JC-1, but not $DiOC_6(3)$ or rhodamine 123, is a reliable fluorescent probe to assess $\Delta\Psi$ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis

Stefano Salvioli[a], Andrea Ardizzoni[a], Claudio Franceschi[a,b], Andrea Cossarizza[a,*]

[a] *Department of Biomedical Sciences, Section of General Pathology, Via Campi 287, 41100 Modena, Italy*
[b] *INRCA, Department of Gerontological Research, Ancona, Italy*

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Abstract The sensitivity and specificity of three fluorescent probes used for cytofluorimetric analysis of mitochondrial membrane potential ($\Delta\Psi$) were studied in the U937 human cell line. First, the role of plasmamembrane in influencing the binding of the probes to mitochondria has been investigated. The depolarization of plasmamembrane with high doses of extracellular KCl had no immediate effects on the loading of JC-1, $DiOC_6(3)$ and rhodamine 123 (R123). However, after a few hours of culture in the presence of KCl, significant changes were observed only in cells stained with $DiOC_6(3)$. Second, a comparative study was performed concerning the effects of agents capable of collapsing $\Delta\Psi$. While adding FCCP to cell cultures resulted in consistent changes in the fluorescence emission of both JC-1 and $DiOC_6(3)$ – but not of R123 – only cells stained with JC-1 responded to valinomycin. On the whole, our data indicate that JC-1 is a reliable probe for analyzing $\Delta\Psi$ changes with flow cytometry, while the others show a lower sensitivity (R123), or a non-coherent behaviour, due to a high sensitivity to changes in plasmamembrane potential [$DiOC_6(3)$]. These data cast some doubts on those studies that, using fluorescent probes that have a low sensitivity to $\Delta\Psi$, hypothesized that the fall in $\Delta\Psi$ is one of the early events, if not one of the main causes, of apoptosis.

Key words: Mitochondrial membrane potential; JC-1; $DiOC_6(3)$; Rhodamine 123; Apoptosis

1. Introduction

The assessment of mitochondrial membrane potential ($\Delta\Psi$) in intact cells is raising a growing interest, considering the role that such organelle has in many physiopathological conditions. In particular, the functionality of mitochondria during apoptosis is still a matter of debate [1–5]. Few years ago, we developed a method for the analysis of $\Delta\Psi$ in intact, living cells by using a fluorescent dye, i.e. the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) [6]. Such molecule exists in a monomeric form, emitting at 527 nm after excitation at 490 nm [7]. In the presence of a high $\Delta\Psi$, JC-1 forms the so called J-aggregates that are associated with a large shift in emission (590 nm) [8,9]. This cytofluorimetric method *(i)* is both qualitative and quantitative; *(ii)* allows the identifications of populations with different mitochondria content; *(iii)* has already been utilized for studying the behaviour of these organelles in a variety of conditions, including apoptosis; *(iv)* has been further validated by analyzing $\Delta\Psi$ at the level of single mitochondria [10–16].

In order to stain mitochondria, any probe has to entry into the cell and to reach the organelles. Its cytoplasmic accumulation is a crucial event, because a critical intracellular concentration is required to obtain an adequate fluorescence signal [17]. Obviously, for lipophilic, cationic molecules, such accumulation mainly depends upon plasmamembrane potential (PMP). With the aim to analyze whether changes in PMP can influence mitochondria stainability, and to ascertain whether such changes can mimic modifications in $\Delta\Psi$, we tested the effect of plasmamembrane depolarization (PD) on cells stained with JC-1 or other different fluorescent probes capable of binding mitochondria, such as rhodamine 123 (R123) and 3,3′-dihexiloxocarbocyanine iodide [$DiOC_6(3)$]. U937 human cells were used and treated with substances able to dissipate PMP such as a KCl-rich medium or the Na^+/K^+ ATPase inhibitor ouabain, or with drugs able to collapse $\Delta\Psi$, such as the K^+ ionophor valinomycin and the proton translocator carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP).

2. Materials and methods

2.1. Cell cultures

U937 human monoblastic leukaemic cell line was cultured in complete medium (RPMI 1640 with 10% heat inactivated foetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine) and kept at 37°C in humidified atmosphere (5% CO_2 in air). Cells were collected during the log phase of growth, washed in phosphate buffered saline (PBS), counted and adjusted to the density of 200 000/ml.

2.2. Calibration curve

Cells were resuspended in PBS and immediately stained with different concentration of JC-1, $DiOC_6(3)$ or R123 (Molecular Probes, Eugene, OR, USA), and incubated for 20 min. at room temperature in the dark. The dyes were dissolved in *N,N*-dimethylformamide (DMF) (Sigma, St. Louis, MO, USA) and stored according to the manufacturer's instruction. The percentage of the organic solvent in the samples never exceeded 1% vol/vol. In some experiments, cells were resuspended in PBS containing different concentrations of KCl (50, 100, 125, and 150 mM) (PBSK). At the end of the incubation period, cells were washed twice with PBS or PBSK, resuspended in a total volume of 400 μl of PBS or PBSK and analyzed.

*Corresponding author. Fax: (39) (59) 428-623.
E-mail: cossariz@unimo.it

Abbreviations: JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; R123, rhodamine 123; $DiOC_6(3)$, 3,3′-dihexiloxocarbocyanine iodide; $\Delta\Psi$, mitochondrial membrane potential; PMP, plasmamembrane potential; PD, plasmamembrane depolarization; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone

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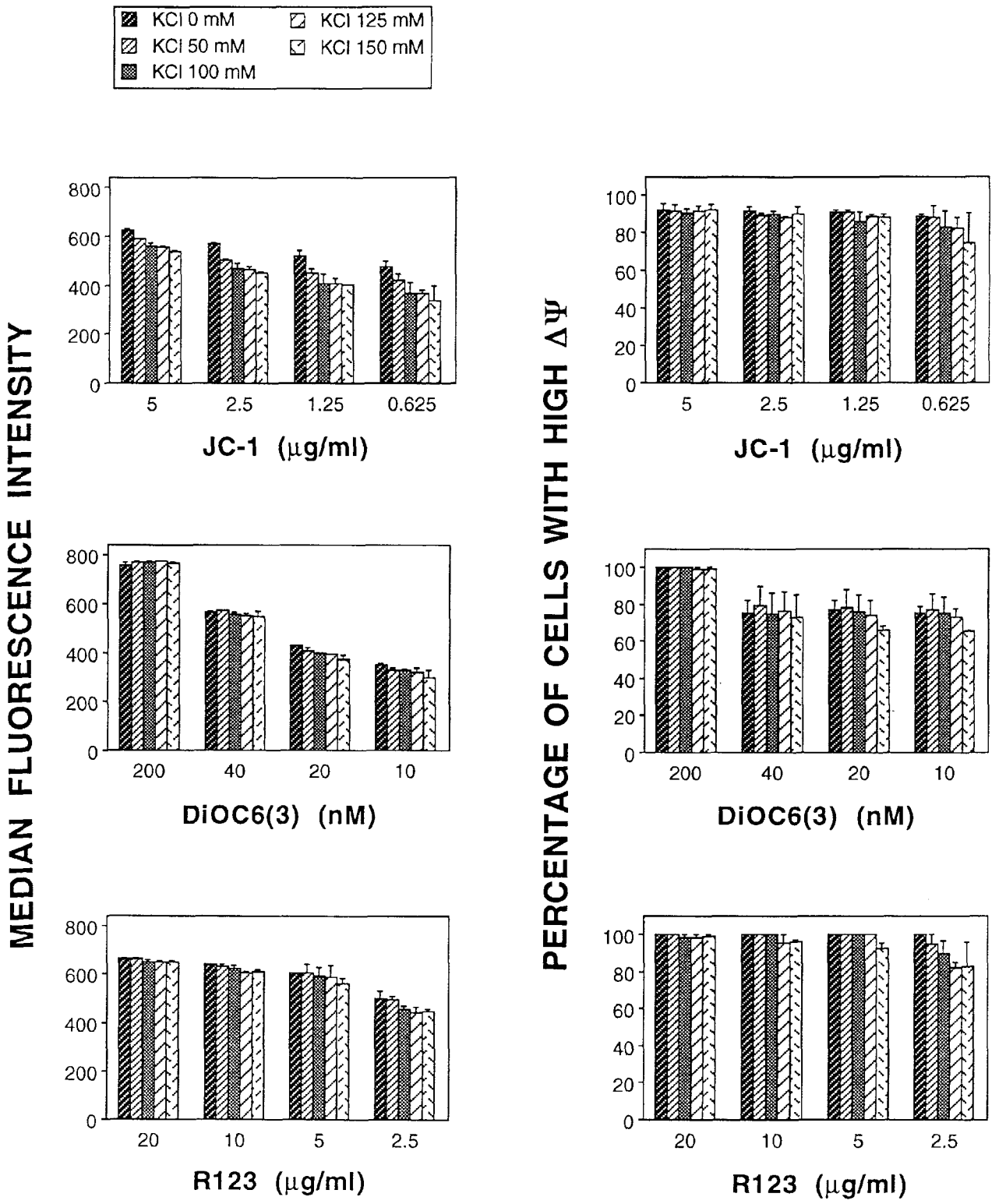

Fig. 1. Effect of a graded and immediate plasmamembrane depolarization on the loading of the dyes. Left column: median fluorescence intensity. Right column: percentage of cells with high $\Delta\Psi$. Data are expressed as mean ± S.E.M. and are referred to 2–5 experiments.

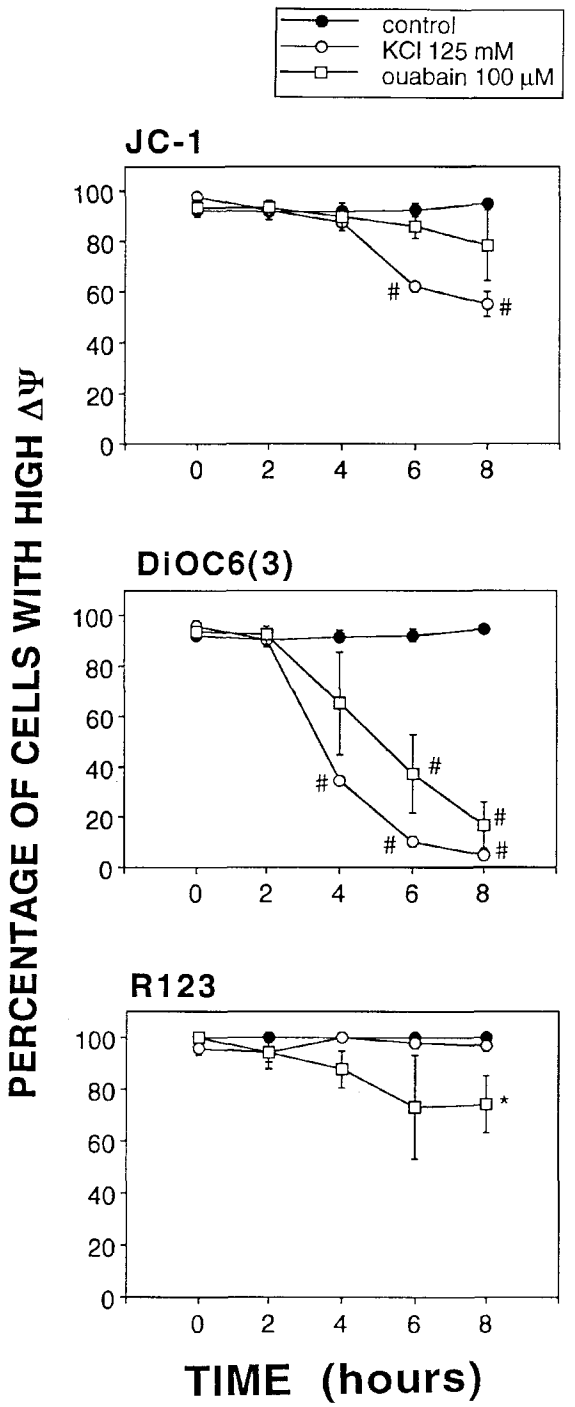

Fig. 2. A prolonged exposure to plasmamembrane depolarizing agents has different effects on the staining with the fluorescent probes. Data are expressed as mean ± S.E.M. and are referred to 4 experiments. *, $P<0.05$; #, $P<0.01$ by two-tailed paired Student's t-test.

2.3. Kinetic studies

Cells were resuspended in fresh medium and seeded in polystyrene culture flask (Corning, New York, USA) together with the following drugs: valinomycin 100 nM, FCCP 250 nM, ouabain 100 μM (all from Sigma), nigericin 1 μg/ml (Molecular Probes). These substances were dissolved in DMF, whose percentage in the samples never exceeded 0.1% vol/vol. Parallel samples were cultured in complete medium plus KCl 125 mM in order to abrogate PMP. Cells were then kept at 37°C in humidified atmosphere for different periods (0, 2, 4, 6, and 8 h). At each time point, 1 ml of cell suspension was collected from the culture flasks, stained with JC-1 5 μg/ml, $DiOC_6(3)$ 40 nM, or R123 10 μg/ml and processed as described above.

2.4. Flow cytometry

The analysis of the samples was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). For cells stained with JC-1, the values of the photomultipliers (PMT) and of the compensations were set as previously described [6]. For the analysis of the cells stained with R123, the FL1 PMT was set at 330 V, FL2 PMT at 150 V. As $DiOC_6(3)$ and R123 have a single wavelength emission, no compensation was required. A minimum of 103 000 cells per sample were acquired in list mode and analyzed with Lysys II software. In the case of JC-1, cells with a high $\Delta\Psi$ were those forming J-aggregates; in the case of $DiOC_6(3)$ and R123 a high $\Delta\Psi$ was attributed to cells with a high fluorescence signal in FL1.

3. Results

Fig. 1 shows the effects of an 'acute', graded PD on the capability of different dyes to load mitochondria. Cells were resuspended in PBS plus KCl 50, 100, 125 or 150 mM, collected and stained with different concentrations of the dyes. When PBS was supplemented with Kcl 125 Mm, i.e. when extracellular K^+ concentration was slightly lower than the intracellular one, the PMP was quite reduced; at 150 mM PMP was completely abrogated [18–20]. Left column shows the median fluorescence intensity of each dye, right column the percentage of cells forming J-aggregates, or having a high fluorescence intensity. Fluorescence intensity was directly correlated to the amount of the dye used, independently from PMP. At all concentrations of any dye, PD had no effect on the mitochondrial loading of the fluorescent probes. On the basis of these results, it was established that in our experimental conditions the minimal effective doses needed to have an optimal cellular loading independently on an immediate PD were 5 μg/ml, 40 nM and 5 μg/ml for JC-1, $DiOC_6(3)$, and R123, respectively. On the other hand, as shown in the right panels, the percentage of cells able to form J-aggregates was unaffected not only by PD but also by the amount of the dye. The same was observed for R123 and $DiOC_6(3)$, as cells were able to maintain a high fluorescence profile independently from PD.

As we observed no relevant changes in cells whose plasmamembrane was 'acutely' depolarized, we asked whether mitochondrial staining could be influenced by a 'chronic', prolonged PD. Interestingly, we found that after a few hours of incubation in KCl-rich medium or in the presence of ouabain, cells stained with $DiOC_6(3)$ changed dramatically their fluorescence patterns (Fig. 2). Cells stained with JC-1 or R123 did not present relevant changes, even if a small decrease in fluorescence was observed after 6 and 8 h. Fig. 3 displays typical examples of the fluorescence distribution of cells stained with the probes used, after the treatment with different agents capable of collapsing PMP or $\Delta\Psi$. A consistent decrease in $DiOC_6(3)$ fluorescence occurred when cells were kept in the presence of ouabain or in a KCl-rich medium, whereas the signal from cells stained with JC-1 and R123 was only marginally affected. Valinomycin and FCCP caused a complete modification of JC-1 fluorescence. $DiOC_6(3)$ fluorescence changed only in cells treated with FCCP. In any case, it is to note that such decrease in fluorescence is much lower than that expected, as the peak of FCCP-treated cells was in a position which is intermediate between those of cells with

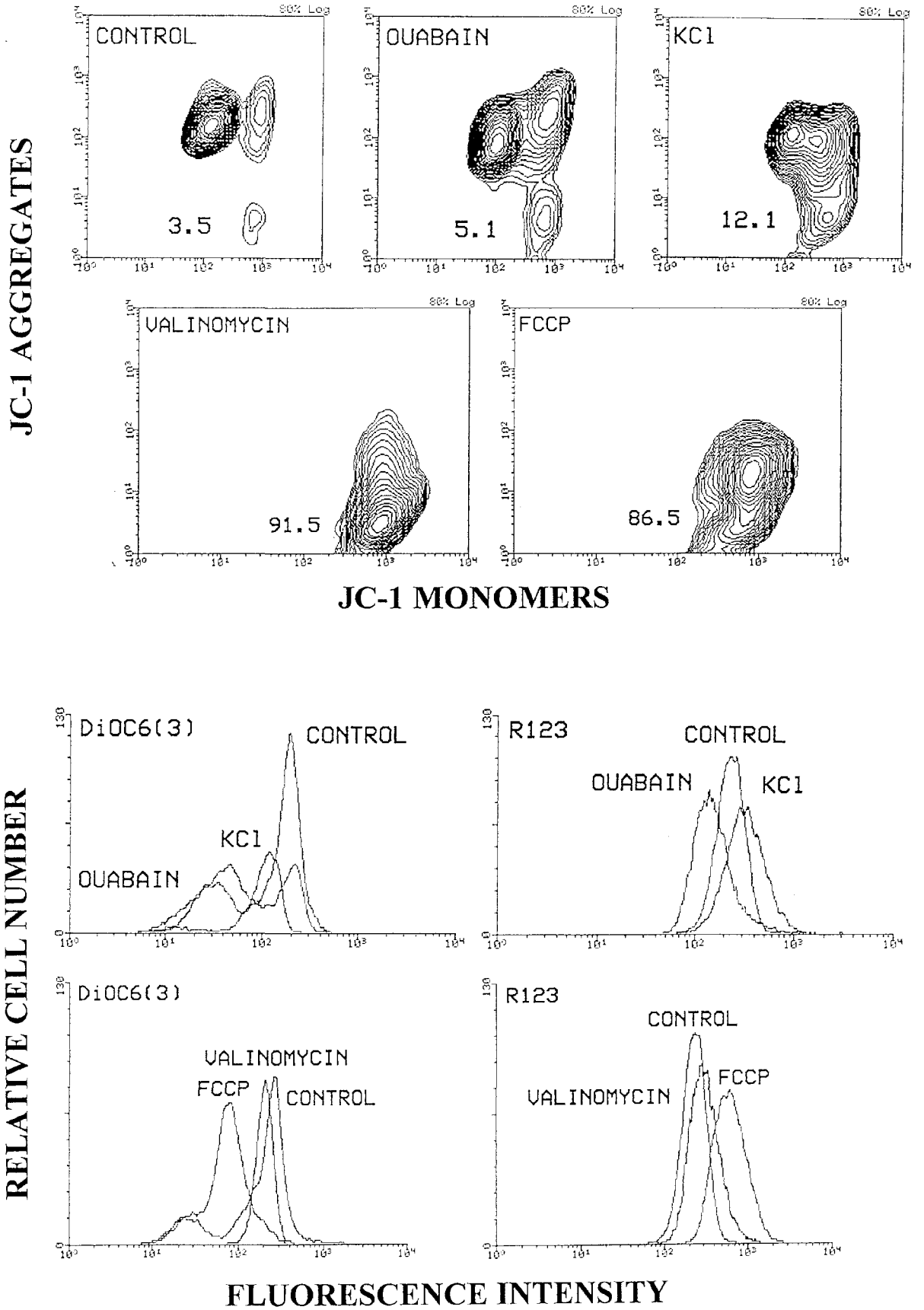

Fig. 3. Representative examples of the fluorescence pattern of cells stained with JC-1 (upper part), $DiOC_6(3)$ or R123 (lower part) after a 4-h incubation with agents affecting PMP (ouabain or a KCl-rich medium) or with agents affecting $\Delta\Psi$ (valinomycin or FCCP). Numbers indicate the percentage of cells with depolarized mitochondria.

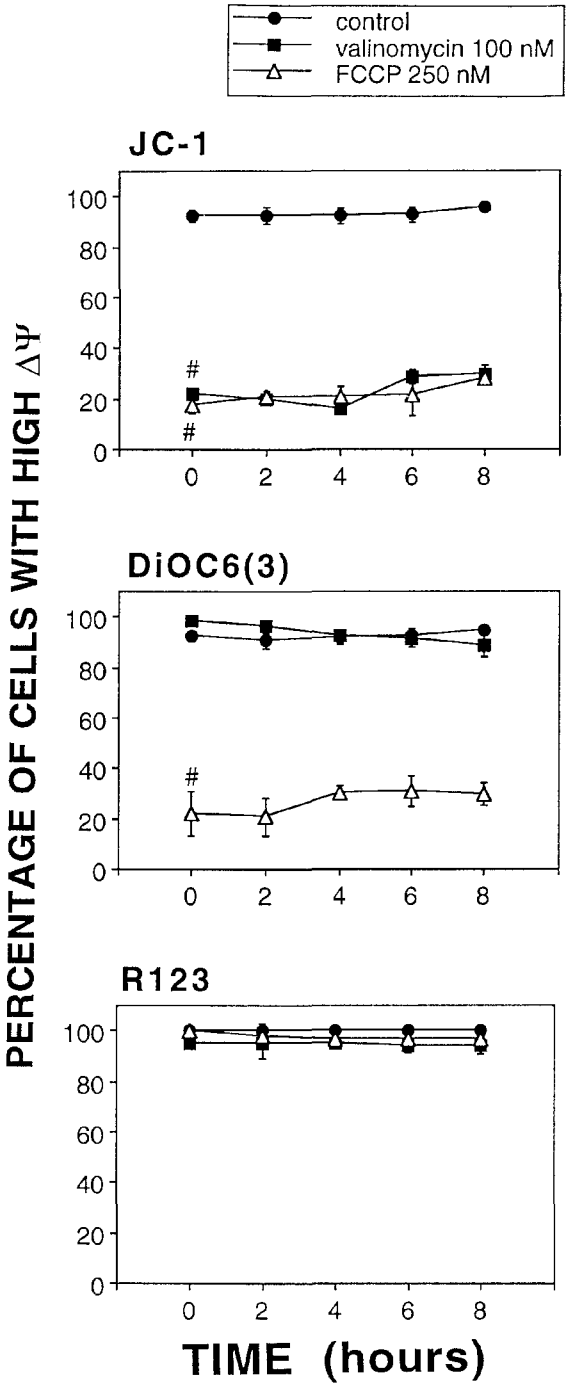

Fig. 4. Effects of a prolonged exposure to ΔΨ depolarizing agents. Data are expressed as mean ± standard error and are referred to 4 experiments. #, in all points; $P < 0.01$ by two-tailed paired Student's *t*-test.

high and low fluorescence. Cells stained with R123 were apparently unaffected by ΔΨ dissipating agents. As far as the effects of a 'chronic' collapse in ΔΨ on the fluorescence signals are concerned, Fig. 4 shows that the changes in the fluorescence distribution did not change with time for the 3 probes investigated. In all cases, the K^+/H^+ ionophor nigericin, that affects intracellular pH but not ΔΨ, had no effect (not shown). Finally, a parallel cytofluorimetric analysis revealed that nigericin, FCCP, valinomycin or different amounts of KCl had no gross effects on cell viability; a moderate increase in cell death (less than 20% vs. 5% in control samples) was only observed in cells treated with ouabain for 8 h (data not shown).

4. Discussion

Until late seventies, fluorimetric methods using cyanine dyes have been utilized to measure the potential of different cell membranes [17,21–23]. Some of these dyes have also been used for measuring ΔΨ [6,24,25]. For example, plasmamembrane potential has been studied by comparing the use of $DiOC_6(3)$ in flow cytometry with results obtained with glass microelectrodes [26]; ΔΨ by analyzing JC-1 in parallel with the distribution of phosphonium ions [16]. In both cases, highly significant correlations were found which allowed to validate the use of flow cytometry for analyzing PMP or ΔΨ, with $DiOC_6(3)$ or JC-1, respectively.

$DiOC_6(3)$ has been recently used for analyzing ΔΨ [2–4], but few methodological data are available concerning the reliability of such molecule for measuring ΔΨ in intact cells. Theoretically, due to the necessity of an adequate intracellular concentration of fluorescent dyes, changes in PMP influence mitochondria stainability, and can mimic changes in ΔΨ. In order to investigate this possibility, we tested the effects of PD on three probes such as JC-1, R123 and $DiOC_6(3)$. JC-1 staining is unaffected by agents that depolarize plasmamembrane, while is strongly affected by drugs that dissipate ΔΨ, confirming that JC-1 is a reliable probe for analyzing ΔΨ changes with flow cytometry.

$DiOC_6(3)$ and R123 seem to be less useful for this purpose because of a non-coherent behaviour. R123 was the first fluorescent probe used for cytofluorimetric studies on ΔΨ, and several authors reported that it behaves as expected in the presence of ΔΨ dissipators and inhibitors of respiratory chain, either in intact cells or isolated mitochondria [24,27,28]. On the contrary, others found that this probe was not fully satisfactory [29]. The binding of R123 to mitochondria occurs with different fluorescence intensities through the formation of putative adducts with some components of the inner mitochondrial membrane. Some of them are freely accessible whatever the energy status of mitochondria, but others are hidden in the energized state and freely accessible in the deenergized form of the organelle. The specific association of R123 to F_0F_1-ATPase could explain the misleading results of membrane potentials measured in the presence of oligomycin [28]. Here we show that, unexpectedly, in U937 cells ΔΨ dissipators had no consistent effects on R123 fluorescence. This behaviour may be explained, at least in part, taking into account the possible presence of several energy-independent R123 binding sites in such cell line, and is similar to previous observations in invertebrate cells [10].

$DiOC_6(3)$ appeared to be more reliable for estimations on PMP rather than studies on ΔΨ. In fact, even if the probe reacted properly when cells were treated with FCCP, such behaviour was not observed in the case of valinomycin. Moreover, a high sensitivity to PD was noted. This was not unexpected, as it is known that this probe can bind several membranes other than mitochondria [30], a phenomenon also reported in the 5th Edition of the Handbook of Fluorescent Probes and Research Chemicals (the 1992–1994 catalogue of the Company that produces and sells this reagent, edited by R.P. Haugland). Moreover, the FCCP-induced de-

crease in $DiOC_6(3)$ fluorescence emission is much lower than that expected if this probe had an exclusive distribution to mitochondria. Indeed, a complete shift towards the left part of the distribution curve, i.e. where all cells with depolarized mitochondria were expected to go, was not noted. This can been explained taking into account that, once released by depolarized mitochondria, $DiOC_6(3)$ could redistribute in other intracellular membranes such as those of endoplasmic reticulum. This could result in a small change in the total cellular fluorescence. Moreover, the marked fluorescence decrease occurring in the presence of plasmamembrane depolarizing agents such as ouabain or high doses of extracellular K^+ indicates a consistent sensitivity of $DiOC_6(3)$ for PMP. Thus, using this probe, it is very difficult, if not impossible, to distinguish between depolarization of plasmamembrane or changes in $\Delta\Psi$ in several physiological or pathological conditions, when both events can take place. In any case, together with different reports from ours and other groups [1,5,31–33], the data here presented cast some doubts on those studies which, using a PMP-sensitive probe such as $DiOC_6(3)$ for analyzing $\Delta\Psi$ [34], generalized that mitochondria depolarization is one of the first events occurring during apoptosis, if not its cause [2–4,35,36]. However, this topic is still a matter of debate [37].

Finally, even if preliminary results in rat thymocytes confirm the behaviour of these probes using the aforementioned experimental conditions, further studies are needed to clarify if similar observations can be extended to other cell types.

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