal dose of dye required to achieve an effective cellular loading was 0.5 µM (Fig. 5A). While performing these experiments, it was noteworthy that at low loading concentrations of JC-1, two cellular subsets could be discriminated on the basis of the green fluorescence signal alone (Fig. 5B), the brightest being sensitive to mClCCP pre-treatment (Fig. 5C). In contrast, at saturating concentrations of JC-1, the dim green fluorescence signal
Fig. 4. Flow cytometric patterns of cardiomyocytes stained with JC-1. Cells were incubated as in Fig. 1 but with 0.5 μM JC-1. After staining cells were detached and analyzed by flow cytometry. (A) Density plots of FSC versus SSC showing the analysis gates R1 and R2 surrounding apoptotic and viable cells, respectively. (B, C) Contour plots of FL1 (JC-1 green fluorescence) versus FL2 (JC-1 orange-red fluorescence) for the events gated within regions R1 (B) and R2 (C). (D, E) JC-1 green (D) and orange-red (E) fluorescence histograms of control (filled tracings) and mClCCP-treated samples (open tracings). mClCCP (5 μM) was added 15 min before dye loading. Histograms depict all the acquired events.

Disappeared while the orange-red fluorescence emission gave a better discrimination between Δψm low and Δψm high subpopulations (Fig. 5D). These results suggest that a careful titration of JC-1 is a pre-requisite to the adequate use of this dye for the evaluation of changes in Δψm in cardiomyocytes.

Fig. 6A shows a representative time course for the uptake of JC-1 (1 μM) by viable cardiomyocytes, as measured by the increase in monomer (green) and aggregate (orange-red) fluoroses. Under these conditions a 15-min period of incubation was enough for the JC-1 monomer to equilibrate between the cell and the bathing medium. However, the formation of JC-1 aggregates was a slow process (t₁/₂ ∼30 min) taking up to 90 min to attain near-steady state. As shown in Fig. 6B, addition of a low concentration of mClCCP (less than 10 μM) to cultures which had been previously incubated with JC-1 for a period long enough to allow aggregate formation to reach near-equilibrium, caused a partial decrease in the orange-red fluorescence emission of viable cells which was accompanied by an increase in green fluorescence. Higher concentrations of mClCCP caused further reductions in the orange-red fluorescence emission but also resulted in a drop of the green fluorescence. These results suggested that at low concentrations of uncoupler, as a result of a drop in Δψm, there was a dissociation of J-aggregates in the mitochondrion, whereas at high concentrations there was an efflux of the dye from the cell, probably as a result of the collapse of the plasma membrane potential (Δφp) (see Discussion).

The effect of mClCCP on the fluorescence emissions of JC-1 was further investigated in experiments which compared cell cultures that had been treated with a low (5 μM) or a high (50 μM) concentration of uncoupler, either before or after dye loading. As shown in Fig. 7, when a low concentration of mClCCP was used, the aggregate fluorescence decreased (upper panel) and the monomer fluorescence increased (lower panel), regardless of the timing of mClCCP addition. Similarly, at a high concentration of mClCCP the aggregate fluorescence decreased in both cases (upper panel). In contrast, at a high concentration of mClCCP the monomer fluorescence (lower panel) decreased when the uncoupler was added after dye loading, but remained unchanged if the uncoupler
was added beforehand. These results suggest that the timing of the addition of the dye is not crucial to reliably assess the usefulness of JC-1 in monitoring changes in $\Delta \psi_m$, provided that a low concentration of uncoupler is used.

### 3.4. Examination of cardiomyocytes by confocal microscopy

Confocal microscopy was used to validate the results obtained by flow cytometry. In images of cardiomyocytes stained with CMXRos mitochondria appeared as thread-like and granular structures concentrated around the nucleus or spreading throughout the cytoplasm (Fig. 8, control). mCICCP pre-treatment did not change significantly the overall intensity of CMXRos fluorescence. However, in this case images lost their definition and the fluorescence appeared more diffusely distributed through the cytoplasm (Fig. 8, mCICCP).

Confocal images of cardiomyocytes stained with JC-1 are shown in Fig. 9. Control cells showed heterogeneous staining of the cytoplasm with both red and green fluorescence co-existing in the same cell (Fig. 9, top panels). Consistent with a mitochondrial localization, the red fluorescence was mostly found in rod-shaped and granular structures distributed throughout the cytoplasm. The green fluorescence followed a similar pattern but overall gave a more blurred image, suggesting also some extra-mitochondrial localization. Superimposition of the green and red fluorescence images revealed a large degree of overlap (orange/yellow colour). A minority of mitochondria, though, exhibited only green fluorescence; these were most conspicuous in areas of cytoplasm surrounding the nucleus. Pre-treatment with mCICCP (Fig. 9, bottom panels) had a dramatic effect on the red fluorescence, which now became very faint. In contrast, it caused a slight but noticeable increase in the green fluorescence intensity, but did not affect the distribution substantially. As a result, the superimposed images showed only a few mitochondria stained orange, with the majority exhibiting green or yellow-green fluorescence.

### 4. Discussion

Cationic lipophilic fluorochromes with delocalised charges have been widely used to assess the functionality of mitochondria in diverse biological scenarios, including differentiation [17], apoptosis [7,10], ageing [8], and heart disease [5]. These dyes which are permeable to the plasma membrane, and therefore can be used on living cells, are generally thought to accumulate specifically in active mitochondria in proportion to the magnitude of their $\Delta \psi_m$ [6,14]. Hence, they have been extensively used to assess changes in this parameter in a variety of cellular models. Nevertheless, their use as sensors of $\Delta \psi_m$ may be potentially misleading; indeed some of these fluorochromes may undergo self-quenching upon accumulation in the mitochondrial matrix, or may show non-specific binding which may or may not be independent of electrical potential [13].
These possible pitfalls prompted us to re-assess some of the most commonly used mitochondrial stains for their ability to detect changes of $\Delta \psi_{\text{m}}$ in cultured cardiomyocytes. In this study we evaluated four dyes using flow cytometry: Rho123, DiOC$_6$(3), JC-1 and CMXRos. We found that only JC-1 reliably detected changes in $\Delta \psi_{\text{m}}$ induced by mitochondrial uncouplers. Furthermore, using confocal microscopy we confirmed the results obtained by flow cytometry.

JC-1 produces two fluorescence emission peaks that reflect the existence of two physical forms of the dye. The monomer, which is the predominant form at low concentrations or at low $\Delta \psi_{\text{m}}$, emits green fluorescence (emission maximum at 527 nm) and the so called ‘J-aggregate’, which is the predominant species at high concentrations of dye or high $\Delta \psi_{\text{m}}$, emits orange-red fluorescence (emission maximum at 590 nm). Earlier observations had suggested that the intensity of the monomer fluorescence is insensitive to changes in $\Delta \psi_{\text{m}}$ [12]. Based on this supposition some investigators have used JC-1 as a ratiometric probe [16,18]. This type of analysis would have had the advantage of making fluorescence measurements of $\Delta \psi_{\text{m}}$ independent of variations in mitochondrial mass or mitochondrial volume. Based on the same premise Mancini et al. [17] have relied on the green fluorescence emission of JC-1 to monitor changes in mitochondrial mass. In contrast, other investigators have reported that upon dissipation of $\Delta \psi_{\text{m}}$, along with the drop

Fig. 6. Kinetics of JC-1 uptake and sensitivity to mClCCP. (A) Cardiomyocyte monolayers were incubated with 1 $\mu$M JC-1. At the indicated times cells were detached and analyzed by flow cytometry. (B) After 90-min incubation as in A, monolayers were washed and incubated for a further 30 min with the indicated concentrations of mClCCP. Values correspond to the relative median green (open circles) and orange-red (filled circles) fluorescence intensities of viable cells. Results from one representative experiment are shown in each case.

Fig. 7. Effect of the timing of mClCCP addition on JC-1 fluorescence emissions. Cardiomyocyte monolayers were incubated with 1 $\mu$M JC-1 for total of 120 min. The indicated concentrations of mClCCP were added 15 min before or 90 min after the initiation of dye loading. Cells were detached and analyzed by flow cytometry. Fluorescence intensities of viable cells were scored relative to a sample treated in parallel with vehicle alone. Values represent the mean±S.D. from three to five experiments; * $P<0.05$ vs. control; ** $P<0.01$ vs. control.
in the intensity of the aggregate fluorescence there is an increase in the intensity of the monomer fluorescence; but no definitive explanation could be advanced to explain this increase [14,15]. In our own experiments using flow cytometry, we found that in cardiomyocytes the disruption of $\Delta \psi_m$ is more reproducibly monitored by examining changes in the aggregate fluorescence alone. For example, while mClCCP caused consistent changes in the orange-red fluorescence emissions of viable cells, it affected the green fluorescence signals less reproducibly, inducing in the majority of cases an increase in fluorescence, though of variable magnitude. For this reason a ratiometric analysis could not be relied upon. The explanation for the lack of reproducibility in the green fluorescence measurement is not obvious, although the contribution of a quenching effect cannot be discounted. The accuracy of the orange-red fluorescence measurement and the specificity of JC-1 were further confirmed in dose–response experiments which demonstrated the ability of the dye to discriminate unambiguously between cells with high and low $\Delta \psi_m$ in a saturable fashion (Fig. 5).

Various investigators have assessed the ability of dyes to detect changes in $\Delta \psi_m$, by adding uncouplers before dye equilibration (e.g. Ref. [10]), whereas others have argued that this procedure cannot distinguish between changes in $\Delta \psi_m$ and inhibition of dye transport across the plasma membrane [25]. The latter possibility could be particularly relevant at high concentrations of uncouplers which also appear to dissipate $\Delta \psi_p$ [25]. In our own experiments with rat cardiomyocytes, the fact that pre-treatment with mClCCP did not alter significantly the fluorescence intensities of either Rho123, DioC$_6$(3) or CMXRos (Fig. 1), the latter having being tested also by confocal microscopy (Fig. 8), suggests that any putative inhibition of dye...

Fig. 8. Confocal micrographs of cardiomyocytes labeled with CMXRos. Cardiomyocytes were grown on coverslips and stained with 25 or 100 nM CMXRos, in the absence (control) or presence of 5 $\mu$M mClCCP.
transport across the plasma membrane of these cells was not substantial enough to affect the eventual extent of dye loading. A similar conclusion can be attained in the case of JC-1, as demonstrated by the fact that, when low concentrations of mClCCP were used, increases in the green fluorescence emission of this dye were comparable, whether induced by adding the uncoupler before or after dye equilibration (Fig. 7). Furthermore, confocal microscopy demonstrated that although pre-treatment of cardiomyocytes with low concentrations of mCICCP prevented the formation of red aggregates in the mitochondria, it did not impair the overall accumulation of green monomers in the cell (Fig. 9). Thus, even if some inhibition of transport across the plasma membrane occurs, this effect is minimized by allowing enough time for dye equilibration. These findings indicate that JC-1 can be used to monitor changes in Δψm regardless of whether these changes are induced before or immediately after dye equilibration, emphasizing the versatility of the technique. In this context, addition of mCICCP before dye equilibration appears to be a more suitable positive control in those situations in which measurement of Δψm is used as an end point to study complex cellular processes.

During these experiments we also found that when the uncoupler was added after dye loading, the decrease in orange-red fluorescence, though still significant (P=0.01), was not as pronounced as when the uncoupler was added beforehand. This could be explained as follows. When the uncoupler is added before dye equilibration, aggregate formation in the mitochondrion is largely prevented due to the pre-established low Δψm. In contrast, when the uncoupler is added after equilibration, the fall in orange-red fluorescence reflects the extent of dissociation of the aggregates that had been formed prior to the fall in Δψm. Thus, these findings may reflect a slow rate of JC-1 aggregate dissociation.

It is now widely recognised that a drop in Δψm takes place during the course of apoptotic cell death [26]. In our flow cytometric profiles apoptotic cells were recognised on the basis of well-established light scatter characteristics [23,27], a finding which we also confirmed by Annexin V staining (Fig. 3). In addition, as previously found in other systems [7,10], these cells showed a diminished stainability with all four mitochondrial dyes tested (Figs. 1 and 4). However, for three of the dyes, namely Rho123, DioC₆(3) and CMXRos, this reduction in staining could not be attributed to the putative fall in Δψm, since the same dyes could not detect the disruption of Δψm induced by mCICCP (Figs. 1, 2 and 8). Attempts to provide a definitive explanation for the differences between our
findings in cardiomyocytes and those reported in other cell types are beyond the experimental reach of this study. A major difference in the intracellular architecture of the cardiomyocyte, or in the macromolecular composition of its mitochondrial membrane, for example in cardiolipin content, could account for these discrepancies. Alternatively, this reduced stainability of apoptotic cardiomyocytes could be due to a drop in $\Delta \psi_m$, a phenomenon recently reported to occur in lymphocytes during the early stages of X-ray-induced apoptosis [28].

Although JC-1 has been previously used to study mitochondria in intact cardiomyocytes [5,13,14,18], there have not been reports on the use of this or any other fluorescent probe to evaluate changes of $\Delta \psi_m$ by flow cytometry in this cell. The study of $\Delta \psi_m$ by flow cytometry has several advantages over other techniques. In particular, it allows the analysis of heterogeneous cell populations and is amenable to multiparametric measurements. Its usefulness in the study of cardiomyocyte pathophysiology is underscored by the fact that it can combine $\Delta \psi_m$ measurements with other parameters associated with cardiomyopathies or cardiac injury, including apoptosis, oxidative stress, and changes in calcium content.

Mitochondria are mechanistically involved in cellular functions that transcend the traditional role of these organelles as the energy factory of the cell. This, together with the realization that the alteration of $\Delta \psi_m$ may have important pathophysiological consequences, has led to a rise in the interest of measuring this variable in a variety of biological settings, including cardiovascular disease. The present study calls for caution against the indiscriminate use of potentiometric dyes to study the functionality of mitochondria in cardiomyocytes.

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Mitochondria are mechanistically involved in cellular functions that transcend the traditional role of these organelles as the energy factory of the cell. This, together with the realization that the alteration of $\Delta \psi_m$ may have important pathophysiological consequences, has led to a rise in the interest of measuring this variable in a variety of biological settings, including cardiovascular disease. The present study calls for caution against the indiscriminate use of potentiometric dyes to study the functionality of mitochondria in cardiomyocytes.


