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Quantitative assay by flow cytometry of the mitochondrial membrane potential in intact cells

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Abstract

Mitochondrial membrane potential, in situ, is an important indicator of mitochondrial function and dysfunction. Because of recent interest in the role of mitochondria in signaling, cell injury and cell death, there is a need for a convenient, sensitive and accurate method for the measurement of the mitochondrial membrane potential, $\Delta\psi_m$, in situ, in a heterogeneous cell population. We have adapted a flow cytometry method for the quantitative measurement of $\Delta\Psi_m$ which utilizes the lipophilic, cationic, fluorescent probe 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$). We developed a new protocol in which cells are equilibrated with very low dye concentrations (<1 nM). Only under these condition, the cell fluorescence appears to be correlated with the magnitude of $\Delta\Psi_m$, as evident from the sensitivity of the fluorescence to low concentrations of uncouplers, ionophores and inhibitors of the mitochondrial proton pumps. The magnitude of the plasma membrane potential, $\Delta\Psi_p$, also affects cell fluorescence, and a procedure that corrects for this effect is outlined. This method offers a distinct advantage over existing methods for estimation of $\Delta\psi_m$ by flow cytometry. 0167-4889/98/$ – see front matter

Keywords: Mitochondria; Membrane potential; Lymphocyte; Flow cytometry

1. Introduction

Recent studies suggest that mitochondria play a role in cell signal transduction by calcium, NO and reactive oxygen species [1–4], and in pathways leading to cell death [5,6]. Mitochondrial dysfunction is believed to contribute to an increasing number of diseases, and in particular, to aging-associated, degenerative diseases of nerve, muscle, and other tissues [7–10].

The mitochondrial membrane potential, in situ, $\Delta\psi_m$, is a sensitive indicator for the energetic state of the mitochondria and the cell [11], and can be used to assess the activity of the mitochondrial proton pumps, electrogenic transport systems, and the activation of the mitochondrial permeability transition [12].

Several cationic, fluorescence probes have been used, in conjunction with different imaging techniques, most recently confocal microscopy, to estimate $\Delta\psi_m$ in different cells [13–15]. However, because these techniques allow measurements only in a few cells at a time, analysis of $\Delta\psi_m$ in a large population of cells is not practical. This is a serious limitation when studying inhomogeneous cell popu-

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lations (e.g. lymphocytes), or when dysfunctional mitochondria are segregated in a fraction of the cell population, as is the case in many mitochondrial disease. Also when studying a slow cellular process that is not synchronized (e.g. apoptosis), quantitative changes of $\Delta\psi_m$ cannot be evaluated reliably from studies of a few single cells.

A quantitative method which is applicable to a large population of cells is based on the distribution of lipophilic cations between cells and the suspending medium, which can be determined with an electrode or by the use of radioactive tracers [16,17]. However, this method requires a very large number of cells (10^7–10^8) for each determination, and is not sensitive to the modulation of $\Delta\psi_m$ in a fraction of the cell population.

Flow cytometry, when used in conjunction with fluorescent $\Delta\psi_m$ probes, could be an ideal method for the investigation of mitochondrial function, in situ. This method could provide estimates of the magnitude of $\Delta\psi_m$ in a large number of individual cells, and is therefore ideally suited for studies of heterogeneous cell populations. Moreover, it requires a relatively small number of cells (10^3–10^5), and is easy to execute rapidly with commercially available flow cytometers.

In recent years, many investigators have used lipophilic cationic probes in flow cytometry assays, in an effort to measure the magnitude of $\Delta\psi_m$ in a variety of cells. In particular, recent studies of the role of mitochondria in apoptosis, were largely based on the use of flow cytometry assays of $\Delta\psi_m$ ([18], reviewed in [6]).

Here we evaluate the utility of the lipophilic, cationic probe, $DiOC_6(3)$, for $\Delta\psi_m$ determinations in spleen lymphocytes. We found that the assay, as commonly used, is flawed, and suggest an improved protocol that provides a sensitive and reliable estimate of $\Delta\psi_m$.

2. Materials and methods

Spleen lymphocytes were prepared from young (3–4-month-old) balb/c or C57BL mice. The spleen was removed and pressed through a nylon mesh in 5 ml HBSS medium (+0.3% BSA). The cell suspension was centrifuged at $800\times g$, for 6 min, at 4°C. The pellet was resuspended in 7 ml of the same medium, centrifuged again and washed once more the same way. The washed cells were resuspended in 5 ml of the same medium and layered on 5 ml Lympholyte M (Accurate), and centrifuged at $1500\times g$ for 20 min, at 25°C. The lymphocytes were removed from the interphase, washed twice in HBSS and suspended in 2 ml of MEM (+1% FCS). The isolated lymphocytes were kept at room temperature for 1–4 h before the experiments. All the experiments shown here were performed at 37°C in MEM medium (+1% FCS, 1 mM Ca^{2+}, 5.5 mM glucose and 2.5 mM glutamine). Similar results were obtained in RPMI medium.

2.1. Cell respiration

Respiration rates were measured by a polarographic oxygen electrode, usually with 1.5×10^7 cells in 1.5 ml, at 37°C.

2.2. Flow cytometry

Flow cytometry and data analysis were carried out on a FACScan instrument (Becton Dickinson), excitation = 488 nm, emission = 530 nm (F1), using the program Lysis.

All incubation media (MEM, HBSS, RPMI), and the following reagents: FCS, oligomycin, valinomycin, myxothiazol, nigericin, ionomycin, rotenone, and carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) were from Sigma. Lympholyte M was from Accurate. Cyclosporin was a gift from Sandoz. $DiOC_6(3)$ was from Molecular Probes.

3. Results

$DiOC_6(3)$ has been used recently as an indicator of $\Delta\Psi_m$ in a large number of flow cytometry studies (cf. [18], reviewed in [6]). However, similar to other lipophilic carbocyanines, $DiOC_6(3)$ is known to be a potent inhibitor of NADH dehydrogenase in isolated mitochondria [19]. Because cells accumulate lipophilic cations, which are concentrated in the cytoplasm by the plasma membrane potential $\Delta\Psi_P$ (see below), it is expected that mitochondrial respiration, in situ, would be even more sensitive to these dyes

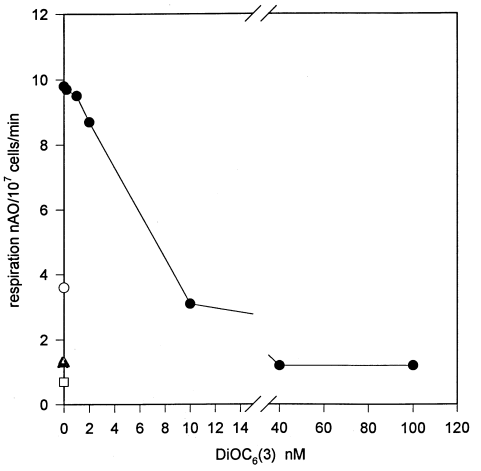

Fig. 1. The effect of $DiOC_6(3)$ on lymphocyte respiration. Assay conditions are described in Section 2. Empty circle, basal respiration; empty square, +oligomycin (0.1 μg/ml); full circles, +oligomycin+CCCP (5 μM) and the indicated $DiOC_6(3)$ concentrations; empty triangle, +oligomycin+CCCP+rotenone (10 nmol/ml).

than in isolated mitochondria. Fig. 1 shows the effect of increasing concentrations of $DiOC_6(3)$ on uncoupler-stimulated lymphocyte respiration. It is observed that the concentrations of dye that has been used previously in assays of $\Delta\psi_m$, namely 40–100 nM (cf. [18]), inhibits cell respiration approximately 90%. This is the same as the maximal inhibition obtained in lymphocytes by potent NADH dehydrogenase inhibitors, such as rotenone (Figs. 1 and 4). Even 2 nM $DiOC_6(3)$ resulted in a small, but significant, inhibition. Since $\Delta\psi_m$ is generated by the electron transport complexes in most cells, and NADH dehydrogenase is the major site of electron entry into the electron transport chain in most cells, it is apparent that this dye cannot be used for reliable estimation of $\Delta\Psi_m$ at concentrations higher than 2 nM. We obtained similar results in cells of a malarial parasite, in which respiration was significantly inhibited above 2 nM [20].

It is also well-known that the $\Delta\psi_m$-driven accumulation of lipophilic cationic dyes on the inner surface of the inner mitochondrial membranes results in extensive, concentration-dependent, quenching of the dye fluorescence (cf. [21]). Indeed, all the methods for $\Delta\psi$ estimation from the fluorescence of cationic probes in suspensions of mitochondria, liposomes and bacterial cells are based on the magnitude of the *quenching* of the suspension total fluorescence, which results from the quenching of the membrane bound dye. In flow cytometry, only the fluorescence of the cell bound dye is measured, and $\Delta\psi_m$ is estimated from the intensity of the cell fluorescence and not from the quenching of the total suspension fluorescence. Therefore, to maximize the response of the cell fluorescence to the magnitude of $\Delta\psi_m$, it is necessary to minimize the quenching of the mitochondrial bound dye. Since the quenching of $DiOC_6(3)$ can be observed in suspensions of isolated mitochondria at the nanomolar concentration range (results not shown), it was necessary to lower the dye concentration to subnanomolar.

In intact cell suspensions, the accumulation of lipophilic cations from the medium to the mitochondria depends not only on $\Delta\psi_m$, which determines the distribution of the cation between the cytosol and the mitochondrial matrix, but also on the magnitude of $\Delta\psi_p$, which determines the cation distribution between the cytosol and the medium [16,17]. Therefore, both $\Delta\psi_m$ and $\Delta\psi_P$ determine the magnitude of the fluorescence at a given dye concentration. The relative contribution of each of these parameters to the total fluorescence signal depends not only on their relative magnitude, but also on the cell density, and the amount of mitochondrial membranes in the cell. In general, reducing the dye concentration (or more precisely the dye/cell ratio) tends to increase the contribution of $\Delta\psi_m$ to the cell fluorescence [22]. Lowering the dye/cells ratio reduces the self-quenching of the mitochondrial bound dye, and increases the ratio of the amount of cell-associated dye to that which is free in the medium. Under these conditions, modulation of $\Delta\psi_p$, would mostly modulate the amount of free dye in the medium, which is relatively small, and therefore would have a relatively small effect on the amount of cell-associated dye.

For example, if only 1% of the dye is free in the medium, then reducing $\Delta\psi_p$ by one third, e.g. from -60 to -40 mV, would increase the free dye in the medium to about 2%, and thus reduce cell fluorescence by less than 1%. If the dye is lipophilic and the mitochondrial membrane potential is high, at least 95% of the cell-bound dye will be in the mitochondria. Reducing $\Delta\psi_m$ by one-third, e.g. -180 to -120 mV, would release more than 90% of the mitochondria-bound dye to the cytosol, most of which would be subsequently released to the medium to

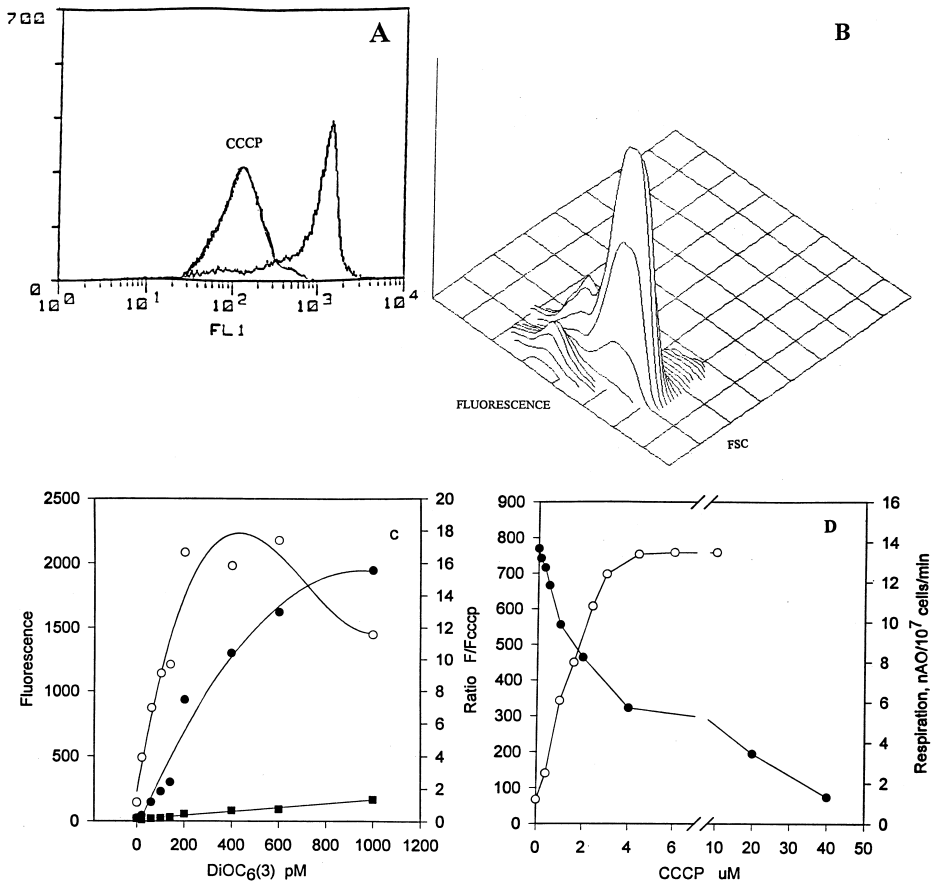

Fig. 2. $DiOC_6(3)$ fluorescence in mouse spleen lymphocytes: effect of dye concentration and CCCP. (A) Typical histograms of cell fluorescence with and without CCCP. 10^6 cells were incubated with 0.2 nM $DiOC_6(3)$ in 1 ml of MEM (+1% FCS) for 20 min at 37°C. Each suspension was divided into two 0.5 ml portions, CCCP (50 μM) was added to one portion, and the incubation continued for an additional 20 min. The cell fluorescence was measured by flow cytometry. Each histogram was obtained from 10^4 cells. (B) Two dimensional histogram of $DiOC_6(3)$ fluorescence and forward light scattering (FSC) in spleen lymphocytes. Incubation conditions as in (A). (C) Fluorescence intensity as a function of the dye concentration. The protocol was the same as in Fig. 1A except for the dye concentrations which vary from 0.01 to 1 nM. The figure shows the average fluorescence of the high fluorescence fraction in controls (full circles); the average fluorescence in the presence of CCCP (full squares) and the ratio of these values (○, right scale). (D) The effect of CCCP on respiration rates and $DiOC_6(3)$ fluorescence. The experiment was carried out on a batch of spleen cells prepared from two mice. For the respiration experiments 1.5×10^7 cells were incubated in 1.5 ml MEM (+1% FCS) medium at 37°C in the oxygen electrode chamber for 20 min. Cyclosporin (1 μM) and oligomycin (30 ng/ml) were added and, after 10 minutes, increasing concentrations of CCCP were added as indicated. The rates of respiration after each addition are shown (○). For the fluorescence measurements, 2×10^7 cells were incubated in 2 ml of MEM medium with 2 nM $DiOC_6(3)$ for 30 min. Cyclosporin (1 μM) and 30 ng/ml oligomycin were added and after 10 min incubation the cells were divided into nine portions and incubated for 20 min with various concentrations of CCCP, as indicated.

retain the equilibrium distribution across the plasma membrane, hence resulting in a very large reduction of cell-associated dye. The magnitude of the reduction of cell fluorescence will depend strongly on cell density, since the extent of the release of dye to the medium depends on the extent to which the free dye concentration in the medium will increase, and also on the dye concentration, which determines the extent of quenching of the mitochondrial bound dye. These considerations suggest that the maximal response of fluorescence to $\Delta\Psi_m$, should be observed at the lowest possible dye/cell ratio.

To be able to correlate the fluorescence intensity with the magnitude of $\Delta\Psi_m$, it is necessary to measure the fluorescence intensity after the dye distribution has reached equilibrium. Since the rate of efflux,

after collapse of $\Delta\Psi_m$, was much higher than the rate of uptake after dye addition (see Fig. 5C), we developed a protocol in which the dye was first incubated with the cells for 20 min to allow the dye to reach near-equilibrium distribution, and then, reagents that modulate $\Delta\Psi_m$ were added. After a further incubation of 20 min, which was sufficient to allow redistribution of the dye in response to the modulation of $\Delta\Psi_m$, the cell fluorescence was measured by flow cytometry (see Fig. 5C for the kinetics of dye uptake and release at optimal dye concentration). When cells were incubated with mitochondrial inhibitors or uncouplers *before* the dye was equilibrated (as is common in most previous protocols, cf. [18]), the transport of the dye into the cells was inhibited, and did not reach the equilibrium distribution even after prolonged incubations (results not shown). Therefore, in these protocols, one measures the effect of the inhibitors on the transport of the dye across the plasma membrane (which is rate limiting) and not on the magnitude of $\Delta\psi_m$.

Fig. 2A shows typical histograms of steady-state mean fluorescence of $DiOC_6(3)$ (0.2 pmol/10^6 cells) in mouse spleen lymphocytes, as obtained by this protocol. Without CCCP most of the cells showed very high fluorescence intensity. However, a broad fraction of intermediate and low fluorescence intensity was observed as well. This pattern of cell fluorescence was stable and remain unchanged for at least 1 h of incubation in MEM medium, at 37°C. The fluorescence histograms of cells that were incubated with a low concentration (5 μM), and a high concentration (50 μM), of CCCP consisted of a single fraction of cells with low fluorescence (about 20 and 10% of control, respectively). At low CCCP concentration, $\Delta\psi_m$ is collapsed, but $\Delta\psi_p$ is not affected, while at high CCCP concentration, both $\Delta\psi_m$ and $\Delta\psi_P$ are collapsed (see below). The fluorescence histogram (without uncoupler) suggests that the cell population is heterogeneous in regard to the magnitude of $\Delta\psi_m$. Fig. 2B shows a two dimensional histogram of $DiOC_6(3)$ fluorescence and forward light scattering (FSC, which is proportional to cell size) in spleen lymphocytes. There is a small fraction of lymphocytes ($\sim$5%) which exhibited both low fluorescence (i.e. low $\Delta\Psi_m$) and low FSC (i.e. small volume). Most of these cells are apparently dead or committed to apoptosis [10]. In addition, the major lymphocyte fraction, with normal FSC, also contained a significant fraction of low $\Delta\psi_m$. The $\Delta\psi_m$ of most of the cells in this fraction could be restored by several reagents (see below).

The selection of 0.2 nM $DiOC_6(3)$ as an optimal concentration for $\Delta\Psi_m$ measurements was based on the titration of the fluorescence intensity as shown in Fig. 2C. When a high concentration of CCCP (i.e. $\Delta\Psi_P = \Delta\psi_m = 0$) was added to cells that were equilibrated with different dye concentrations, there was a linear relationship between the fluorescence intensity and the dye concentration, as expected from a simple partition of the dye between the cell membranes and the medium. However, the dependence of the mean fluorescence of the major lymphocyte fraction on the dye concentration in cells that maintain high $\Delta\psi_m$ and $\Delta\psi_P$ (e.g. without CCCP) is more complicated. The fluorescence rises sharply up to about 200 pM and then continues to rise moderately, slowly approaching saturation. The same data were plotted as the ratio of the mean fluorescence of the major cell fraction in suspensions without CCCP to the mean fluorescence of cells incubated with CCCP (50 μM) (empty circles, right scale). If there was no quenching, the magnitude of the ratio of the intensities (F/F_{cccp}) should be correlated with the magnitude of $\Delta\psi_m+\Delta\psi_P$, regardless of the dye concentration, since it is proportional to the ratio of the concentration of the cationic dye in the cell with and without $\Delta\psi_m+\Delta\psi_p$. The observed increase in the ratio, at low concentrations (from 0.04 to 0.2 nM), was found to be the result of an increased rate of uptake at higher dye concentrations. Only above 0.2 nM was the dye fully equilibrated after a 40 min incubation (see Fig. 5C). Between 0.2 and 0.6 nM, the ratio was approximately constant. Above 0.6 nM, the ratio declined as a result of the concentration-dependent quenching of the mitochondrial bound dye. Similar results were obtained with the malarial parasite, except that the optimal concentration was somewhat higher [20].

Within the 0.2–0.6 nM range, where the ratio is maximal, the fluorescence intensity exhibited the highest sensitivity to the magnitude of $\Delta\Psi_m$. To avoid complications from inhibition of electron transport, we used the lowest concentration in this range, namely 0.2 nM (or more precisely 0.2 pmol/10^6 cells). The only drawback of using such a low

concentration of dye was that the time of equilibration is relatively slow $t_{0.5}$ of about 7 min, see Fig. 5C).

A test of the sensitivity of this assay to the modulation of the magnitude of $\Delta\Psi_m$ was obtained from parallel titrations, with the protonophore CCCP, of the lymphocytes' rate of respiration and the fluorescence intensity. A typical example is shown in Fig. 2D. Lymphocyte respiration was first inhibited by oligomycin, which inhibits oxidative phosphorylation at ATP synthase, and the uncoupler CCCP relieved this inhibition and stimulated the rate of respiration well above the basal rate (see Fig. 1). This stimulation, which results from the reduction of $\Delta\Psi_m$ [23], was paralleled by a decrease of the fluorescence intensity (Fig. 2D). Even very low concentrations of CCCP, which stimulated respiration only slightly, resulted in a significant reduction of the fluorescence intensity. The stimulation of respiration was maximal at about 5 μM CCCP, which is apparently sufficient to collapse $\Delta\psi_m$. Reduction of the fluorescence intensity to its lowest value required a much higher concentration of CCCP, apparently due to the collapse of $\Delta\Psi_P$ (see below). These results suggest that the ratio F/F_{cccp} is sensitive to the magnitude of $\Delta\Psi_m$ over its entire range. However, this titration, while demonstrating the sensitivity of the fluorescence, under these conditions, to the magnitude of $\Delta\psi_m$, does not provide a quantitative estimate of the magnitude of $\Delta\psi_m$.

It is possible to obtain a more quantitative estimate of $\Delta\Psi_m$ by analyzing the effect of the K^+-ionophore valinomycin [21] on the fluorescence intensity. Fig. 3A shows a titration of the $DiOC_6(3)$ fluorescence ratio (F/F_{cccp}) with valinomycin in MEM medium which contains 5.5 mM K^+. Very low (nM) concentrations of valinomycin collapsed $\Delta\Psi_m$ and reduced F/F_{cccp} from a value of 10.4 in the absence of valinomycin to about 2.3 at 100 nM. However, increasing the valinomycin concentration to the μM range enhanced the fluorescence and increased the fluorescence ratio to 5. This complicated behavior is the result of the differential effect of valinomycin on the K^+ permeability of the mitochondrial inner membrane and the plasma membrane [24]. Low concentrations of valinomycin (nM) appear to be sufficient to increase greatly the mitochondrial K^+ permeability [21,24], but not

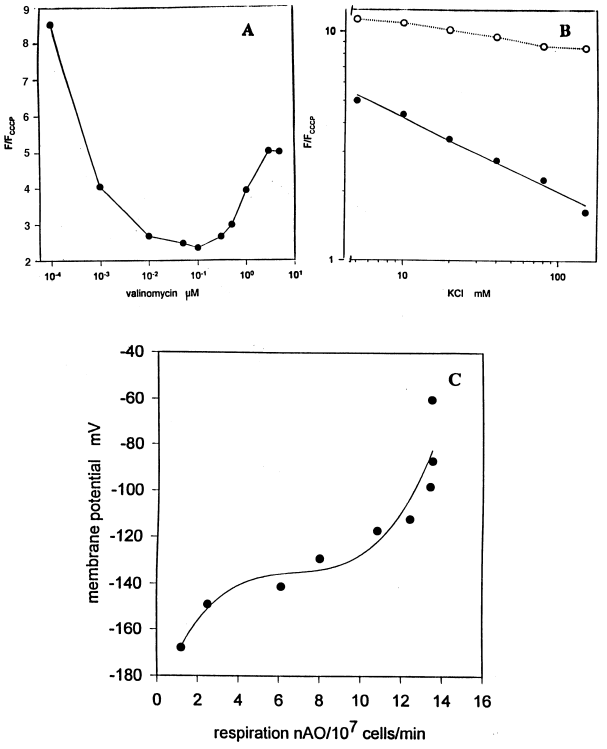

Fig. 3. Relationships between the magnitude of $\Delta\Psi_m$ and $DiOC_6(3)$ fluorescence. (A) The effect of valinomycin on the fluorescence ratio in MEM medium. Cells were incubated as in Fig. 2A but with different concentrations of valinomycin, F_{cccp} was 50 μM. (B) The effect of KCl concentrations on the fluorescence ratio. The protocol was the same as in (A) except that valinomycin concentration was either 0 (empty circles) or 3 μM (full circles) and KCl as indicated. (C) The relationship between $\Delta\psi_m$ and the rate of uncoupled respiration. The data are taken from Fig. 2D. $\Delta\psi_m$ is calculated from the corrected ratio F/F_{cccp} according to Eq. 1.

that of the plasma membrane [24]. When the mitochondrial inner membrane becomes permeable to K^+ (at 0.1 μM valinomycin), $\Delta\Psi_m$ collapses because the concentration of K^+ in the mitochondrial matrix is similar to the concentration of K^+ in the cytosol ($\sim$100 mM). However, when the concentration of valinomycin is increased, the plasma membrane also becomes permeable to K^+, which leaks out of the cell. At high concentrations of valinomycin (>1 μM), the concentration of K^+ in the cytosol is gradually reduced to the concentration in the medium (5.5 mM), which partially restores $\Delta\Psi_m$, because the K^+ gradient across the mitochondrial inner membrane has increased. Since $\Delta\psi_P$ is largely determined by the K^+ gradient across the plasma mem-

brane, $\Delta\psi_P = 0$, at high valinomycin concentration (Fig. 3A).

Increasing the medium K^+ concentration, at high valinomycin concentration (3 μM), increased cytosolic K^+ and therefore collapsed $\Delta\psi_m$. Fig. 3B shows that in the presence of 3 μM valinomycin KCl reduced the ratio F/F_{cccp}. It is observed that the log of the ratio F/F_{cccp} decreased linearly with log $[K^+]$. This response is identical to the $\Delta\Psi_m$ response obtained in isolated mitochondria treated with valinomycin and increasing concentrations of K^+ [21]. In the presence of valinomycin, $\Delta\Psi_m$ depends on the equilibrium distribution of K^+ and approximately obeys the Nernst equation ($\Delta\Psi = 60\log\{[K^+]_m/[K^+]_{ex}\}$) (see [21] for discussion of this procedure of calibration). Assuming that $\Delta\psi_m$ obeys the Nernst equation and that $[K^+]_m$ is constant, a 10-fold change in $[K^+]_{out}$ should produce a 60 mV difference in $\Delta\Psi_m$. Therefore, from the slope of Fig. 3B:

$$\Delta\Psi_m = \log (F/F_{cccp})/0.0054 \text{ mV} \quad (1)$$

The slope of the calibration curve is sensitive to the dye/cell ratio because the slope depends on the distribution of the dye between the cells and the medium. Since most of the lipophilic dye is in the cells, the relative decrease in the dye external concentration, following an increase in $\Delta\Psi_m$, is larger than the relative increase in the cell content, which reduces the slope of the calibration curve.

To evaluate the effect of $\Delta\psi_p$ on the fluorescence, we examined the effect of KCl in the absence of valinomycin, since high KCl depolarizes the plasma membrane potential in lymphocytes [22,24]. The addition of KCl decreased the fluorescence ratio from about 10.7 to 8.7 (Fig. 3B). These results indicate that the magnitude of the plasma membrane potential contributes significantly to the fluorescence ratio even under conditions that maximize the response to $\Delta\psi_m$ (i.e. very low dye/cell ratio, see Section 4). A similar effect of KCl on the fluorescence was observed in lymphocytes incubated with low concentrations of CCCP (5 μM), which suggests that this concentration of CCCP collapses $\Delta\psi_m$, but not $\Delta\psi_P$. At high concentrations of CCCP (50 μM), KCl had no effect on the fluorescence, suggesting that at this concentration both $\Delta\psi_m$ and $\Delta\psi_P$ have collapsed (results not shown).

The fluorescence ratio of the major fraction in lymphocyte suspensions from young mice (10^6 cells/ml), incubated in MEM (+1% FCS) medium with 0.2 nM $DiOC_6(3)$, varied from preparation to preparation in the range of 6–17. Taking into account the variation in the slope of the calibration curves, and correcting for the contribution of $\Delta\Psi_P$ (see below), these measurements resulted in a range of $\Delta\psi_m$ values between 130 and 190 mV, which is well within the range of $\Delta\Psi_m$ estimated by other methods [13,16,17]. The average potential in the major fraction of spleen lymphocytes from 12 individual mice was 163 ± 8 mV (S.E.M.). To demonstrate the use of this calibration, we show the dependence of lymphocytes' uncoupled respiration rate on membrane potential, when attenuated by increasing concentrations of CCCP (Fig. 3C). This figure is derived from the parallel titration shown in Fig. 2D, using Eq. 1, while correcting for the contribution of $\Delta\Psi_P$ to the fluorescence ratio (see below). The results are in good agreement with similar titrations in isolated mitochondria [21,23].

The mitochondrial membrane potential is generated by the redox proton pumps under most conditions (aerobic respiration), but can also be generated from ATP hydrolysis by ATP synthase. A method which is sensitive to the magnitude of $\Delta\psi_m$ should respond to modulation of the activities of these proton pumps. Under aerobic conditions the potential which is generated by the redox pump is utilized to drive ATP synthesis by proton flow through ATP synthase. Inhibition of oxidative phosphorylation by specific inhibitors of ATP synthase (e.g. oligomycin) results in a small but significant increase of $\Delta\psi_m$ in isolated mitochondria. Fig. 4A shows the effect of oligomycin on the fluorescence histogram. It is seen that oligomycin significantly increases the fluorescence of the major lymphocyte fraction, suggesting active oxidative phosphorylation (i.e. 'state 3'). Specific inhibitors of the proton pumping electron transport complexes, e.g. rotenone (complex I), myxothiazol and antimycin (complex III), and cyanide (complex IV), were without effect on the fluorescence, at low concentrations, unless oligomycin was also added (Fig. 4A). This indicates that lymphocytes can generate sufficient ATP by glycolysis to support $\Delta\psi_m$ [25]. Fig. 4B shows parallel titrations of lymphocyte respiration (in the presence of oligomycin and CCCP) and the F/F_{cccp} ratio (in the presence

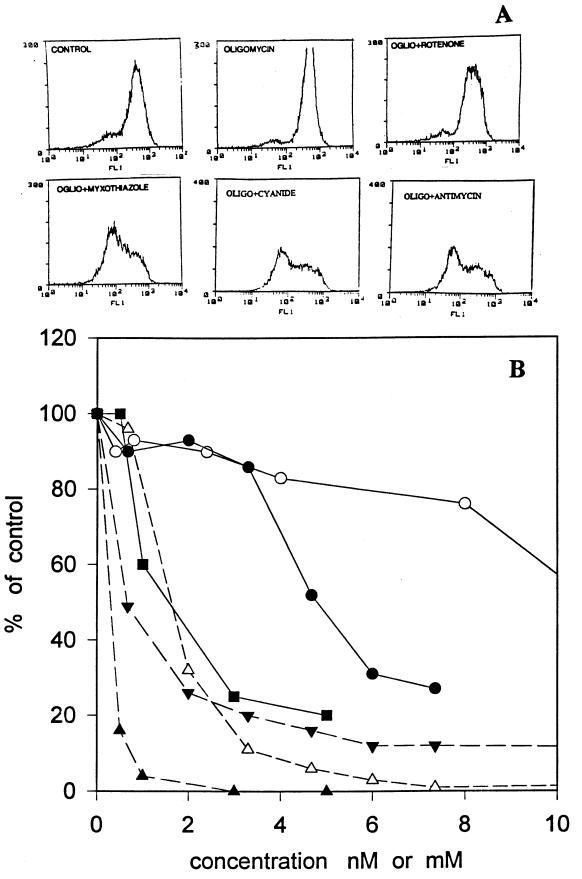

Fig. 4. The effect of inhibitors of the mitochondrial proton pumps on $\Delta\Psi_m$. (A) Fluorescence histograms of lymphocytes incubated with oligomycin (0.1 μg/ml), rotenone (10 nM), myxothiazol (10 nM), antimycin A (10 nM), and cyanide (10 mM). Assay conditions as in Fig. 2A. (B) Titrations of respiration (in the presence of oligomycin (0.1 μg/ml) and CCCP (5 μM)) and the F/F_{cccp} ratio (in the presence of oligomycin) by rotenone, myxothiazol, and cyanide. Respiration assay as described in Section 2: full triangles, myxothiazole (nM); empty triangles, cyanide (mM); inverted triangles, rotenone (nM). $DiOC_6(3)$ fluorescence ratio assay (F/F_{cccp}) as in Fig. 2A: full squares, myxothiazol (nM); full circles, cyanide (mM); empty circles, rotenone (nM). The titration of respiration and F/F_{cccp} for each inhibitor was carried out with the same batch of lymphocytes.

of oligomycin) with the respiratory inhibitors rotenone, myxothiazol and cyanide. Rotenone was the least efficient inhibitor of the F/F_{cccp} ratio in lymphocytes (empty circles), while myxothiazol (full squares) and cyanide (full circles) were equally efficient. This is also true for inhibition of respiration. Rotenone inhibited less than 90% of the respiration (inverted triangles), while myxothiazol (full triangles) and cyanide (empty triangles) inhibited respiration completely. Because $\Delta\psi_m$ does not depend strongly on the rate of respiration, rotenone is not an efficient inhibitor of $\Delta\psi_m$ in lymphocytes. Apparently there is sufficient activity of alternative dehydrogenases in lymphocytes that can support significant electron transport rates, at least for short incubation periods.

The maximal reduction of the F/F_{cccp} ratio that is obtained by cyanide+oligomycin (′80%) indicates the contribution of $\Delta\psi_m$ to the total signal, and is comparable to the reduction by 0.1 μM valinomycin (Fig. 3A), and also to that obtained by low concentration (1–5 μM) of CCCP (Fig. 2D). These observations indicate that these treatments should be used to obtain the reference F value for estimation of the magnitude of $\Delta\psi_m$ instead of 50 μM CCCP. Alternatively, F/F_{cccp} can be measured at high KCl concentrations (Fig. 3B), although plasma membrane depolarization could lead to activation of processes that modulate $\Delta\psi_m$. The magnitudes of each of these ratios (e.g. F/F_{cccp} (5 μm), $F/F_{oligomycin+cyanide}$, $F/F_{valinomycin}$ (0.1 μM) can be compared directly to each other or converted to an estimate of $\Delta\psi_m$ by calibration with valinomycin+K^+ (Fig. 3B).

The activation of the mitochondrial permeability transition (PT) abolishes $\Delta\psi_m$ [12]. Cyclosporin inhibits PT activation and restores $\Delta\psi_m$, and can be used to estimate the activation state of PT. We have observed considerable activation of PT in spleen lymphocytes from old mice [10]. Fig. 5A shows that the addition of cyclosporin to a lymphocyte suspension (from young mice) also significantly reduced the size of the fraction of cells with low fluorescence, eliminated the fraction with intermediate fluorescence and increased the average fluorescence intensity of the fraction with high fluorescence, which became significantly more uniform in fluorescence intensity. The dependence of the mean fluorescence ratio (from all the fractions) on the cyclosporin concentration is shown in Fig. 5B (full symbols). The maximal increase in the mean fluorescence ratio was obtained at 1 μM. The averaged effect of 1 μM cyclosporin on spleen lymphocytes from 6 young mice was an increase of $\Delta\Psi_m$ (calculated from the mean fluorescence ratio of all the lymphocytes) from 163 ± 8 to 176 ± 6 mV (see also [10]).

Cyclosporin also inhibits the multi-drug-resistance pump (MDR), which ejects many types of lipophilic cations from cells [27], and this activity could affect

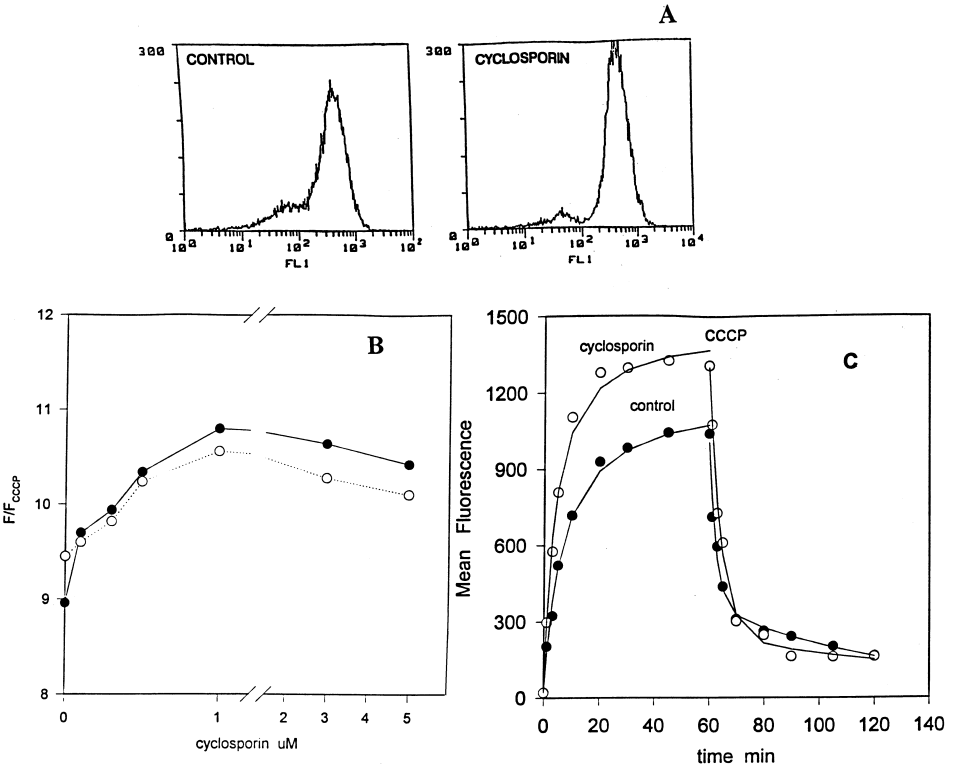

Fig. 5. The effect of cyclosporin on $\Delta\Psi_m$. (A) The effect of cyclosporin on the fluorescence histogram. Assay conditions are as in Fig. 2A except that cyclosporin (1 μM) was added instead of CCCP. (B) The effect of cyclosporin concentration on the fluorescence ratio. Assay conditions are as in (A), except for the concentration of cyclosporin as indicated. Without reserpine (full circles); with 1 μg/ml reserpine (empty circles). (C) The effect of cyclosporin on the kinetics of the uptake and the CCCP-induced release of $DiOC_6(3)$. 3×10^6 lymphocytes were incubated at 37°C in 3 ml MEM (+1% FCS) with 1 μM cyclosporin or without for 15 min. Then, 0.2 nM $DiOC_6(3)$ was added and samples were withdrawn for flow cytometry at the indicated times. After 60 min, 50 μM CCCP was added and additional samples were withdrawn at the indicated times. The results shown are the average intensity with (○) and without (●) 1 μM cyclosporin. The uptake kinetics was fitted to a hyperbolic function with $t_{0.5} = 6.9$ min for the control and 4.0 min with cyclosporin. The efflux kinetics was fitted to a sum of two exponential decays.

the steady state distribution of $DiOC_6(3)$. To test this possibility, we studied the effect of other inhibitors of the MDR pump in comparison to the cyclosporin effect. Verapamil and reserpine, two potent inhibitors of the MDR pump [26], enhanced $DiOC_6(3)$ fluorescence. However, their effect was smaller than cyclosporin, affecting mostly the size of the low fluorescence fraction. Moreover, cyclosporin further enhanced the fluorescence of reserpine treated lymphocytes. Fig. 5B shows the concentration dependence of the cyclosporin effect on reserpine treated lymphocytes (empty circles). It is observed that the enhancement of the fluorescence, which can be attributed to the effect of cyclosporin on the MDR pump, is fully saturated at a low cyclosporin concentration (0.1 μM), while the major effect on the fluorescence ratio is saturated at 1 μM, with or without reserpine. Unlike cyclosporin reserpine did not stimulate cell respiration. Therefore, the reserpine-insensitive fluorescence enhancement by cyclosporin, which is associated with a stimulation of cell respiration [10], could be attributed to the inhibition of the mitochondrial PT.

Fig. 5C shows the effect of cyclosporin on the kinetics of $DiOC_6(3)$ uptake and the CCCP-induced efflux. Cyclosporin enhanced both the rate and the extent of dye uptake, as measured by the increased mean fluorescence. The uptake kinetics could be fitted to a simple hyperbolic function with $t_{0.5}$ of 6.9 min in control and 4.0 min in cyclosporin-treated cells. The efflux kinetics, after addition of high concentration of CCCP, was biphasic. The fast phase correspond to the collapse of $\Delta\psi_m$, while the slow phase correspond to the collapse of $\Delta\Psi_P$. Cyclospor-

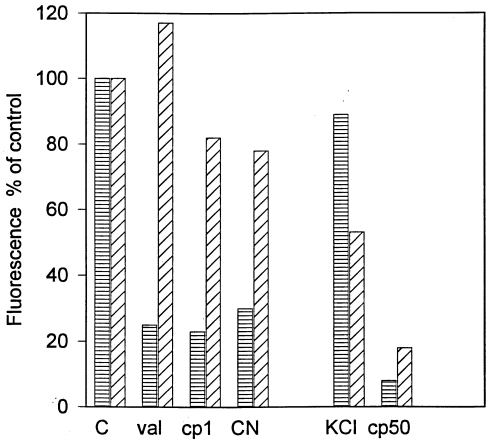

Fig. 6. Comparison of the response of $DiOC_6(3)$ fluorescence to modulation of $\Delta\psi_m$ and $\Delta\psi_p$ with 0.2 and 40 nM dye. Assay conditions are as in Fig. 2A except for the dye concentrations: horizontal lines, 0.2 nM; diagonal lines, 40 nM. Control (no addition), C; valinomycin (0.1 μM), val; CCCP (1 μm), cp1; cyanide (10 mM), CN; KCl (100 mM), KCl; CCCP (50 μM), cp50.

in did not inhibit the overall rate of efflux of the dye, and hence its effect on the fluorescence could not be attributed to inhibition of the MDR pump. The major effect of cyclosporin on the kinetics of the efflux was to increase the amplitude of the fast phase, which is compatible with an increase of $\Delta\psi_m$ in a fraction of the cells. These results demonstrate that this assay can detect activation of PT in a small fraction of the cell population.

Fig. 6 shows a comparison of the fluorescence response to various modulators of $\Delta\psi_m$ and $\Delta\Psi_P$, as sensed by 0.2 and 40 nM $DiOC_6(3)$, in the same batch of cells. Low concentrations of valinomycin (0.1 μM, val), which collapses $\Delta\psi_m$ (Fig. 3A), reduced the cell fluorescence to 25% of control at low dye concentration, but actually stimulated the fluorescence at high dye concentration. The combination of oligomycin and cyanide (CN), which also collapses $\Delta\psi_m$ (Fig. 4), reduced the fluorescence to 31% of control at low dye concentration, but at high dye concentration the reduction was much smaller (79% of control). Similarly, CCCP at low concentration (1 μM, cp1), which also collapses $\Delta\psi_m$ (Fig. 2D), reduced the fluorescence to 23% of control at the low dye concentration but reduced the fluorescence only slightly, to 82% of control, at the high dye concentration. In contrast, 100 mM KCl, which collapses $\Delta\psi_p$ had only a small effect on the fluorescence at low dye concentration, but reduced the fluorescence nearly 50% in high dye concentration. CCCP, at 50 μM, which collapses both $\Delta\psi_m$ and $\Delta\psi_p$, reduced the fluorescence to 8% of control at low dye concentration and 17% of control at the high dye concentration. These results suggest that at high concentrations, the dye is much more sensitive to $\Delta\Psi_p$ than to $\Delta\Psi_m$.

4. Discussion

Several fluorescent probes have been used previously, in conjunction with flow cytometry, to measure $\Delta\Psi_m$ in lymphocytes. The most widely used probe is rhodamine 123 (reviewed in [27]). However, this probe was used mostly as a qualitative probe, and under most conditions it is not sensitive to the metabolic state of the mitochondria. This dye is also used frequently for measuring the activity of the MDR pump [26]. In fact, often the dye distribution is determined by the activity of the MDR pump and not by $\Delta\Psi_m$ (cf. [28]). In our hands, rhodamine 123 fluorescence was highly quenched in spleen lymphocytes, even in nM concentrations, and hence did not respond to modulations of $\Delta\Psi_m$. At very low concentrations, both the uptake of the dye by the cells and the efflux were very slow; equilibration took many hours, and the release of the accumulated dye from the mitochondria to the cell by attenuation of $\Delta\psi_m$ actually resulted in an increase of cell fluorescence (Rottenberg, unpublished results).

$DiOC_6(3)$ has been used in a series of recent studies to probe $\Delta\Psi_m$ in cells undergoing apoptosis (cf. [18], reviewed in [6]). However, in all of these studies it was used qualitatively, without establishing that the magnitude of the fluorescence is correlated with the magnitude of $\Delta\psi_m$. Very high concentrations of uncouplers reduced the fluorescence, but this appears to be due to the collapse of $\Delta\psi_p$ (see below). Similarly, the effects of very high concentrations of mitochondrial inhibitors do not appear to result from specific inhibition of mitochondrial proton pumps. We found that the previously employed dye concentrations, i.e. 40–100 nM, not only inhibited respiration, but also resulted in extensive quenching of the mitochondrial bound dye, thus greatly attenuating the sensitivity of the fluorescence to $\Delta\psi_m$. This is

shown clearly in the experiments presented in Fig. 6 that suggest that at 40 nM dye the fluorescence responds strongly to modulation of $\Delta\psi_p$ and only weakly to modulation of $\Delta\psi_m$. It is very likely that because of the lipophilicity of the dye, even at high dye concentrations, most of the cell fluorescence comes from mitochondrial bound dye. However, because of the strong quenching of the mitochondrial-bound dye, the fluorescence is not very sensitive to the amount of bound dye, and hence to the magnitude of $\Delta\psi_m$.

Previously, $DiOC_6(3)$ fluorescence has been used to estimate the plasma membrane potential, $\Delta\psi_p$ without consideration of $\Delta\Psi_m$ (cf. [29]). It has already been demonstrated long ago that the same dye responds largely to the plasma membrane potential at high concentration, where the mitochondrial-bound dye is almost completely quenched, and largely to $\Delta\Psi_m$, at low concentrations, where mitochondrial-bound dye is less quenched and dominates the signal [22]. Long preincubation with high concentrations of CCCP decreased the fluorescence intensity also at high dye concentration (Fig. 6), which was interpreted by other investigators as evidence for sensitivity to $\Delta\psi_m$, but this response is probably the result of the CCCP-induced hydrolysis of ATP, as well as a direct effect on the plasma membrane permeability, which together results in a collapse of $\Delta\psi_p$. The unreliability of both rhodamine 123 and $DiOC_6(3)$, as currently used, for assessing $\Delta\psi_m$ has been pointed out recently by other investigators (cf. [30]). The inadequacy of the uncritical use of uncouplers as indicators of the mitochondrial contribution to compartmentation of cell ions has also been pointed out recently in relation to calcium compartmentation [31].

In the new protocol for the determination of $\Delta\psi_m$, collapsing $\Delta\Psi_m$ reduced the fluorescence intensity of the major lymphocyte fraction by up to 85%, which allows for accurate measurement of a small modulation of $\Delta\Psi_m$. Also, as the titrations with valinomycin and CCCP suggest, the fluorescence is sensitive to $\Delta\Psi_m$ over the entire range. Moreover, because flow cytometry allows measurement from individual cells, one can detect small changes, in a very small fraction of cells, which is not possible with conventional methods that provide the average $\Delta\Psi_m$ of all the cells in the suspension. In particular, the methods in which $\Delta\Psi_m$ is calculated from the distribution of lipophilic cations between the medium and the total population of cells [16,17] are insensitive to modulation of $\Delta\Psi_m$ in the fraction with low $\Delta\Psi_m$, because this calculation produces a weighted average that is strongly biased towards the cells with high $\Delta\psi_m$.

All the mitochondrial inhibitors and ionophores that we tested (e.g. CCCP, valinomycin, nigericin, oligomycin, cyclosporin, rotenone, antimycin A, myxothiazol, and cyanide) produced effects on $\Delta\Psi_m$, in situ, which were very similar to their effects on $\Delta\Psi$ in isolated mitochondria, and were strongly correlated with their effects on cell respiration, suggesting that this assay provides a sensitive measure of $\Delta\Psi_m$, in situ.

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