Mitochondrial membrane potential in single living adult rat cardiac myocytes exposed to anoxia or metabolic inhibition

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1. The relation between mitochondrial membrane potential ($\Delta\Psi_m$) and cell function was investigated in single adult rat cardiac myocytes during anoxia and reoxygenation. $\Delta\Psi_m$ was studied by loading myocytes with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), a fluorescent probe characterized by two emission peaks (539 and 597 nm with excitation at 490 nm) corresponding to monomer and aggregate forms of the dye.

2. De-energizing conditions applied to mitochondria, cell suspensions or single cells decreased the aggregate emission and increased the monomer emission. This latter result cannot be explained by changes of JC-1 concentration in the aqueous mitochondrial matrix phase indicating that hydrophobic interaction of the probe with membranes has to be taken into account to explain JC-1 fluorescence properties in isolated mitochondria or intact cells.

3. A different sensitivity of the two JC-1 forms to $\Delta\Psi_m$ changes was shown in isolated mitochondria by the effects of ADP and FCCP and the calibration with K^+ diffusion potentials. The monomer emission was responsive to values of $\Delta\Psi_m$ below 140 mV, which hardly modified the aggregate emission. Thus JC-1 represents a unique double sensor which can provide semi-quantitative information in both low and high potential ranges.

4. At the onset of glucose-free anoxia the epifluorescence of individual myocytes studied in the single excitation (490 nm)–double emission (530 and 590 nm) mode showed a gradual decline of the aggregate emission, which reached a plateau while electrically stimulated (0·2 Hz) contraction was still retained. The subsequent failure of contraction was followed by the rise of the emission at 530 nm, corresponding to the monomer form of the dye, concomitantly with the development of rigor contracture.

5. The onset of the rigor was preceded by the increase in intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) monitored by mag-indo-1 epifluorescence. Since under these experimental conditions intracellular $[Ca^{2+}]$ and pH are fairly stable, the increase in $[Mg^{2+}]_i$ was likely to be produced by a decrease in ATP content.

6. The inhibition of mitochondrial ATPase induced by oligomycin during anoxia was associated with a rapid and simultaneous change of both the components of JC-1 fluorescence, suggesting that $\Delta\Psi_m$, instead of producing ATP, is generated by glycolytic ATP during anoxia.

7. The readmission of oxygen induced a rapid decrease of the monomer emission and a slower increase of the aggregate emission. These fluorescence changes were not necessarily associated with the recovery of mechanical function. Even in those cells which hypercontracted during reoxygenation a further addition of FCCP induced a decrease in $\Delta\Psi_m$ and an increase in $[Mg^{2+}]_i$, indicating that residual mitochondrial function and ATP production can exist even in irreversibly damaged cells.

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We have studied the response of the mitochondrial membrane potential ($\Delta\Psi_m$) to anoxia in single rat cardiac myocytes. The heart is dependent upon adequate O_2 delivery for normal function, as it derives most of its metabolic energy from oxidative phosphorylation. The mitochondrial proton gradient ($\Delta\mu H^+$), of which $\Delta\Psi_m$ forms by far the largest part, is the most direct measure of the state of energization of the mitochondria (Mitchell, 1979). However, it is rarely measured *in situ* in heart preparations. Instead, adenine nucleotide phosphorylation potential is often measured, as it can be derived from measurements of ATP, creatine phosphate and inorganic phosphate (P_i) – which are accessible to the NMR technique (Radda, 1986). However, phosphorylation potential may not be a good surrogate for $\Delta\mu H^+$ under pathophysiological conditions. For instance it has been suggested that the activities of both the adenylate translocase (Hütter, Alves & Soboll, 1990) and F_1-ATPase are depressed during ischaemia (Rouslin, 1983*a*). In this case the inhibition of mitochondrial respiration would be associated with a partial maintenance of ATP content, at least within the mitochondrial matrix. An opposite situation could be generated during post-ischaemic reperfusion when $\Delta\Psi_m$ is used for Ca^{2+} uptake. Since this process, utilizing the same source of energy, namely $\Delta\Psi_m$, competes with oxidative phosphorylation, the restoration of mitochondrial respiration may not be paralleled by the recovery of ATP content. Accordingly, myocardial ATP contents have been shown not to correlate with the degree of functional recovery during post-ischaemic reperfusion (Neely & Grotyohann, 1984). More recently, in hepatocytes the evolution toward cell death has been shown to be predicted by a collapse in $\Delta\Psi_m$ rather than ATP depletion (Snyder, Pastorino, Thomas, Hoek & Farber, 1993). Mitochondria are themselves sensitive to damage following ischaemia and post-ischaemic reperfusion. Thus, a decreased function of NADH dehydrogenase has been reported in mitochondria extracted from ischaemic hearts (Rouslin 1983*b*; Hardy, Clark, Darley-Usmar, Smith & Stone, 1991). Furthermore, it appears from NMR studies that in isolated and perfused hearts, under ischaemic conditions, oxygen consumption does not correlate with performance, which is consistent with some degree of mitochondrial uncoupling (Neubauer, Hamman, Perry, Bittl & Ingwall, 1988).

Although mitochondria play a central role in the onset and evolution of ischaemic damage to the heart, little is known about their function in intact cells under these conditions. Most of our knowledge on mitochondrial function derives from studies performed on isolated mitochondria. Those studies which have attempted to investigate $\Delta\Psi_m$ in intact cells or tissues have generally used techniques which are based on the cellular distribution of lipophilic cations (Rottenberg, 1979; Chen, 1988). Their accumulation in the mitochondrial matrix is driven by the membrane potential according to the Nernst equation. Recently, a carbocyanine derivative, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), has been introduced as a fluorescent probe which can be utilized, at least theoretically, to monitor $\Delta\Psi_m$ in living cells (Smiley *et al.* 1991). It has been suggested that the ability of JC-1 to probe the membrane potential depends on the reversible formation of J-aggregates, which are responsible for the shift in the emitted light from 530 (corresponding to JC-1 monomer) to 590 nm when excited at 490 nm (Reers, Smith & Chen, 1991).

We investigated the use of JC-1 to monitor the time course of the changes in $\Delta\Psi_m$ and to relate these changes to the function of single adult rat cardiac myocytes exposed to anoxia and reoxygenation. A study with similar objectives has recently been published by Duchen, McGuinness, Brown & Crompton (1993). Our results show that (i) the two emission peaks of JC-1 respond to $\Delta\Psi_m$ changes with different sensitivities; (ii) $\Delta\Psi_m$ decreases at the onset of anoxia when contractile activity is still retained and collapses at the onset of rigor; and (iii) during reoxygenation a partial recovery of $\Delta\Psi_m$ occurs in recovering myocytes and in a subset of irreversibly damaged myocytes. Preliminary accounts of these results have been published (Di Lisa, Silverman, Stern, Blank, Gambassi & Hansford, 1992).

METHODS

Characterization of JC-1 fluorescence properties

JC-1 was dissolved (1 mg ml^{-1}) in dimethyl sulphoxide, fractionated in small aliquots and stored at −20 °C. Under these conditions the optical and physical properties of the probe were constant over a long period of time (> 1 month). The fluorescence spectra of the dye (0·5 μM) dissolved in various solvents were collected by means of a Delta-Scan 1 (Photon Technology International, Inc., Princeton, NJ, USA). This instrument was equipped with two separate photomultipliers for collecting simultaneously fluorescence emitted at two different wavelengths during kinetic studies. The cuvette mount was thermostated and equipped with magnetic stirring. Fluorescence in non-particulate experiments was recorded in the right-angle mode and with mitochondria or cells in the front-face mode.

Measurements of JC-1 fluorescence in mitochondria

Rat heart mitochondria were isolated by using the Nagarse digestion technique (Hansford, 1978), which harvests both subsarcolemmal and interfibrillar mitochondria (Palmer, Tandler & Hoppel, 1977). The fluorescence emission of JC-1 excited at 490 nm was monitored simultaneously at 530 and 590 nm in order to compare the results obtained in isolated mitochondria with those derived from the fluorescence microscopy of individual myocytes. The effects of ADP or carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP) addition on JC-1 fluorescence were assayed by incubating 0·25 mg of mitochondrial protein in buffer I comprising (mM): 120 KCl, 20 K-Hepes, 1 EGTA, 1 sodium pyruvate, 1 sodium malate; and 310 nM JC-1; pH 7·2. The final volume was 2 ml and the temperature was 25 °C. It has to be pointed out that even minor changes in either JC-1 concentration or the amount of mitochondrial protein reduced the reproducibility of the results, especially with respect to the emission at 590 nm. Calibration of JC-1 fluorescence with K^+ diffusion potentials was performed on mitochondria (0·25 mg mitochondrial protein ml^{-1})

incubated in buffer II consisting of 200 mM sucrose, 20 mM mannitol, 1 mM EDTA, 20 mM Na-MOPS; and 1 μM rotenone, 2·5 μM oligomycin; pH 7·2 at 25 °C, and 310 nM JC-1 in the presence of KCl concentrations ranging from 0·1 to 20 mM. The potassium diffusion potential was created by the subsequent addition of 20 nM valinomycin (Reers *et al.* 1991). The membrane potential ($\Delta\Psi_m$ in millivolts) was calculated according to the Nernst equation and assuming that the matrix potassium concentration was equal to 120 mM (Rossi & Azzone, 1969).

Cardiac myocyte isolation and loading with JC-1

Single cardiac myocytes were isolated from ventricles of 2- to 3-month-old Wistar rats as previously described (Capogrossi, Kort, Spurgeon & Lakatta, 1986). The animals were decapitated by use of a guillotine. Cells were suspended (1 mg protein ml^{-1}) in a Hepes–saline (HS) buffer (comprising (mM): 154 NaCl, 5 KCl, 1·2 $MgSO_4$, 1·2 NaH_2PO_4, 1·0 $CaCl_2$ and 10 Na-Hepes; pH 7·4 at 23 °C) to which 11 mM D-glucose had been added.

Cells were loaded with JC-1 (Molecular Probes) essentially as described by Smiley *et al.* (1991). Briefly, concentrated JC-1 was dissolved (10 μg ml^{-1}) in 1 ml of warm (37 °C) HS buffer supplemented with 10% calf serum. This solution was gently added to 2 ml of cell suspension (1 mg cell protein ml^{-1}) in a plastic vial. After 10 min incubation at 37 °C under orbital shaking, cells were washed by sedimentation and resuspension in HS buffer free of JC-1. Modifications of this loading procedure produced unsatisfactory results. Longer incubations decreased markedly the number of rod-shaped cells, whereas higher concentrations of JC-1 resulted in a decrease in fluorescence changes upon de-energization, which was especially evident in the aggregate band.

Measurement of cell length and fluorescence

A small portion (40 μl) of the suspension of JC-1-loaded cells was placed in an experimental chamber, which was mounted on the stage of a custom-designed inverted microscope (Zeiss IM 35). The myocyte to be studied was illuminated with red (650–750 nm) light, and its image visualized using a solid-state television camera (model kP-120V; Hitachi Denshi America, Woodbury, NY, USA). An image of the cell was also projected onto a line-scan diode array (Reticon, Sunnyvale, CA, USA) allowing measurement of cell length. Full details of the instrumentation are given in a previous report (Spurgeon *et al.* 1990).

Epifluorescence was excited by a xenon arc lamp, and an interference filter (Oriel, Stratford, CT, USA) was used to select an excitation wavelength of 490 ± 5 nm. A 510 nm long-pass dichroic mirror (Zeiss) directed the exciting light to a ×100, 1·3 numerical aperture UV fluorglycerin-immersion objective lens (Nikon). Fluorescent light was collected from the whole microscope field, which contained a single myocyte. The fluorescent light was subsequently split by a 550 nm dichroic mirror and directed to two photomultiplier tubes equipped with bandpass interference filters (Oriel) selecting 530 ± 5 and 590 ± 5 nm corresponding to the peak emissions of the monomer and aggregate forms of the indicator, respectively. The fluorescence intensities detected by the two photomultiplier tubes were used to calculate the 530/590 nm emission ratio. Although, as detailed in the Discussion, JC-1 cannot be considered a ratiometric dye, the utilization of the ratio between the two wavelengths can still be useful in minimizing artifacts due to cell motion or fluctuations in the excitation source.

Mag-indo-1 fluorescence

Intracellular Mg^{2+} ($[Mg^{2+}]_i$) was measured using the Mg^{2+}-sensitive probe mag-indo-1 as previously reported (Silverman *et al.* 1994). Briefly, cells were loaded by incubating suspensions of cells with the membrane-permeant acetoxymethyl ester of mag-indo-1 (mag-indo-1 AM; Molecular Probes). Cells were then resuspended in HS buffer free of probe and were allowed a further 60 min for de-esterification of mag-indo-1 AM before the study. Mag-indo-1 was excited at 350 ± 5 nm and the fluorescence emission was collected at 391–434 nm, corresponding to the Mg^{2+}-bound form of mag-indo-1, and at 477–507 nm, corresponding to the Mg^{2+}-free form. The absolute fluorescence of individual mag-indo-1 loaded cells was 29 times that of unloaded myocytes, making insignificant the possible contribution due to NADH changes (Silverman *et al.* 1994). $[Mg^{2+}]_i$ was monitored as the ratio of Mg^{2+}-bound/Mg^{2+}-free forms of the probe (Silverman *et al.* 1994).

Exposure to anoxia

Individual cells were studied in a specially developed chamber (Stern *et al.* 1988) on the stage of the inverted microscope described above. Humidified, warmed ultra-high purity argon (99·9995%; Matheson, Rutherford, NJ, USA) entered a conical well from a toroidal manifold through a circumferential slit lying above an optical quality quartz dish (Mindrum Precision Products, Cucamonga, CA, USA) containing the cells. The gas flow rate was precisely controlled to yield a stable laminar layer of argon preventing the back diffusion of oxygen into the chamber ($P_{O_2} < 0{\cdot}02$ mmHg). During anoxia, myocytes were superfused with HS buffer equilibrated with ultrapure argon. In addition, glucose was omitted and 0·5 mM octanoate represented the only exogenous oxidizable substrate. All cells were studied at room temperature (23 °C).

Metabolic inhibition

The concentrations of metabolic inhibitors to be used were chosen on the basis of the changes induced in either cell length or fluorescence, and assuming a close relationship between ATP depletion and contracture (Haworth, Hunter & Berkoff, 1981). The minimum concentrations which induced rigor contracture consistently were 1 μM FCCP and 2 μM rotenone in the presence of 0·15 mM iodoacetate. A particular case was represented by oligomycin, commonly used as an inhibitor of the mitochondrial ATPase, but which also inhibits *in vitro* sarcolemmal Na^+–K^+-ATPase (Fahn, Koval & Albers, 1966). We titrated the effect of oligomycin in our model system as the minimum concentration required for the abolition of the rise in $[Mg^{2+}]_i$ after FCCP addition, which we found to be 30 μM. The titration proved to be necessary since, consistent with an ouabain-like effect, oligomycin potentiated the contractile performance and, after prolonged treatment (> 20 min), induced intracellular Ca^{2+} overload and eventually hypercontracture. In other terms, low concentrations of oligomycin resulted in an increase, rather than a decrease, in the rate of ATP depletion.

RESULTS

JC-1 fluorescence in solution

JC-1 fluorescence was profoundly modified by interaction with different solvents (Fig. 1). In aqueous media of high ionic strength (0·15 M KCl), the JC-1 emission scan (excitation = 490 nm) was characterized mostly by a single emission peak at 597 nm, corresponding to the formation of J-aggregates. On the other hand, in less polar solvents such as DMSO, acetonitrile or ethanol, aggregation of JC-1 molecules was prevented, resulting in the appearance of a

single emission peak, which corresponds to JC-1 monomers (Fig. 1*A*). The increase in hydrophobicity resulted in a slight shift of the monomer spectrum. The emission peaks were 527 nm in KCl, 530 nm in acetonitrile, 531 nm in ethanol and 539 nm in DMSO. The behaviour of JC-1 in aqueous KCl solution could be differently modified by hydrophobic or polar interactions. The addition of broken mitochondrial membranes resulted in the appearance of the monomer emission with minor changes in the aggregate band (Fig. 1*B*). Similar results could be obtained by adding phospholipids or sonicated mitochondria to a solution of JC-1 in aqueous KCl. The aggregate emission was reduced by the addition of dextran sulphonate, a hydrophilic polyanion (Fig. 1*C*).

JC-1 in isolated mitochondria

It has been previously shown that in isolated mitochondria the fluorescence emission corresponding to J-aggregates can be used to monitor $\Delta\Psi_m$ changes induced by ADP

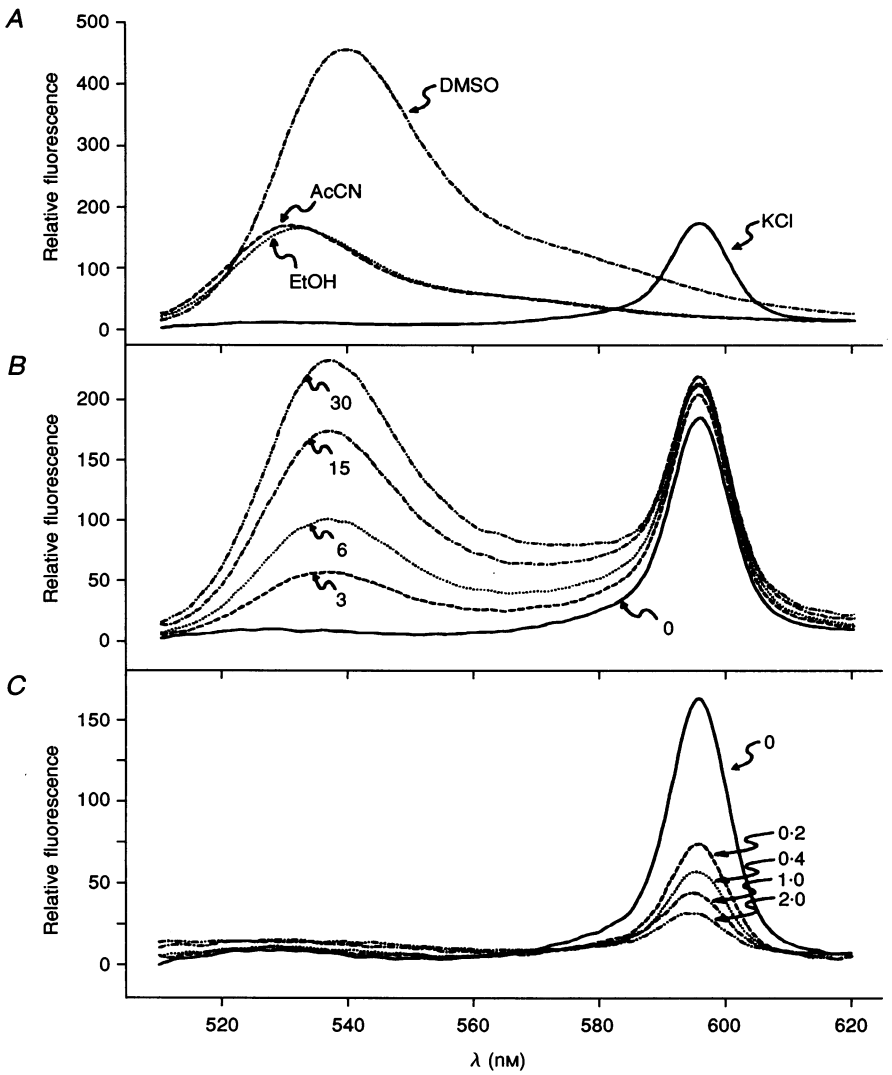

Figure 1. Effects of polar or hydrophobic interactions on the fluorescence emission spectrum of 0·5 μM JC-1

A, fluorescence spectra of JC-1 dissolved in various solvents. KCl, 0·15 M aqueous KCl; EtOH, ethanol; AcCN, acetonitrile; DMSO, dimethyl sulphoxide. Excitation was set at 490 nm and the cuvette mount was thermostated at 25 °C. The results shown in the lower panels were obtained by using a solution of 0·5 μM JC-1 in aqueous 0·15 M KCl, 10 mM Hepes, pH 7·5, to which was added broken mitochondrial membranes (*B*) or dextran sulphonate (*C*). Numbers correspond to micrograms mitochondrial protein per millilitre and milligrams of dextran sulphonate per millilitre in *B* and *C*, respectively. Rat heart mitochondria isolated as described in Methods were disrupted by the following procedure. After isolation the final pellet was suspended in a hypotonic solution (50 mM KCl, 10 mM Hepes, pH 7·5) and subjected to three cycles of freezing and thawing. The resulting suspension was sonicated six times with 5 s bursts separated by 15 s intervals.

phosphorylation, respiratory chain inhibition or uncoupling of respiration (Reers *et al.* 1991). JC-1 solution behaviour forced us to follow not only the aggregate but also the monomer emission (Fig. 2). When rat heart mitochondria were added to buffer I in the presence of 310 nM JC-1, the emission at 590 nm showed an initial rapid increase which was followed by a slower phase resulting in the achievement of a near steady state after 5–7 min. During this period the emission at 530 nm did not show major changes. The addition of 0·25 mM ADP induced a rapid drop in the emission at 590 nm, which was followed by the recovery of the steady state. Oxidative phosphorylation, which is known to generate a decrease of about 30 mV in $\Delta\Psi_m$ (Jensen, Gunter & Gunter, 1986), did not induce any change in the emission at 530 nm. A further ADP addition induced a similar response of the aggregate band, whereas the monomer emission was still not responsive. However, when mitochondria were uncoupled by FCCP addition, both the wavelengths changed, so that the decrease of the emission at 590 nm was simultaneous with the enhancement of the emission at 530 nm.

From these results it could be inferred that small changes in $\Delta\Psi_m$ affect only the fluorescence associated with the J-aggregates, whereas a collapse of $\Delta\Psi_m$, such as that induced by a protonophore such as FCCP, modifies the monomer band as well as the aggregate band. This interpretation was validated by calibrating the changes of the two emission wavelengths with diffusion potentials created by addition of valinomycin, in the presence of different gradients of K^+ ions (Fig. 3). In particular, we investigated the dissipation of various K^+ diffusion potentials brought about by FCCP. The emission corresponding to J-aggregates decreased after FCCP addition for KCl concentrations between 0·1 and 0·5 mM, which correspond to $\Delta\Psi_m$ values ranging from 182 to 140 mV. At higher KCl concentrations the aggregate changes were hardly discernible. On the other hand, the emission at 530 nm was varied by the whole range of KCl concentrations tested.

JC-1 in cardiac myocytes

The increase of the monomer fluorescence concomitant with the decrease of the aggregate fluorescence was observed also in suspensions of cardiac myocytes treated with FCCP (results not shown), as has been recently reported (Duchen *et al.* 1993). However, the measurements performed on cell suspensions are affected by several variables, such as cell viability, amount of dye taken up and cell damage due to mechanical stirring. In addition, the lack of electrical stimulation of cells in suspension rules out from the experimental protocol any relation with myocyte contraction. In order to override these limitations, the JC-1 epifluorescence of individual myocytes was studied on the stage of an inverted microscope in the single excitation (490 nm)–dual emission (530 and 590 nm) mode.

When myocyte superfusion was started, there was a decrease of the emitted light in both channels, which was probably related to the washout of the excess JC-1. After an equilibration period lasting about 10 min, steady state values were achieved. However, large variations in the amount of dye loading were recorded among cells even in those showing similar size and morphology. The fluorescence

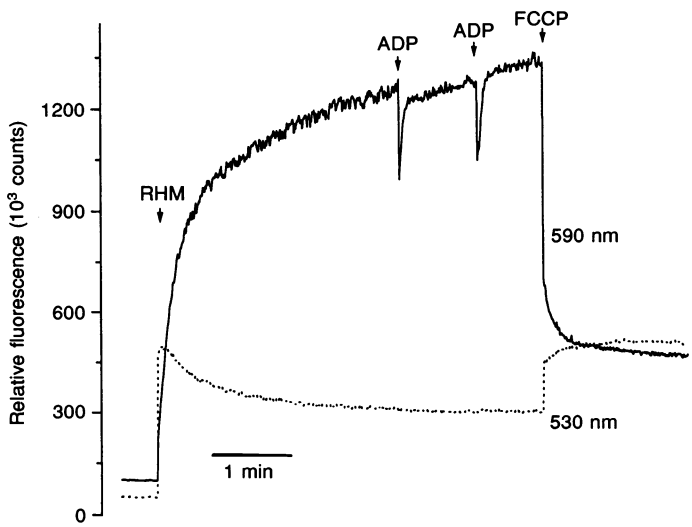

Figure 2. Effects of ADP-stimulated respiration and uncoupled respiration (FCCP) of mitochondria on the fluorescence of JC-1

The indicator JC-1 was present at 310 nM prior to the addition of rat heart mitochondria (RHM; 0·25 mg protein ml^{-1}). The incubation medium contained (mM): 120 KCl, 10 Hepes, 5 succinate, 1 potassium phosphate; and 1 μM rotenone; pH 7·2. The excitation wavelength was 490 nm and the fluorescence was monitored front-face simultaneously at 590 (J-aggregate) and 530 nm (JC-1 monomers) at 37 °C.

intensities did not show any appreciable variations under normoxic conditions when the frequency of stimulation was changed from 0 to 2 Hz or the extent of shortening was enhanced by either increasing Ca^{2+} in the superfusing medium to 2·5 mM or adding 10 nM isoproterenol (results not shown).

In electrically paced myocytes, FCCP induced a precipitous failure of contraction which was followed by rigor contracture within 5–7 min (Fig. 4). The emission at 590 nm showed a gradual decrease which started before the arrest of contraction and attained a plateau at the development of rigor or soon after. An identical time course was shown by the increase in the monomer emission (530 nm). The third panel in Fig. 4, as well in the following figures, shows the behaviour of the 530/590 nm fluorescence ratio, which clarifies the JC-1 response by improving the signal/noise ratio.

Anoxia and reoxygenation

Single cells, loaded with JC-1 and electrically stimulated at 0·2 Hz, were made anoxic and JC-1 fluorescence and cell length were monitored simultaneously. Immediately at the onset of anoxia, the emission at 590 nm began to decrease, reaching a plateau within 16–25 min (Fig. 5). Following these changes, cell contraction amplitude progressively decreased and contraction ceased. It is worth mentioning that from cell to cell the time to contractile failure shows a large variability (Stern *et al.* 1988) which is likely to be the result of differences in glycogen content and/or glycogenolytic rate. Three to five minutes after the arrest of contraction, cells rapidly shortened to roughly two-thirds of their original length as they underwent rigor contracture, which is commonly accepted as an index of ATP depletion. Just before or at the onset of rigor the emission at 530 nm rapidly increased reaching a plateau when the contracture was fully developed. During this phase the emission at 590 nm showed only a variable degree of artifactual increase due to the large cell motion.

On the basis of the observations made both with JC-1 in solution and with isolated mitochondria, the clear separation between the time courses of changes in the aggregate and the monomer bands can be interpreted as a biphasic response of $\Delta\Psi_m$ in anoxic myocytes. The respiratory chain inhibition owing to the lack of oxygen might be expected to force mitochondria to utilize the glycolytically produced ATP in order to maintain, although at lower values, their membrane potential. Consequently, the complete collapse of $\Delta\Psi_m$ would follow the failure of glycolysis and ATP depletion induced by glycogen exhaustion. Indeed, in rotenone-treated cells in the presence of glucose, $\Delta\Psi_m$ collapsed as a result only of the addition of iodoacetate (results not shown). Mitochondria can utilize the ATP produced in the cytosol by inverting the direction of the reaction operating under normal conditions, i.e. ATP synthesis. In order to demonstrate that an active mitochondrial ATPase is necessary for the maintenance of $\Delta\Psi_m$ during the early anoxic phase, myocytes were superfused with oligomycin for 5 min before the exposure to anoxia (Fig. 6). During the normoxic (pre-anoxic) phase, although myocyte shortening was potentiated

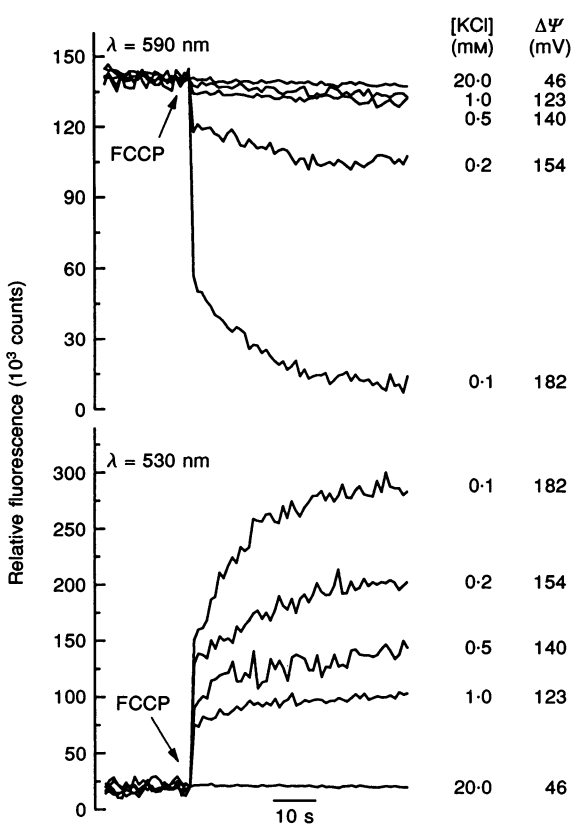

Figure 3. Calibration of the fluorescence of JC-1 in rat heart mitochondria using potassium diffusion potentials

Isolated mitochondria (0·25 mg of protein ml^{-1}) were incubated in a buffer containing 200 mM sucrose, 20 mM mannitol, 20 mM NaMOPS, 1 mM NaEDTA, 1 μM rotenone, 2·5 μM oligomycin, 300 nM JC-1, 20 nM valinomycin, pH 7·2, and one of the concentrations of K^+ indicated in the figure. After achieving a steady-state fluorescence, FCCP was added. Different values of fluorescence at the moment of addition of FCCP have been superimposed in the figure. The excitation wavelength was 490 nm. Note that the fluorescence emission at 530 nm, corresponding to the JC-1 monomer, is responsive to values of $\Delta\Psi_m$ below 140 mV, whereas the 590 nm emission, corresponding to the JC-1 aggregate, shows very little sensitivity in this range of $\Delta\Psi_m$.

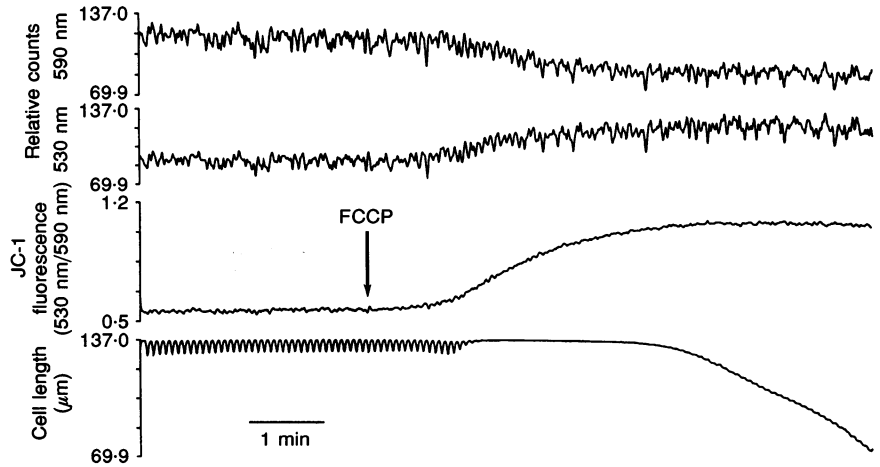

Figure 4

Effect of the uncoupling of respiration on JC-1 fluorescence and cell length of an electrically stimulated (0·2 Hz) single rat cardiac myocyte.

by the concomitant inhibition of Na^+–K^+-ATPase, both the components of JC-1 fluorescence were unchanged (results not shown). In oligomycin-treated cells anoxia was followed by early and synchronous changes of both the components of JC-1 fluorescence, which were partially restored upon reoxygenation (Fig. 6). The increase in emission at 530 nm was not associated with asystole or rigor, which is the converse of results from myocytes not treated with oligomycin.

Neither cell length nor fluorescence was modified by the prolongation of anoxia following rigor. When oxygen delivery was re-established within a short time after rigor (10 min in Fig. 5), myocytes re-lengthened and gradually recovered the ability to twitch. Immediately upon reoxygenation the emission at 530 nm fell abruptly and then, with a slower rate of decrease, approached the pre-anoxic values. The emission at 590 nm showed a large variability. As shown in Fig. 5, during reoxygenation the aggregate fluorescence was almost unchanged except for a later minor decrease. Conversely, in the example of Fig. 7, the readmission of oxygen induced a rapid but partial increase of the emission at 590 nm. The monomer emission showed a decrease superimposable on that reported in Fig. 5. However, major differences exist between the cell

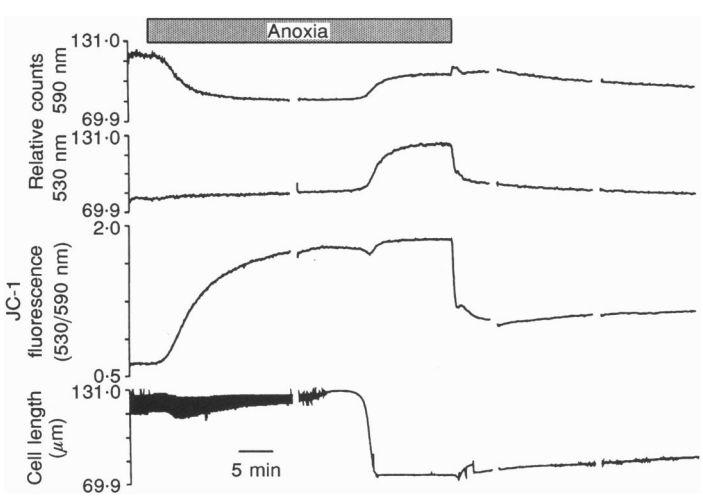

Figure 5. Record of JC-1 fluorescence and cell length prior to, during and following exposure to anoxia

The cell was electrically stimulated at 0·2 Hz. The onset of rigor contracture is indicated by the rapid decrease in cell length seen soon after the failure of contraction. Reoxygenation was performed 10 min after the development of rigor contracture. This cell showed the typical patterns of reversibility since it retained its morphology and partially recovered the ability to twitch upon the readmission of oxygen.

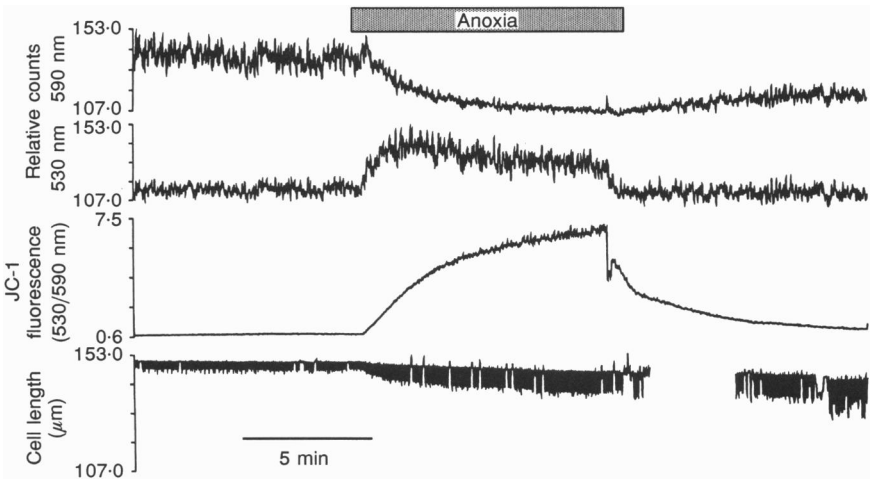

Figure 6. Effect of anoxia on JC-1 fluorescence and contractile function in the presence of mitochondrial ATPase inhibition

For this purpose, the anoxic phase was preceded by a 5 min superfusion with 30 μM oligomycin.

exemplified by Fig. 5 and that represented by Fig. 7. Since in this latter experiment anoxia was maintained for 30 min after rigor contracture, reoxygenation resulted in irreversible damage as indicated by (i) hypercontracture to a rounded form; (ii) loss of sarcomere pattern; and (iii) lack of electrically stimulated contraction. Thus, from JC-1 fluorescence it appears that $\Delta\Psi_m$ is partially restored during reoxygenation independently of the recovery of mechanical function. This suggestion was further validated by the addition of FCCP after the myocyte had been irreversibly damaged by reoxygenation (Fig. 7). The response to the uncoupling agent was characterized by sudden 'antiparallel' changes in the two emission wavelengths, i.e. enhancement of the monomer and decrease of the aggregate fluorescences.

In order to gain further information about the bioenergetics of anoxic single myocytes, the results concerning $\Delta\Psi_m$ were compared with the observations obtained with another indicator of energy metabolism, namely Mg^{2+}. The intracellular concentration of this cation ($[Mg^{2+}]_i$) was studied in mag-indo-1-loaded myocytes. We have previously shown that, under physiological conditions, $[Mg^{2+}]_i$ was scarcely affected by changes in intracellular $[Ca^{2+}]$ and pH (Silverman *et al.* 1994). On the other hand, conditions associated with a decrease of ATP, which is the most

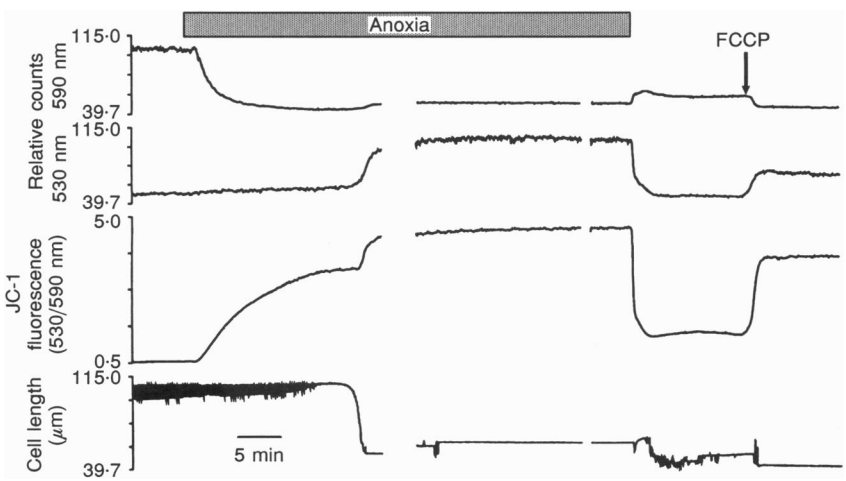

Figure 7. Record of JC-1 fluorescence and cell length during prolonged anoxia and reoxygenation followed by FCCP addition

This myocyte hypercontracted when oxygen was readmitted 30 min after rigor development. FCCP addition is associated with changes in JC-1 fluorescence without any further modification of cell function or morphology.

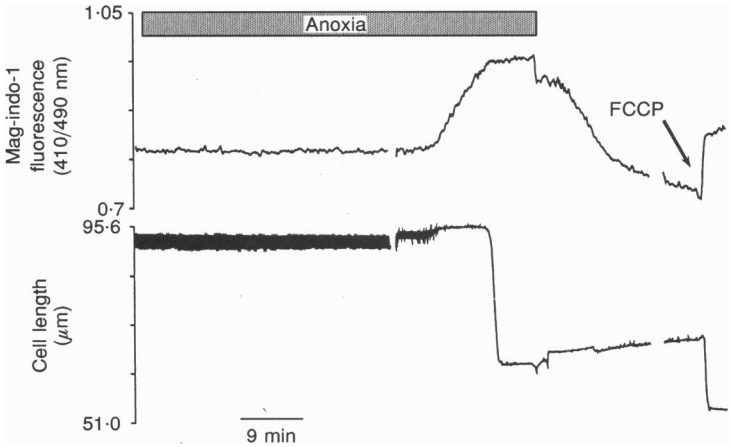

Figure 8. Record of $[Mg^{2+}]_i$ and cell length during anoxia and reoxygenation followed by FCCP addition

$[Mg^{2+}]_i$ was monitored by reference to the ratio of emission at 410 nm to 490 nm in mag-indo-1-loaded myocytes.

abundant intracellular Mg^{2+} chelator, resulted in a more than twofold increase in $[Mg^{2+}]_i$ (Silverman *et al.* 1994).

During the early phase of anoxia, when the stimulated contraction was preserved, there was no change in $[Mg^{2+}]_i$; it began to rise as contraction failed and rose steadily until the onset of rigor contracture (Fig. 8), from a control value of $1{\cdot}31 \pm 0{\cdot}12$ mM to $2{\cdot}78 \pm 0{\cdot}21$ mM. When cells were reoxygenated 10 min after rigor, $[Mg^{2+}]_i$ gradually returned to preanoxic values as the cells partially re-lengthened and contraction resumed. The subsequent exposure of reoxygenated cells to uncoupling agents again induced rigor contracture and $[Mg^{2+}]_i$ rise (Fig. 8). Reoxygenation after longer periods of anoxia was always associated with a decrease in $[Mg^{2+}]_i$ even if cells hyper-contracted into rounded dysfunctional forms. Further, even in these irreversibly damaged cells, $[Mg^{2+}]_i$ increased in response to subsequent FCCP addition (results not shown).

DISCUSSION

It has been proposed that, in a process driven by $\Delta\Psi_m$, JC-1 monomers should accumulate within the aqueous matrix space resulting in the formation of J-aggregates once the critical J-aggregate concentration is exceeded (Reers *et al.* 1991). Accordingly, a decrease in membrane potential has been related to a fall in the aggregate fluorescence (Reers *et al.* 1991). However, a more complex picture emerges from our results (Figs 2–7). In the present study and in two other recent reports (Duchen *et al.* 1993; Minezaki, Suleiman & Chapman, 1994), de-energization procedures were consistently associated not only with the expected decrease of the fluorescence at 590 nm, but also with an increase in the monomer fluorescence at 530 nm. Upon de-energization, if the probe that had accumulated in the matrix aqueous phase had been totally released in the aqueous cytosolic space, the resulting dilution could not have produced an increase in monomer fluorescence. Therefore, the interaction of JC-1 with hydrophobic components, i.e. mitochondrial membranes, has to be considered to explain the whole range of JC-1 fluorescence changes induced by de-energization. In solution, the fluorescence peaks assigned to the monomer and aggregate were sensitive to the presence of agents which provide hydrophobic or polar interactions (Fig. 1). The interaction of JC-1 with mitochondrial membrane components could alter the fluorescence properties associated with the monomer and aggregate. This postulated interaction with membrane components could itself be dependent on $\Delta\Psi_m$ in a manner which either differs from, or is similar to, the $\Delta\Psi_m$ driven uptake of JC-1 into the mitochondrial matrix.

The responses of monomers and aggregates to de-energization differed with respect to the time course (Figs 5 and 7) and the range of sensitivity to $\Delta\Psi_m$ changes (Figs 2 and 3). When $\Delta\Psi_m$ was high ($\geqslant 140$ mV), aggregate fluorescence was sensitive to changes in energization while monomer fluorescence was insensitive. In contrast, at lower $\Delta\Psi_m$ values the monomer fluorescence was sensitive to changes in energization while the aggregate fluorescence showed very little sensitivity. These characteristics suggest that JC-1 may act as a unique double sensor for low and high potential ranges. It should be emphasized that fluorescence signals depending on $\Delta\Psi_m$ cannot be calibrated in intact cells (Farkas, de Wei, Febbroriello, Carson & Loew, 1989) and data are generally presented as absolute or percentage changes of relative fluorescence intensities (Farkas *et al.* 1989, Duchen & Biscoe, 1992; Duchen *et al.* 1993; Minezaki *et al.* 1994). The potential quantitative information available as a result of the different sensitivities

of the monomer and aggregate fluorescence to $\Delta\Psi_m$ is excluded by the use of JC-1 as an emission ratio probe. This use has been previously suggested, based on the apparent insensitivity of the monomer fluorescence to membrane potential (Reers *et al.* 1991). In fact, it appears that both the monomer and aggregate fluorescence provide useful information about $\Delta\Psi_m$. JC-1 may be used as an emission ratio probe under conditions in which the monomer or aggregate fluorescence remains constant. The results reported in the present paper do not provide a definite mechanism for the different sensitivity of aggregates and monomers to $\Delta\Psi_m$ changes. It is possible that a slight decrease of $\Delta\Psi_m$ might promote the decrease in aggregate emission by reducing the concentration of JC-1 within the mitochondrial matrix below the critical concentration which is required for aggregation. However, it is then difficult to explain the rise in monomer emission which accompanies a large decrease of $\Delta\Psi_m$. In fact, it is unlikely that only the JC-1 molecules which remain within mitochondria after the initial de-energization are the ones which interact with the membrane. Alternatively, it is tempting to speculate that the decrease in aggregate emission might be due to polar interaction of JC-1 with negative charges on the inner leaflet of the inner mitochondrial membrane. Upon $\Delta\Psi_m$ collapse, the massive release of JC-1 from mitochondria together with the disappearance (or decrease) of membrane charges would force the probe to interact with the inner core of the membrane giving rise to monomer emission.

Few attempts have been made to characterize $\Delta\Psi_m$ modifications induced by a lack of oxygen in intact cells. One of the earlier studies showed that in hepatocytes $\Delta\Psi_m$ is retained for 30 min after the onset of anoxia owing to the processes of mitochondrial cation extrusion and anion sequestration (Aw, Anderson & Jones, 1987). In particular it was shown that Ca^{2+} is released and adenine nucleotides are retained within the matrix space suggesting the inhibition of adenylate translocase (Aw *et al.* 1987). However, neither mitochondrial Ca^{2+} release nor adenylate translocase inhibition appeared to be involved in $\Delta\Psi_m$ maintenance in anoxic myocytes. In the same experimental model used in the present study, mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) was unchanged during anoxia until rigor contracture developed (Miyata, Lakatta, Stern & Silverman, 1992). The prolongation of anoxia after rigor was associated with the rise of both $[Ca^{2+}]_m$ and cytosolic Ca^{2+} ($[Ca^{2+}]_c$) which showed almost identical values (Miyata *et al.* 1992). Thus, in cardiac myocytes mitochondrial Ca^{2+} release did not occur either during the initial phase of anoxia, when $\Delta\Psi_m$ was maintained, or in the later stage, when $\Delta\Psi_m$ collapsed and rigor developed. Furthermore, the inhibition of adenylate translocase does not seem to occur in anoxic myocytes. Conversely, the results of the present study suggest that during the initial phase of anoxia adenylate translocase activity was preserved and glycolytic ATP was utilized to drive ATP synthase backwards, allowing maintenance of $\Delta\Psi_m$. This mechanism is supported by the following evidence: (i) $\Delta\Psi_m$ is maintained until rigor develops and ATP is exhausted; (ii) $\Delta\Psi_m$ collapsed immediately if ATP synthase is inhibited; and (iii) mitochondria are able to utilize ATP produced by glycolysis. These result differ somewhat from those of Duchen *et al.* (1993), who found relatively minor decreases in $\Delta\Psi_m$, as measured with JC-1, in the early stages of anoxia of cardiac myocytes; only following the onset of rigor contraction was there found to be a pronounced depolarization. It is hard to read more into the difference between this and the present work, as only the 530/580 nm ratio is presented by Duchen *et al.* (1993), whereas much of the information in the present report comes from the individual wavelengths. Clearly, both reports support the idea of partial maintenance of $\Delta\Psi_m$ by glycolytic ATP during the early stages of anoxia.

The rapid fall in ATP content resulting from glycogen exhaustion seems to be the cause of rigor development (Haworth *et al.* 1981). A direct and elegant demonstration of the relation existing between ATP loss and contracture has been obtained recently in myocytes injected with luciferase, superfused with iodoacetate and exposed to NaCN (Bowers, Allshire & Cobbold, 1992). Anoxia cannot be studied with the luciferase procedure, since the luciferase-catalysed oxidation of D-luciferin requires oxygen. We used the fluorescence of mag-indo-1 to monitor $[Mg^{2+}]_i$, as an indirect and qualitative measure of ATP hydrolysis (Silverman *et al.* 1994). Inside the cells the majority of the Mg^{2+} ions are bound to ATP, so that a fall in ATP content is reflected by antiparallel increases in $[Mg^{2+}]_i$. Other factors, such as pH and $[Ca^{2+}]$, can influence mag-indo-1 fluorescence and $[Mg^{2+}]_i$, but no major variations of the intracellular concentrations of H^+ and Ca^{2+} were observed during anoxia prior to rigor development (Silverman *et al.* 1991; Miyata *et al.* 1992). Thus, the increase in $[Mg^{2+}]_i$, which started after the failure of contraction and plateaued at the onset of the rigor (Fig. 8), should reflect a fall in ATP content (Silverman *et al.* 1994).

It has been demonstrated that mitochondrial ATPase constitutes a relevant site for ATP hydrolysis in the anoxic myocardium (Rouslin, Ericsson & Solaro, 1986). The rapid and complete fall of $\Delta\Psi_m$ that we observed in oligomycin-treated anoxic myocytes (Fig. 6) supports the notion of a mitochondrial utilization of ATP for $\Delta\Psi_m$ preservation. This mechanism does not seem to pertain exclusively to cardiac myocytes. The acceleration in anoxia-induced mitochondrial depolarization after oligomycin administration has been shown also in type I cells of carotid bodies (Duchen & Biscoe, 1992).

It could be argued that mitochondria hydrolyse their own (matrix) ATP while mechanical activity is maintained by cytosolic ATP in the absence of ATP exchange between intracellular compartments. This hypothesis is ruled out by two different observations. First, the simultaneous failure

of cell shortening and $\Delta\Psi_m$ strongly suggests that these two processes are maintained by the same pool of ATP. Secondly, under aerobic conditions, if glycolysis was inhibited by iodoacetate, the addition of rotenone, which was without effect in untreated myocytes, was immediately followed by rigor development and $\Delta\Psi_m$ loss (F. Di Lisa & R. G. Hansford, unpublished results).

Mitochondrial failure has been associated with irreversibility of ischaemic damage. Our data show that mitochondrial function is retained even when cell morphology and function are irreversibly impaired (Figs 7 and 8). However, these results do not exclude a possible role for mitochondria in determining the hypercontracture, which most likely represents an irreparable event in this model. Actually, the appearance of rounded dysfunctional forms seems to require respiring and phosphorylating mitochondria. Myocytes exposed to the uncoupling agent FCCP underwent rigor contracture (Fig. 4) and never developed hypercontracture. In addition, if the respiratory chain was inhibited with rotenone during anoxia and reoxygenation, cells retained the rigor state indefinitely (results not shown), as occurs if anoxia is not followed by reoxygenation. These data are in accordance with previous observations (Ganote, McGarr, Liu & Kaltenbach, 1980) which showed that the extent of myocardial damage is limited by mitochondrial inhibitors. These findings suggest that the restoration of ATP production by mitochondrial oxidative phosphorylation is essential for cell recovery, but it can also contribute to those processes which produce cell necrosis. This apparent paradox could be related to the different ATP requirements of contraction and relaxation at the myofilament level. In fact three major possibilities can be envisaged: (i) under conditions of mitochondrial inhibition (cyanide) or uncoupling, ATP is virtually absent and cells or tissues are kept 'frozen' in a rigor state; (ii) when reoxygenation is accompanied by adequate recovery of mitochondrial function, the restoration of ATP content allows cell elongation or tissue relaxation and intracellular $[Ca^{2+}]$ rapidly returns to control levels; and (iii) when mitochondrial function is impaired, low levels of ATP impair Ca^{2+} homeostasis and enhance the formation of rigor bonds which, in the absence of sufficient energy for the relaxation process, produce the hypercontracture. Indeed, Ca^{2+} appears to increase the ATP requirements for the maintenance or the recovery of the elongated morphology (Altschuld *et al.* 1985).

The association between myocardial damage and intracellular calcium overload has been related to mitochondrial dysfunction since the pioneer observation of Shen & Jennings (1972). Oxidative phosphorylation is likely to be reduced by a massive mitochondrial uptake of Ca^{2+} since both the processes utilize the same source energy, namely $\Delta\Psi_m$. The reduced ATP availability is followed by a failure of the mechanisms which extrude Ca^{2+} from the sarcoplasm, creating a vicious cycle of calcium overload and mitochondrial impairment. Alternatively, or in addition, the rise in $[Ca^{2+}]_i$ could promote the opening of the cyclosporine-sensitive mitochondrial permeability transition pore (MTP), leading to a sudden $\Delta\Psi_m$ dissipation. It has been proposed that inappropriate MTP opening during post-ischaemic reperfusion is a key event in the ensuing tissue damage (Crompton & Costi, 1990). In fact, $\Delta\Psi_m$ dissipation caused by MTP opening could well result in massive and abrupt release of the accumulated Ca^{2+} into the cytosol leading to cell death. This mechanism is not supported by the present results, since $\Delta\Psi_m$ and ATP contents, as indirectly traced by $[Mg^{2+}]_i$, are partially restored during reoxygenation, in a process which is independent of functional or morphological recovery. In the same experimental model used in the present study, during reoxygenation $[Ca^{2+}]_m$ is similar to, or slightly less than, that measured during the rigor phase, whereas $[Ca^{2+}]_c$ gradually declines (Miyata *et al.* 1992). MTP opening might rather occur during anoxia. It has been demonstrated that the membrane transition is a direct function of $\Delta\Psi_m$ with a voltage-gated mechanism (Bernardi, 1992). Thus it could be hypothesized that a drastic reduction in glycolytic ATP production curtails the possibility of maintaining $\Delta\Psi_m$, the decline of which below a critical threshold would promote MTP opening. Such a mechanism could underlie the rapid $\Delta\Psi_m$ collapse and $[Ca^{2+}]_m$ rise which occur concomitantly with the rigor development. On the other hand, conditions which give rise as a primary event to cytosolic calcium overload could lead to an abnormal elevation of $[Ca^{2+}]_m$ followed by MTP opening and $\Delta\Psi_m$ collapse. Indeed, it has been recently demonstrated that Ca^{2+} readmission to myocytes previously bathed in the absence of Ca^{2+} gives rise to a biphasic response of $\Delta\Psi_m$ consisting of a transient increase followed by a rapid and persistent collapse (Minezaki *et al.* 1994).

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