Changes in mitochondrial function induced in isolated guinea-pig ventricular myocytes by calcium overload

K. K. Minezaki, M.-S. Suleiman and R. A. Chapman*

British Heart Foundation Research Group, School of Veterinary Science, University of Bristol, Southwell Street, Bristol BS2 8EJ

1. Changes in $[Ca^{2+}]_i$ and pH_i, mitochondrial membrane potential (Ψ_m) and mitochondrial [NADH] have been measured independently using fluorescent techniques in single isolated guinea-pig ventricular myocytes subjected to Ca^{2+} overload.

2. The changes in NADH autofluorescence on the inhibition or uncoupling of respiration are consistent with the signal emanating from the mitochondrial NADH.

3. Removal of Ca^{2+} and Mg^{2+} from the bathing Tyrode solution induced a modest fall in both $[Ca^{2+}]_i$ and pH_i, a small slowly developing depolarization of Ψ_m and an initial fall followed by a rise in mitochondrial [NADH].

4. In myocytes that maintained an intact sarcolemma, return to Ca^{2+}-containing fluid elicited a strong but brief intracellular acidification, a rise in $[Ca^{2+}]_i$ which generally recovered more slowly to stabilize above the initial level in Tyrode solution, a steep fall in mitochondrial [NADH] and a brief transient recovery followed by a large sustained depolarization of Ψ_m. NADH autofluorescence and mitochondrial depolarization often reached values that were not further increased by uncoupling respiration although recovery of NADH was elicited by inhibitors of respiration.

5. These changes were reduced when the Ca^{2+} overload was less severe as evidenced by a reduced hypercontracture upon Ca^{2+} repletion. A similar reduction could be routinely achieved by elevation of $[Mg^{2+}]_o$ during the period of Ca^{2+} depletion.

6. These results suggest that the well-established depletion of energy-rich phosphates that occurs on Ca^{2+} overload is due to the combined effects of the failure of the citric acid cycle to provide sufficient mitochondrial NADH for the respiratory chain and an uncoupling of respiration from ATP production due to depolarization of Ψ_m. The former effect could result from the depletion of sarcoplasmic amino acids and the latter from increased Ca^{2+} cycling across the mitochondrial wall provoked by the elevated $[Na^+]_i$ and $[Ca^{2+}]_i$.

The responses of cardiac muscle to metabolic inhibition suggest that glycolysis and/or β-oxidation can provide sufficient ATP to sustain the quiescent heart and maintain the transmembrane ionic gradients but oxidative phosphorylation is required to meet the needs of the contracting heart (Cobbold & Bourne, 1984; Allen, Morris, Orchard & Pirolo, 1985; Bowers, Allshire & Cobbold, 1992). Oxidative phosphorylation is coupled with the demands of the cell by the intramitochondrial dehydrogenases associated with the citric acid cycle (Denton & McCormack, 1980), the mitochondrial ATP synthetase (Das & Harris, 1990), both regulated by intramitochondrial Ca^{2+}, as well as by feedback from the breakdown products of ATP (Balaban, 1992). There is ample evidence to suggest that changes in sarcoplasmic $[Ca^{2+}]$ are relayed into the mitochondria but the change within the mitochondrial matrix depends on the interaction between the entry of Ca^{2+}, via a uniport driven by the H^+-dependent mitochondrial membrane potential, and the egress of Ca^{2+} via the electrically neutral Na^+–Ca^{2+} antiport in the mitochondrial membrane (for recent reviews see Hansford, 1985; McCormack, Halestrap & Denton, 1990). This means that changes in intramitochondrial $[Ca^{2+}]$ (and effects on the citric acid cycle) may occur when the sarcoplasmic levels of Ca^{2+}, Na^+ or H^+ are altered. Indeed, Cox & Matlib (1993) have recently shown that the activity of the mitochondrial Na^+–Ca^{2+} exchange can influence oxidative phosphorylation in isolated cardiac mitochondria.

*To whom correspondence should be addressed.

A loss of control of sarcoplasmic Ca^{2+} and pH coupled with a fall in ATP are commonly associated with the onset of damage to cardiac muscle. A condition where this occurs is the so-called calcium paradox where a steep rise in sarcoplasmic $[Ca^{2+}]$ precedes the fall in energy-rich phosphates and therefore contrasts with the changes seen on metabolic inhibition (see Chapman & Tunstall, 1987; for review). In the Langendorff-perfused rat heart the removal of Ca^{2+} from the bathing medium leads to a marked fall in oxygen consumption and glucose utilization. This is associated with little change or even a small increase in energy-rich phosphates (Penpargkul & Scheuer, 1969; Schaffer & Tan, 1985; Bulkley, Nunnally & Hollis, 1987) suggesting that a reduction in oxidative energy production follows the fall in energy demand. On return to Ca^{2+}-containing Tyrode solution there is evidence of a mismatch between the demand for energy and its production because a strong but transient recovery of glucose utilization and a weak transient recovery of oxygen consumption accompany the decline in energy-rich phosphates (Schaffer & Tan, 1985; Bulkley *et al.* 1987).

Mitochondrial NADH (and FADH) produced by the citric acid cycle and β-oxidation provides 'fuel' for the electron transport chain to generate the chemiosmotic gradient that drives the synthesis of ATP. As a result the $NADH/NAD^+$ ratio has been widely used as a measure of the energy flux of a cell (Chance, 1976). Early work relied on chemical measurements of specific redox couples to calculate a ratio (Schaffer & Tan, 1985, used the $[\text{glutamate}]/\{[\alpha\text{-ketoglutarate}][NH_4^+]\}$ quotient). This method, however, is likely to be unreliable when applied to the heart (Hassinen, 1986); particularly as a substantial efflux of the amino acids used for the calculation occurs during Ca^{2+} depletion (Suleiman & Chapman, 1993). In several types of mammalian cell and isolated mitochondria changes in autofluorescence (at wavelengths of between 420 and 540 nm, when excited by light at a wavelength of 350 nm) have been positively correlated with changes in mitochondrial [NADH] (Chance & Baltscheffsky, 1958; Chance, Schoener, Oshino, Itshak & Nahase, 1979; Nuutinen, 1984; Eng, Lynch & Balaban, 1989; Duchen & Biscoe, 1992*a*). Although this signal does not distinguish between NADH and NAD(P)H or between cytosolic and mitochondrial NADH, about 80 % of the autofluorescence would seem to originate from mitochondrial NADH in the heart (Eng *et al.* 1989). The contribution of extramitochondrial NADH should be further reduced when pyruvate is used as the metabolic substrate because glycolysis is bypassed. In this work changes in mitochondrial [NADH] have been measured in this way and correlated with changes in $[Ca^{2+}]_i$, pH_i and mitochondrial membrane potential determined separately using fluorescent indicators loaded into the cells. The contraction of the myocytes was continuously monitored and the integrity of the cell membrane was determined by the ability of the cell to exclude the dye Trypan Blue. The changes in mitochondrial NADH levels, measured in this way, differ markedly from those calculated for whole heart and suggest that energy demand is also important in determining the response of the heart to changes in $[Ca^{2+}]_i$.

Preliminary reports of this work have appeared in abstract form (Minezaki, Suleiman & Chapman, 1992; 1993*a*, *b*).

METHODS

Cell isolation

Adult male guinea-pigs (200–350 g body weight) were killed by cervical dislocation and the hearts quickly removed. Ventricular myocytes were isolated as described previously (Rodrigo & Chapman, 1991) except that 40 mM taurine was included in the Tyrode solution used for final perfusion and mechanical dispersion. This method produces myocytes with an increased resistance to the calcium paradox (Chapman, Chatamra & Little, 1991).

Experimental procedure

Isolated myocytes were stored in the taurine-containing Tyrode at room temperature and used within 8 h. They were placed in a Perspex perfusion chamber in which the floor was formed by a glass coverslip. The volume of the chamber was less than 0·3 ml and the flow of the solutions through the chamber was 1·0 ml min^{-1}. The temperature of the fluid in the chamber and that of the incoming solutions was controlled at 37 °C (± 1 °C) as described previously (Chapman, 1993). Once the myocytes had settled on the floor of the chamber, perfusion with normal Tyrode solution was started. The normal Tyrode solution contained (mM): 140 NaCl, 2·5 KOH, 2·5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 sodium pyruvate, 5 Hepes with a pH of 7·4. The Ca^{2+}- and Mg^{2+}-free Tyrode solution was made by omitting the Ca^{2+} and Mg^{2+} salts and the pCa was buffered to 7 or 8 using a mixture of 4 mM Ca-EGTA and 4 mM Tris-EGTA (Chapman, Coray & McGuigan, 1983). In a few experiments sodium pyruvate was replaced by 10 mM D-glucose while in others 20 mM NH_4Cl was added as a solid or $[Mg^{2+}]_o$ was elevated without compensation for the change in tonicity.

Myocytes were viewed with the aid of a TV camera attached to a Nikon Diaphot inverted microscope (Nikon, Telford, Shropshire, UK) and illuminated with the microscope light through a low-pass 700 nm filter. At the end of each experiment the integrity of the sarcolemma was tested by a few minutes perfusion with Tyrode solution containing 0·03 % Trypan Blue. About 80 % of the myocytes subjected to Ca^{2+} depletion and repletion were able to exclude the dye from the sarcoplasm and the results from these myocytes will be presented in detail.

Fluorescence measurements

The epifluorescence of single myocytes was measured with a photon-counting system (Newcastle Photometric Systems, Newcastle upon Tyne, UK) attached to a Nikon Diaphot inverted microscope. A low-pass filter (600 nm) was placed in front of the photomultipliers to cut out red light from the microscope light. NADH autofluorescence was excited at 350 nm and the emission was measured between 420 and

540 nm. Changes in NADH concentration of 1 μM in an aqueous solution could be detected using this method. Changes in sarcoplasmic [Ca^{2+}] were monitored using the fluorescent probe fura-2 loaded into the myocytes by incubation in a 5 μM solution of the acetoxymethyl (AM) ester form for 30 min at 35 °C. The two excitation filters were 350 and 380 nm bandpass and the emission filtered by a 420 nm low-cut filter. An approximate calibration of the change in the fura-2 fluorescence ratio was made with 150 mM KCl solution with Ca^{2+} activity buffered over the range 10^{-7} to 10^{-5} M (the Ca^{2+} activity was calculated according to the method of Chapman *et al.* 1983). This method gives a relatively accurate measurement of the size of the change in $[Ca^{2+}]_i$ but the actual values are approximate (Williams & Fay, 1990). This problem was particularly noticeable in myocytes exposed to Ca^{2+}-free Tyrode solution when the fluorescence ratio could fall to values lower than those obtained during calibration, suggesting that this approximate calibration underestimates the real $[Ca^{2+}]_i$.

Changes in sarcoplasmic pH (pH_i) were made in myocytes loaded with the AM ester form of the agent 2′,7′-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein (BCECF; 2·5 μM for 30 min at 35 °C). The myocytes were excited at 440 and 490 nm and the emission was measured at 530 nm. Calibration of the BCECF fluorescence ratio was achieved by exposure of the myocyte to a solution of 150 mM KCl containing 10 μM nigericin and varying the pH over the range 5·5–8·5 (Thomas, Buchsbaum, Zimniak & Racker, 1979).

To determine changes in the mitochondrial membrane potential (Ψ_m) myocytes were loaded with either rhodamine-123 (rhod-123) or carboxycyanine(5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Lemasters, DiGuiseppi, Niemann & Herman, 1987; Reers, Smith & Chen, 1991). Incubation at a relatively low concentration of 1 μg ml^{-1} rhod-123 for 30 min at 35 °C achieved sufficient loading to allow changes in fluorescence (excited at 488 nm and measured at 530 nm) to be detected when carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to the perfusate, but the myocytes still showed an increased fragility. The response obtained with CCCP suggested that the probe was not only leaving the mitochondria but also the cell. JC-1 has been successfully used as a probe to monitor Ψ_m in a variety of mammalian cells (Reers *et al.* 1991; Smiley *et al.* 1991). This agent can be used ratiometrically and therefore measurements are less affected by changes in probe concentration. On entering the cytoplasm, fluorescence peaks at 530 nm but then falls to stabilize after a few minutes. This fall is mirrored by a rise in fluorescence of 590 nm. This change is due to the accumulation of the JC-1 in the mitochondria and its subsequent forced aggregation into complexes. The partitioning of the dye is dependent on Ψ_m which is therefore measured by the ratio of the fluorescence 530/590 nm. Cardiac myocytes were loaded with JC-1 by incubation at 37 °C at a concentration of 5 μM in Tyrode solution for 30 min at 37 °C. No calibration of these signals was made although work with isolated mitochondria show a linear relationship between Ψ_m and fluorescence.

Chemicals

CCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP), oligomycin, rotenone, amytal and Trypan Blue were obtained from Sigma Chemical Co., Poole, Dorset; fura-2 AM, BCECF AM, rhod-123 and JC-1 were obtained from Molecular Probes Inc. (Eugene, OR, USA).

Analysis and statistics

The epifluorescence, as measured by the photomultipliers, was recorded and stored as counts per 0·2 s. Mathematical manipulation of files and the production of the figures were done using the software package Fig.P (Biosoft, Cambridge, UK). To ease transfer, large data files were decimated by averaging up to five successive points. For comparison of experiments, data are expressed throughout as means ± S.E.M. with (n) as the number of observations. Statistical comparisons were made using ANOVA or Student's t test, as appropriate, using Statview (Abacus, Berkeley, CA, USA) on a MacIntosh SE/30.

RESULTS

Control experiments

NADH autofluorescence

In a variety of cells the autofluorescent signal between 420 and 540 nm when excited by light at 350 nm has been shown to originate primarily from mitochondrial NADH (Eng *et al.* 1989; Duchen & Biscoe, 1992*a*). In guinea-pig myocytes this autofluorescence was increased by blockade of the electron transport chain by rotenone (to block the NADH dehydrogenases irreversibly), amytal (to prevent the reduction of ubiquinone) and cyanide (to block the oxidation of the cytochromes) while the uncoupling of respiration by the proton ionophores CCCP and FCCP decreased the NADH autofluorescence (Fig. 1 and Table 1). The response to CN^- was rapid and when exposure was for less than 2 min, recovery was also rapid and some cases showed a brief undershoot. With longer exposure the recovery showed a second slower phase and sometimes damped oscillation (Fig. 1*A*). A reversible increase was also seen with amytal while 10 μM rotenone caused a slowly developing irreversible increase in NADH autofluorescence (Table 1). Exposure to CCCP induced a fall in NADH autofluorescence which reached a maximum at 5 μM (Fig. 1*B* and Table 1). A similar fall was seen when 5 μM CCCP was applied together with 2 μM oligomycin to block ATP synthetase ($n = 6$), or when 5 μM FCCP was applied alone ($n = 3$) or together with oligomycin ($n = 5$).

Intracellular [Ca^{2+}]

Work with the fluorescent indicator quin-2 and with Ca^{2+}-sensitive microelectrodes has shown that $[Ca^{2+}]_i$ in isolated mammalian cardiac myocytes falls on exposure to Ca^{2+}-free Tyrode solution and rises steeply and often irreversibly on return to normal Tyrode solution (Lambert, Johnson, Lamka, Brierley & Altschuld, 1986; Rodrigo & Chapman, 1991). In guinea-pig myocytes, isolated with the aid of taurine, the fura-2 ratio gives an approximate value for $[Ca^{2+}]_i$ of 84 ± 2 nM ($n = 32$) for cells bathed in normal Tyrode solution. On exposure to a bathing fluid free of Mg^{2+} at a pCa of 7, the fura-2 fluorescence ratio decreased and the approximate free $[Ca^{2+}]_i$ approached steady values close to the limit of resolution of the calibration technique

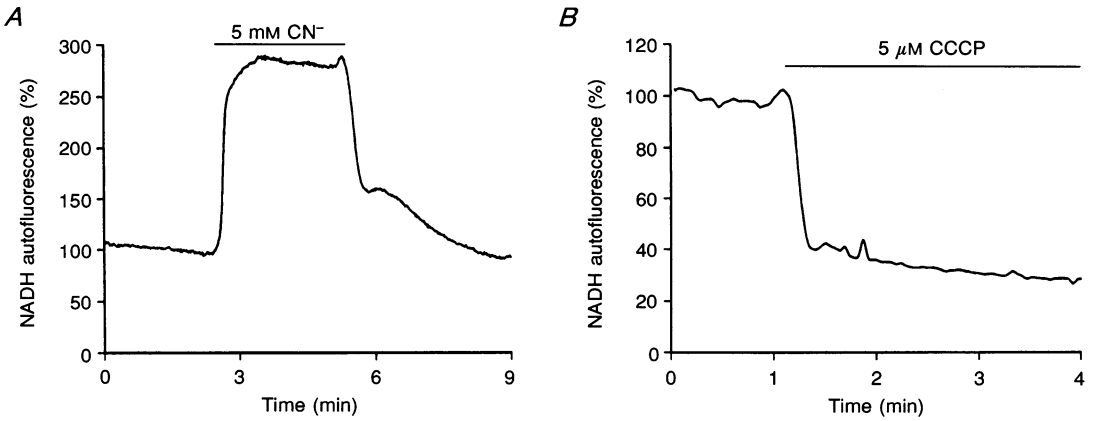

Figure 1. Typical changes in NADH autofluorescence from an isolated myocyte when respiration was fully inhibited by cyanide or fully uncoupled with CCCP
NADH autofluorescence recorded from a single isolated guinea-pig ventricular myocyte expressed as a percentage of the steady level at the beginning of the experiment in response to 5 mM CN^- (*A*) which induced a large reversible increase and the subsequent application of 5 μM CCCP (*B*) which induced a marked sustained and irreversible decline. The period of exposure to the inhibitor is indicated by a horizontal bar in each case. Data from experiments of this type were used to calculate the relative oxygen consumption.

(Fig. 2*A*). On return to normal Tyrode solution, the fura-2 fluorescence ratio invariably showed a rapid increase which reached a peak within 1–3 min. However, the size and form of the change varied from myocyte to myocyte. Of the thirty myocytes treated in this way, seven showed a modest increase in the fura-2 ratio (from 0·6 ± 0·1 to 1·4 ± 0·3) associated with a weak contracture. Subsequently there was a good recovery but in no case did it return to the level seen previously in normal Tyrode solution (Fig. 2*C*). The remaining myocytes all showed a large increase in the fura-2 fluorescence ratio (> 3·0; equivalent to a $[Ca^{2+}]_i$ of 1 μM) and a strong contracture. Of these, four showed a sudden fall in fluorescence at both wavelengths and were later found not to exclude Trypan Blue, six showed little recovery in fura-2 fluorescence ratio over the next 15 min (Fig. 2*B*), and in thirteen the ratio recovered within 10 min to reach a nadir at about twice the level seen previously in normal Tyrode solution (in 4 of these a secondary rise occurred; Fig. 2*A* and Table 1). In experiments where Ca^{2+} was removed from the bathing fluid in the presence of

Table 1. The collected results of the change in autofluorescence, approximate $[Ca^{2+}]_i$ and pH_i for isolated guinea-pig ventricular myocytes exposed to a variety of conditions in Tyrode solution containing pyruvate

Bathing solution	Relative NADH autofluorescence (%)	Approximate $[Ca^{2+}]_i$ (nM)	pH_i
Tyrode	100	84 ± 2·0 (32)	7·35 ± 0·05 (7)
2 mM CN^-	130 ± 5 (5)	—	—
5 mM CN^-	243 ± 12 (6)	—	—
5 mM amytal	172 ± 13 (8)	—	—
10 μM rotenone	173 ± 15 (3)	—	—
1·5 μM CCCP	72 ± 3 (11)	—	—
5 μM CCCP	37 ± 3 (6)	—	—
pCa 7, 0 Mg^{2+}	143 ± 4 (33)	—	7·26 ± 0·04 (7)
5 μM CCCP	44 ± 4 (8)	—	—
5 mM amytal	181 ± 6 (3)	—	—
Ca^{2+} repletion after pCa 7, 0 Mg^{2+}	49 ± 4 (12)	954 ± 103 (peak)(10) 138 ± 23 (final)	6·46 ± 0·08 (7) —
pCa 7, 10 mM Mg^{2+}	116 ± 5 (5) 70 ± 4 (2)	162 ± 27 (final)(6)	—

All data are from myocytes able to exclude the dye Trypan Blue at the end of the experiment.
Means ± S.E.M. with the number of observations in parentheses.

10 mM Mg^{2+} the fall in fura-2 ratio was similar to that obtained in the absence of Mg^{2+}. On return to Tyrode solution (with either 10 or 1 mM Mg^{2+}) the rise in the fluorescence ratio was more consistent, being smaller and showing a good recovery but to a level above that seen during the initial period of perfusion with normal Tyrode solution and was similar to that seen in Fig. 2*C* (Table 1). The myocytes did not develop a strong irreversible contracture but generally showed a period of spontaneous mechanical activity associated with transient changes in the fura-2 fluorescence ratio.

Intracellular pH

In myocytes loaded with the probe BCECF, the mean resting pH_i was $7{\cdot}35 \pm 0{\cdot}05$ ($n = 7$). The application of Tyrode solution containing an additional 20 mM NH_4Cl produced a typical alkalinization on application and acidification on removal. Exposure to Tyrode solution free of Mg^{2+} at a pCa of 7 induced a small slowly developing acidification which stabilized after about 5 min with a mean value of $7{\cdot}26 \pm 0{\cdot}04$ ($P < 0{\cdot}5$, paired t test when compared with the pH_i in normal Tyrode solution in the

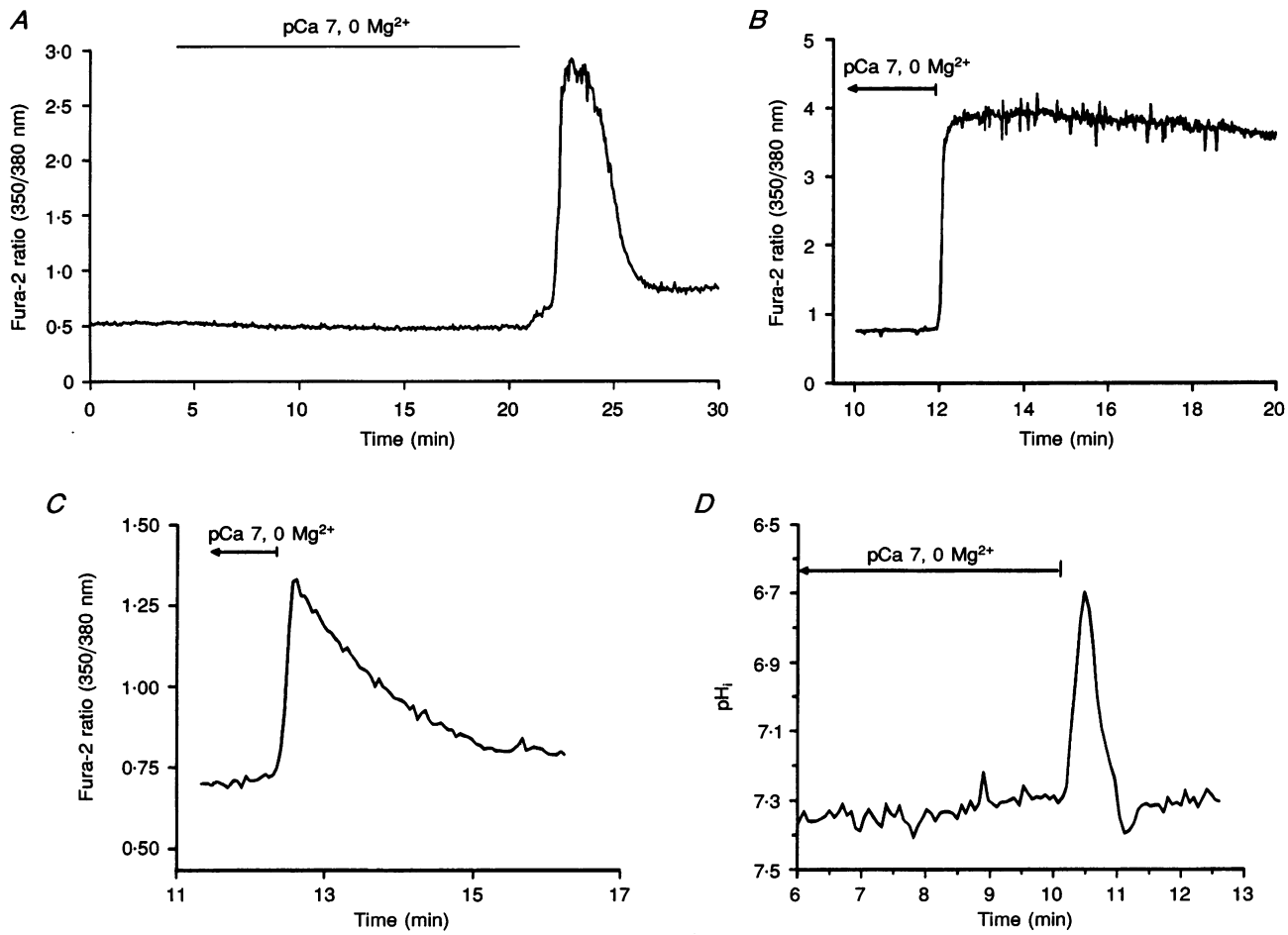

Figure 2. The change in $[Ca^{2+}]_i$ as indicated by the fura-2 fluorescence ratio and pH_i (from the BCECF fluorescence ratio) in isolated guinea-pig ventricular myocytes during Ca^{2+} depletion and repletion

A, the change in fura-2 fluorescence ratio when the bathing solution was altered from normal Tyrode solution to one with the pCa buffered to 7 with EGTA in the absence of added Mg^{2+} (as indicated by the labelled horizontal bar) followed by return to normal Tyrode solution which induced a large transient increase. *B* and *C* show the extremes which the change in fura-2 fluorescence ratio on return to normal Tyrode solution can take, with *B* showing a large sustained increase and *C* a smaller more transient increase. In *A* and *B* a strong irreversible hypercontracture developed as the fura-2 ratio increased while in *C* the myocyte showed a period of spontaneous rhythmic mechanical activity without developing a contracture. *D*, the transient change in pH_i (measured from the change in BCECF fluorescence ratio) induced on Ca^{2+} repletion when the myocyte developed a weak contracture. Note the expanded time scale in *A*, *B* and *C* with time zero at the beginning of perfusion with pCa 7, Mg^{2+}-free Tyrode solution.

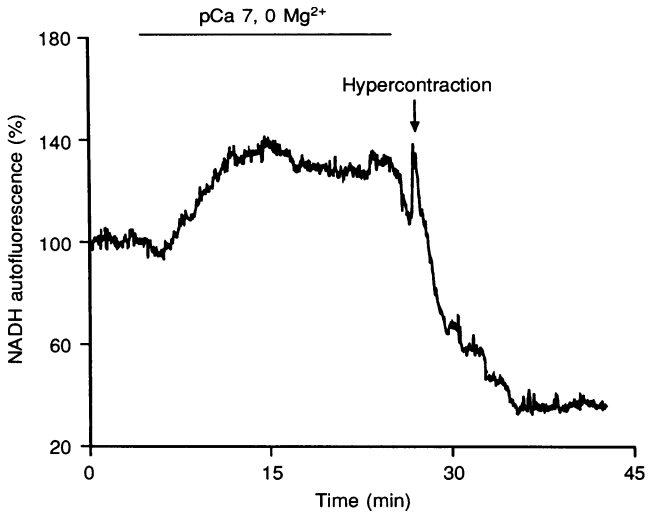

Figure 3. A typical change in relative NADH autofluorescence of a single guinea-pig ventricular myocyte on exposure to Tyrode solution without Mg^{2+} and with the pCa buffered at 7 followed by return to normal Tyrode solution
Exposure to pCa 7, Mg^{2+}-free Tyrode solution induces a small transient fall which is followed by a rise to a sustained high level. On return to normal Tyrode solution a rapid marked fall in autofluorescence occurred. The onset of the hypercontracture that induced a small artifact is indicated by the labelled arrow. The period of Ca^{2+} and Mg^{2+} deprivation is indicated by the horizontal bar.

same myocytes). On return to normal Tyrode solution (after 10 min Mg^{2+}-free, Ca^{2+}-free perfusion) a rapid large but transient acidification developed. The peak acidification was reached in less than 1 min at a mean pH_i of 6·46 ± 0·08 ($P < 0·0001$, paired t test; compared with pH_i in Tyrode solution or during Ca^{2+} depletion). The pH_i returned to a value not significantly different from that during Ca^{2+} deprivation within 2 min (Fig. 2*D*).

Changes in NADH autofluorescence on changing extracellular Ca^{2+} and/or Mg^{2+}

Calcium depletion

In myocytes exposed to Tyrode solution with pCa buffered to 7 or 8 with EGTA, the NADH autofluorescence fell initially, to be followed by an increase which eventually exceeded the value seen in Ca^{2+}-containing Tyrode solution after 10 min (Table 1 and Fig. 3). The duration and

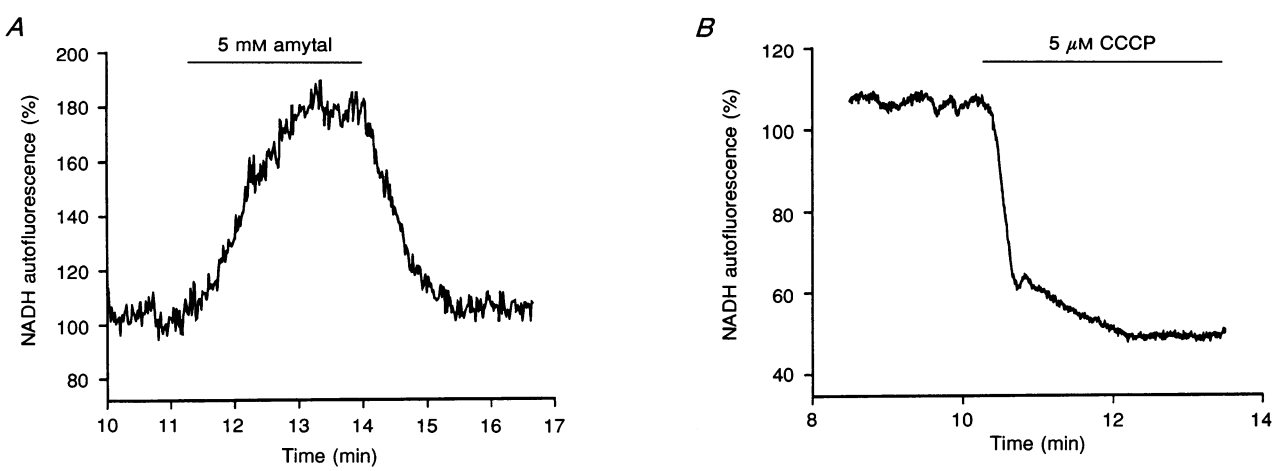

Figure 4. Typical changes in NADH autofluorescence when respiration was blocked by amytal or uncoupled with CCCP in an isolated myocyte perfused with Tyrode solution lacking divalent cations
The effects of inhibition of respiration with 5 mM amytal (*A*) to induce an increased NADH autofluorescence and the uncoupling of respiration with 5 μM CCCP (*B*) to produce a fall in NADH autofluorescence in myocytes bathed by pCa 7, Mg^{2+}-free Tyrode solution. Note that the relative NADH autofluorescence had already increased during perfusion with the Tyrode solution free of divalent cations which had begun at time zero. The period during which the inhibitor was present in the Tyrode solution is indicated by the horizontal bar.

magnitude of the initial period of reduced fluorescence was variable; in twenty-six of forty-three myocytes it had risen above the value seen initially in Tyrode solution within 5 min, in six this occurred after 10 min and in the remainder it stayed depressed. Once the fluorescence had stabilized, responses to respiratory inhibitors or uncouplers caused the NADH autofluorescence to reach levels similar to those seen when such agents were applied in Tyrode solution (Table 1 and Fig. 4). Similar changes in NADH autofluorescence occurred when the pCa of the bathing Tyrode solution was increased to 7 in the presence of 10 mM $[Mg^{2+}]_o$ (Table 1).

Calcium repletion

Return to normal Tyrode solution after a period of perfusion with a solution of pCa 7, Mg^{2+}-free solution resulted in a marked decrease in NADH autofluorescence (Table 1, Figs 3 and 5). In myocytes that developed a marked hypercontracture this fall reached levels similar to those caused by respiratory uncouplers (i.e. < 50 %) and was not further decreased by the application of the CCCP (Fig. 5*B* and Table 1).

The application of respiratory inhibitors after Ca^{2+} repletion still resulted in an increase in NADH autofluorescence which reached values similar to those obtained before Ca^{2+} depletion and repletion (Fig. 5*A*). This shows that the citric acid cycle was able to build up NADH when respiration was blocked. In two (of fourteen) myocytes where fall in NADH autofluorescence reached only 60 % of control and no hypercontracture developed, a slow spontaneous recovery of the autofluorescence was seen. A similar reduced fall followed by recovery of NADH autofluorescence and absence of hypercontracture was seen in myocytes on Ca^{2+} repletion after exposure to pCa 7 Tyrode solution containing 10 mM Mg^{2+} (Table 1).

The effects of the replacement of pyruvate by D-glucose on NADH autofluorescence

An eventual rise in the NADH autofluorescence after removal of Ca^{2+} from the bathing fluid was not seen in carotid body type I cells when D-glucose was the substrate (Duchen & Biscoe, 1992*a*). On switching from pyruvate to glucose, a small maintained fall in NADH autofluorescence was seen in guinea-pig ventricular myocytes (88 ± 3 % of control, $n = 10$). Exposure to 5 mM CN^- or 5 μM CCCP caused changes in relative autofluorescence similar to those seen in the presence of pyruvate. The effect of the removal of divalent cations from the bathing medium showed some differences in that the initial fall in NADH autofluorescence, on Ca^{2+} depletion, was more prolonged in the presence of D-glucose (in 4 myocytes it failed to exceed control levels of NADH autofluorescence after 5 min although a final rise to stabilize at levels well above control occurred within 15 min, while in another the autofluorescence remained depressed). The changes in Ca^{2+} repletion were similar to those seen when pyruvate was present in the bathing medium.

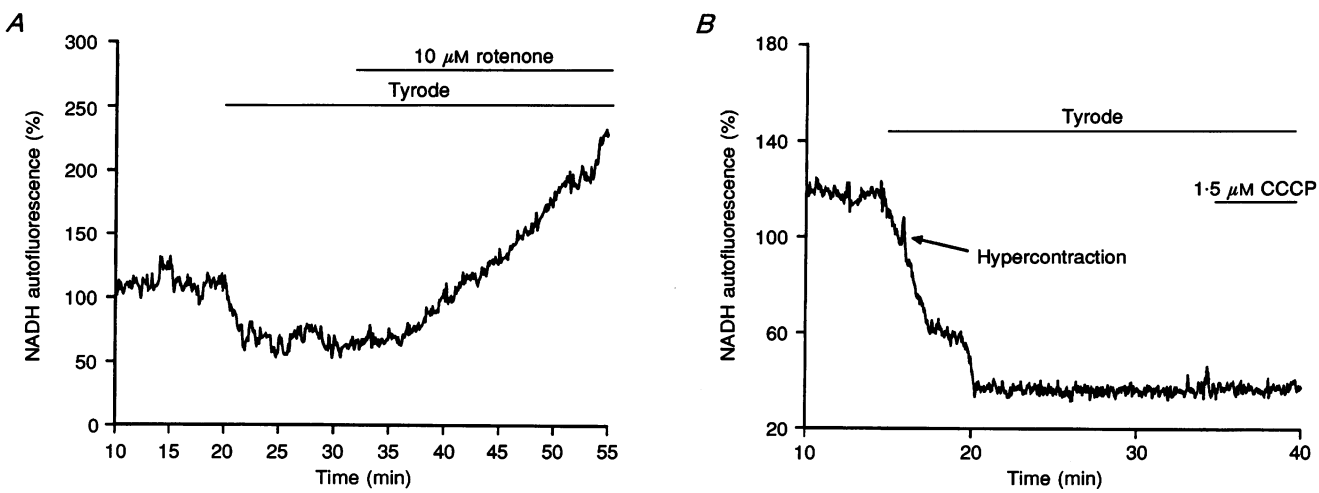

Figure 5. Typical changes in NADH autofluorescence induced by the application of rotenone to block respiration and CCCP to uncouple respiration in a myocyte subjected to Ca^{2+} overload
The effects of the application of the 10 μM rotenone (*A*) to inhibit respiration and 5 μM CCCP (*B*) to uncouple respiration on the relative NADH autofluorescence of isolated guinea-pig myocytes made sometime after return to normal Tyrode solution after exposure to Tyrode solution without Mg^{2+} and with a pCa of 7. The time scale is labelled to show the elapsed time since the beginning of perfusion with Mg^{2+}-free, pCa 7 fluid. The periods during which the bathing fluid was returned to normal Tyrode solution are indicated by the horizontal bars, as are the periods of exposure to rotenone and CCCP. Both myocytes developed a strong hypercontrature on Ca^{2+} repletion which produced a noticeable artifact in *B* as indicated by the labelled arrow.

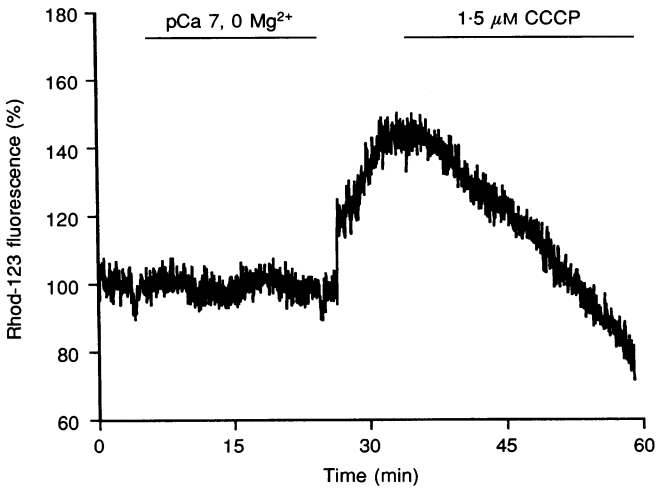

Figure 6. The change in relative rhod-123 fluorescence in a myocyte exposure to Mg^{2+}-free Tyrode solution with a pCa of 7 followed by return to normal Tyrode solution
Once the fluorescence had stabilized on return to Tyrode solution 1·5 μM CCCP was applied. The myocyte developed a strong hypercontracture within the first minute of reperfusion with normal Tyrode solution.

Changes in mitochondrial membrane potential as determined by the fluorescence measurements

Rhodamine (rhod-123) fluorescence has been widely used to monitor changes in mitochondrial membrane potential in isolated cells (e.g. Lemasters *et al.* 1987; Duchen & Biscoe, 1992*b*). As noted in the Methods, the loading of rhod-123 into guinea-pig myocytes increased their fragility. Even with a reduced loading concentration only 30 % of the myocytes were able to exclude Trypan Blue at the end of the experiment. In those myocytes which maintained an intact cell membrane the fluorescence was low and generally declined slowly with time. The application of 5 μM CCCP ($n = 8$) or FCCP ($n = 4$), to collapse the mitochondrial membrane potential, induced a brief small initial increase which lasted less than 2 min followed by a slower decrease in rhod-123 fluorescence which reached background levels within 30 min. The initial increase in relative rhod-123 fluorescence was much smaller than that reported for carotid body type I cells (Duchen & Biscoe, 1992*b*), while the delayed fall was similar to that reported for hepatocytes (Lemasters *et al.* 1987). In hepatocytes the fall in fluorescence has been interpreted as being due to rhod-123 leaving the depolarized mitochondria and the cell; indeed an increase in the rhod-123 fluorescence in the surrounding fluid was seen. Cardiac myocytes would seem to give responses that are intermediate between carotid body type I cells and hepatocytes. Little change in rhod-123 fluorescence was seen on removal of divalent

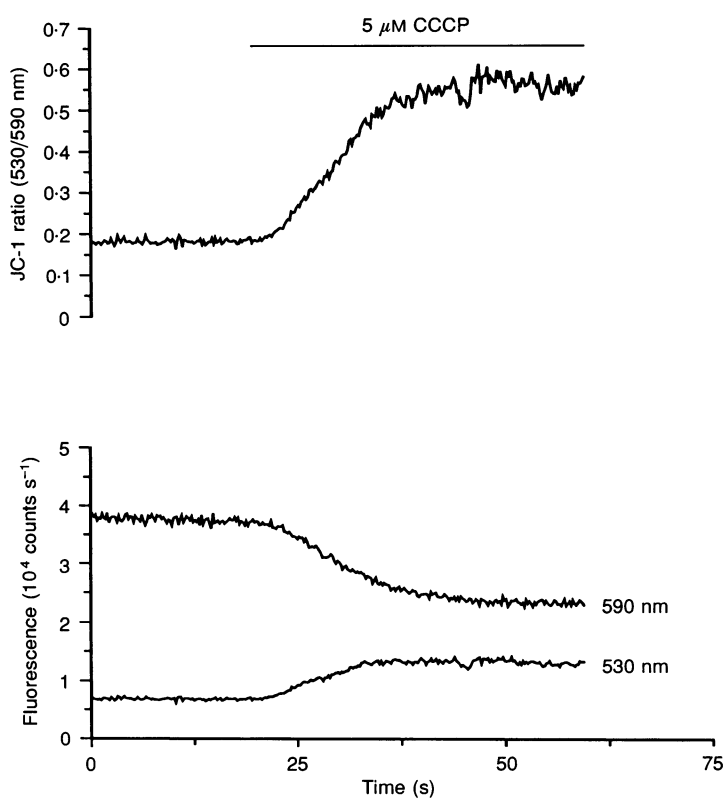

Figure 7. The change in mitochondrial membrane potential induced by the application of the proton ionophore CCCP to an isolated myocyte as detected by the fluorescent indicator JC-1
The change in JC-1 fluorescence ratio (upper trace) and the changes at the two measured wavelengths of 530 and 590 nm (lower traces) from isolated guinea-pig myocytes in response to 5 μM CCCP in normal Tyrode solution.

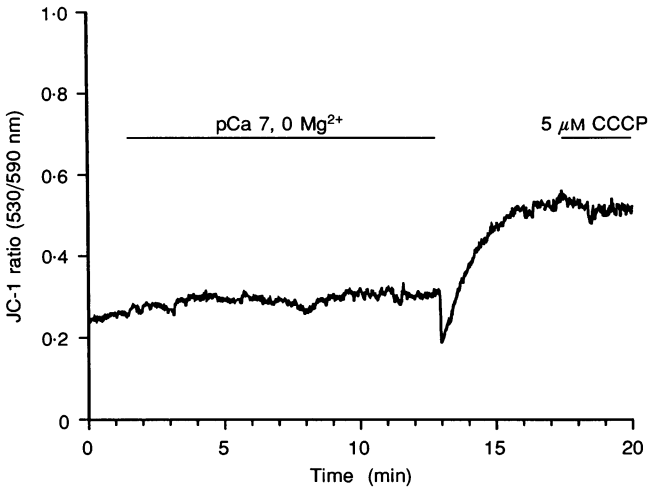

Figure 8. The change in mitochondrial membrane potential induced by Ca^{2+} depletion followed by Ca^{2+} repletion, as detected by JC-1 fluorescence ratio in guinea-pig ventricular myocytes
Initially normal Tyrode solution was replaced by one lacking Mg^{2+} and with a pCa of 7 after which perfusion with normal Tyrode solution was resumed. On return to normal Tyrode solution a strong hypercontracture developed while the fluorescence ratio fell initially but was then followed by a sustained rise. Once the JC-1 fluorescence ratio had stabilized at a higher level, application of 5 μM CCCP had no further effect.

cations from the solution bathing cardiac myocytes but on return to normal Tyrode solution there was invariably an increase in rhod-123 fluorescence (to $132 \pm 20\%$ of the initial value in Tyrode solution; $n = 16$). This increase was maintained for varying lengths of time and was followed by a large but slower decrease in fluorescence which developed within 1 min in eight myocytes and was then not affected by the subsequent addition of CCCP ($n = 4$). A spontaneous decrease occurred within the next 5 min in a further four myocytes, while in the remainder a similar decrease was provoked by the application of 5 μM CCCP (Fig. 6).

The ratiometric fluorescent probe for mitochondrial membrane potential, JC-1, did not increase myocyte fragility (only 2 of 20 myocytes showed a sudden loss of fluorescence at both wavelengths and were unable to exclude Trypan Blue at the end of an experiment) and there was little evidence of significant loss of the probe from the cells. Oligomycin, which by inhibition of the ATP synthetase may hyperpolarize the mitochondrial membrane, had no effect on the JC-1 fluorescence ratio in the four myocytes tested. The application of 5 μM CCCP produced a rapid fall in fluorescence at 590 nm and a rise at 530 nm and thereby a marked increase in fluorescence ratio (from $0{\cdot}18 \pm 0{\cdot}01$ to $0{\cdot}60 \pm 0{\cdot}10$ in 5 myocytes; $P < 0{\cdot}01$, paired t test) consistent with depolarization of the Ψ_m (Fig. 7). Removal of bathing divalent cations resulted in a slowly developing increase in JC-1 fluorescence ratio (from $0{\cdot}14 \pm 0{\cdot}05$ to $0{\cdot}24 \pm 0{\cdot}04$; $n = 8$; $P < 0{\cdot}0005$, paired t test). On return to Tyrode solution there was a transient decrease in JC-1 fluorescence ratio which returned the fluorescence ratio close to the initial level found in Tyrode solution ($0{\cdot}17 \pm 0{\cdot}02$; $n = 6$, not significantly different from the initial value in Tyrode solution) followed by a large and sustained increase (to $0{\cdot}55 \pm 0{\cdot}13$; $n = 6$; $P < 0{\cdot}001$, paired t test) which was not further increased by the application of CCCP (Fig. 8). The transient decrease in JC-1 fluorescence ratio that occurs immediately on Ca^{2+} repletion suggests that the small depolarization that occurs during Ca^{2+} depletion is initially reversed which would be consistent with the transient increase in O_2 consumption reported by Schaffer & Tan (1985) for rat heart. However, it should be noted that JC-1 fluorescence is affected by pH (Reers *et al.* 1991) and a strong transient acidification of the cytoplasm occurs at this time (Fig. 2*D*). Three myocytes failed to develop a hypercontracture on Ca^{2+} repletion, although the change in JC-1 fluorescence ratio during the phase of Ca^{2+} depletion was similar. On Ca^{2+} repletion they showed a small rise in JC-1 fluorescence ratio after a delay of 1–2 min (to $0{\cdot}27 \pm 0{\cdot}02$) followed by a recovery over the next 5–10 min to a value similar to that seen initially in Tyrode solution.

DISCUSSION

Although the myocytes prepared with taurine in the enzymatic isolation media show a greater resistance to cellular disruption on Ca^{2+} overload, the resting values of $[Ca^{2+}]_i$ and pH_i and the changes associated with Ca^{2+} depletion and subsequent Ca^{2+} repletion are similar to those reported previously for the heart (e.g. Lambert *et al.* 1986; Chapman & Suleman, 1986; Rodrigo & Chapman, 1991).

In taurine-isolated myocytes the magnitude of the Ca^{2+} overload (the accompanying contracture) and recovery has been found to be variable, when Ca^{2+} is returned to the bathing medium after a period of Ca^{2+} deprivation. In a number of cells the Ca^{2+} overload is severe and leads to hypercontracture and even disruption of the cell membrane. Elevation of $[Mg^{2+}]_o$ during Ca^{2+} depletion, which reduces the loading of the myocytes with Na^+ by inhibition of the Na^+ current through the L-type Ca^{2+} channels (Chapman, Fozzard, Friederlander & January, 1986; Rodrigo & Chapman, 1991), reduces in the rise in $[Ca^{2+}]_i$ and allows study of the intracellular events over a wide range of Ca^{2+} overload. None of the cells studied fully recovered from the Ca^{2+} overload. The failure of the fura-2 ratio to recover fully on repletion after Ca^{2+} depletion may indicate an elevated $[Ca^{2+}]_i$ associated with the failure of $[Na^+]_i$ to recover fully when the Na^+ influx through the Ca^{2+} channels is blocked (see Fig. 2 of Rodrigo & Chapman, 1990) and its effect on the Na^+–Ca^{2+} exchange or entry of the probe into intracellular organelles.

[NADH] and relative oxygen consumption

The changes in NADH autofluorescence on inhibition or uncoupling of respiration in guinea-pig ventricular myocytes are similar to measurements made on other cells (Eng *et al.* 1989; Duchen & Biscoe, 1992*a*). Previous workers have calculated the mitochondrial NADH/NAD^+ ratio (also called the relative oxygen consumption) from the maximum (when respiration is fully inhibited by cyanide; ΔF_{CN}) and minimum (when respiration is fully uncoupled with CCCP; ΔF_{CCCP}) levels of NADH autofluorescence as follows: $(NADH/NAD^+) = \Delta F_{CCCP}/(\Delta F_{CCCP} + \Delta F_{CN})$. In guinea-pig myocytes 5 mM CN^- was required to produce the maximal reduction (minimum O_2 consumption) and 5 μM CCCP the maximal oxidation (O_2 consumption is maximal) and yields a ratio of $0{\cdot}31 \pm 0{\cdot}03$ ($n = 6$) in experiments where both agents were applied to the same myocyte. This ratio is somewhat smaller than that for rat ventricular myocytes, chromaffin cells or dorsal root ganglion cells, but larger than that for carotid body type I cells. However, the concentrations of the inhibitors used in these studies produce submaximal responses in guinea-pig myocytes. It should be noted that this ratio is, however, much smaller than that calculated from biochemical measurements by Schaffer & Tan (1985) for the beating perfused rat ventricle.

Measurement of mitochondrial membrane potential

The monitoring of changes in Ψ_m during Ca^{2+} depletion and repletion yielded complicated and variable results when rhod-123 was used. A major problem was the increased fragility of myocytes incubated with rhod-123, which was not reduced by lowering the loading concentration. However, in myocytes that remained intact, the response to respiratory uncouplers was intermediate between those reported for hepatocytes and carotid body type I cells (Lemasters *et al.* 1987; Duchen & Biscoe, 1992*b*). These changes would be consistent with the rhod-123 leaving first the depolarized mitochondria and then the cell, which means that the cardiac cell membrane is relatively permeable to rhod-123. This could account for the increased fragility (rhod-123 is toxic at high concentrations). The changes in rhod-123 fluorescence seen on Ca^{2+} repletion are similar to those seen when CCCP is applied and suggest that a depolarization of Ψ_m is associated with Ca^{2+} overload. JC-1, a new ratiometric fluorescent probe for Ψ_m, did not affect myocyte variability and yielded responses on exposure to respiratory uncouplers similar to that reported for a variety of other cells and which are consistent with a depolarization of Ψ_m with no obvious evidence of exit of the probe from the cell (Reers *et al.* 1991; Smiley *et al.* 1991). The small slowly developing depolarization of Ψ_m indicated by the change in JC-1 fluorescence ratio on Ca^{2+} depletion suggests that a mild depression of respiration occurs. On Ca^{2+} repletion a transient recovery is quickly followed by a marked depolarization, especially when the Ca^{2+} overload is severe and the failure of respiratory uncouplers to have further effect suggests that respiration is fully uncoupled.

[NADH] changes on Ca^{2+} depletion

The removal of the Ca^{2+} bathing isolated guinea-pig ventricular myocytes, in the absence or presence of Mg^{2+} and with either pyruvate or D-glucose as the metabolic substrate, results in an initial fall which is more often than not followed by a rise in NADH autofluorescence to levels above that obtained during the preceding perfusion with Tyrode solution. NADH autofluorescence remains reduced for much longer when glucose is the substrate. A similar fall in NADH autofluorescence is seen in carotid body type I cells when D-glucose is the substrate, but in these experiments the period of Ca^{2+}-free perfusion may have been too short to observe the secondary rise. The difference in response to Ca^{2+} deprivation when D-glucose or pyruvate is used as the metabolic substrate requires further investigation but it is unlikely that profound changes in the metabolic responses of the myocytes occurred during Ca^{2+} depletion because the responses to respiratory inhibitors and uncouplers persist (Table 1 and Fig. 4). The mitochondrial NADH/NAD^+ ratio is a reflection of the difference between NADH reduction by the activity of the citric acid cycle and its oxidation by the respiratory chain. Both are likely to be affected by a fall in $[Ca^{2+}]_i$; the former by the Ca^{2+}-dependent intramitochondrial dehydrogenases and the latter by changed energy demand feeding back on respiration and effects on the ATP synthetase. The biphasic change in NADH autofluorescence on Ca^{2+} depletion could be interpreted by the fall in $[Ca^{2+}]_i$ affecting both the citric acid cycle and respiration, with the former responding earlier. The increase in the NADH/NAD^+ ratio (or the quotient for the relative oxygen consumption) is consistent with the known fall in oxygen consumption (Penpargkul & Scheuer, 1969; Schaffer & Tan, 1985) but the myocytes are generally quiescent and would do little work on contraction. Furthermore, the rise in $[Na^+]_i$, which occurs during Ca^{2+} depletion, would be expected to activate the Na^+ pump. For a rise in mitochondrial [NADH] to occur, the effects on energy demand and on oxidative phosphorylation must exceed those on the mitochondrial dehydrogenases. At the present time an explanation for this unexpected change is elusive, unless, as McCormack *et al.* (1990) suggested, the activation of the mitochondrial dehydrogenases by Ca^{2+} is reduced in hearts bathed with pyruvate. What should be remembered, however, is that resting tension falls in cardiac tissue on exposure to Ca^{2+}-free media and during Ca^{2+} depletion ATP and creatine phosphate levels have been reported to rise (Bulkley *et al.* 1978; Schaffer & Tan, 1985). A rise in ATP should depress respiration by negative feedback at several steps in the chain (Balaban, 1992) and this is consistent with the mild depolarization of Ψ_m indicated by the change in the JC-1 fluorescence ratio (Fig. 8).

Changes induced by Ca^{2+} overload

In the present experiments the changes in $[Ca^{2+}]_i$, pH_i, NADH autofluorescence and Ψ_m associated with the Ca^{2+} overload, induced by reperfusion with Tyrode solution after a period of Ca^{2+} deprivation, have been measured independently. For comparison of the separate measurements the only points of reference are time, the disruption of the cell membrane or the presence or strength of the hypercontracture (as affected by a raised $[Mg^{2+}]_o$ during Ca^{2+} deprivation). The intracellular acidification and rise in $[Ca^{2+}]_i$ would seem to occur very soon after Ca^{2+} repletion. The recovery of pH_i was found to be more complete and faster. The maintained depolarization of the Ψ_m and fall in mitochondrial [NADH] develop more slowly. Comparison of myocytes exposed to Ca^{2+} deprivation in the presence or absence of Mg^{2+} suggest that the greater the rise in $[Ca^{2+}]_i$ the stronger is the hypercontracture and the larger is the fall in pH_i, NADH autofluorescence and Ψ_m, suggesting that all are related to the degree of Ca^{2+} overload. When Ca^{2+} overload is large mitochondrial NADH is completely depleted and the mitochondria fully depolarized because CCCP is without further effect. The citric acid cycle is still active because NADH autofluorescence is increased on the inhibition of respiration. This suggests that during Ca^{2+} overload the demands of respiration exceed the ability of the citric acid cycle to provide NADH. This fall in mitochondrial [NADH] again contrasts with the rise that is calculated by Schaffer & Tan (1985) in the intact rat heart.

There is little doubt that demand for energy will rise steeply because of the combined effects of the hypercontracture and the activation of Na^+ and Ca^{2+} pumps. The failure of the citric acid cycle to meet the demands of respiration could result from an inadequate reactivation of the dehydrogenases because either the rise in mitochondrial Ca^{2+} is attenuated or essential metabolites have become depleted. The rise in mitochondrial Ca^{2+} may be reduced in spite of the marked rise in sarcoplasmic $[Ca^{2+}]$ because $[Na^+]_i$ is also elevated and takes several minutes to recover on Ca^{2+} repletion (Rodrigo & Chapman, 1991). The increased influx through the mitochondrial Ca^{2+} uniport provoked by the rise in sarcoplasmic $[Ca^{2+}]$ should be countered by an increased efflux provoked by the effect of the elevated $[Na^+]_i$ on the mitochondrial Na^+–Ca^{2+} exchange (as reviewed by McCormack *et al.* 1990). A rise in intramitochondrial $[Ca^{2+}]$ sufficient for a significant reactivation of the dehydrogenases is unlikely to occur until $[Na^+]_i$ has fallen. Another limit is placed on the reactivation of the citric acid cycle by the rise in $[Na^+]_i$ which induces a marked fall in the essential metabolites alanine, glutamate and aspartate particularly during Ca^{2+} depletion (Suleiman & Chapman, 1993).

The inability of the citric acid cycle to provide sufficient mitochondrial NADH for oxidative phosphorylation will contribute to the depletion of ATP and creatine phosphate that occurs after Ca^{2+} repletion (Boink, Ruigrok, Maas & Zimmerman, 1976; Bulkley *et al.* 1978; Schaffer & Tan, 1985). A more profound effect on ATP synthesis will result from the fall of Ψ_m which will uncouple respiration from ATP synthesis. This could result from the increased Ca^{2+} cycling across the mitochondrial wall because the egress via the Na^+–Ca^{2+} exchange, unlike the Ca^{2+} entry, is electrically neutral (Crompton, Capano & Carafoli, 1976) so that at each cycle of a Ca^{2+} ion there will be a net inward movement of two positive charges across the inner mitochondrial membrane. Indeed, the absence of an effect of CCCP on the JC-1 fluorescence or NADH autofluorescence after Ca^{2+} repletion suggests that respiration is fully uncoupled when Ca^{2+} overload is extreme (Figs 5*B* and 8).

The outcome of these changes will be, in spite of the effect on ATP synthetase (Das & Harris, 1990), a marked increase in the demand for ATP on Ca^{2+} overload which will not be met by oxidative phosphorylation. This would account for the marked increase in glucose utilization reported by Schaffer & Tan (1985). These features together with an elevated $[Na^+]_i$ mean that, apart from a maintained acidification (and there is rise in lactate in rat heart when glucose is the substrate; Schaffer & Tan, 1985), a myocyte experiences conditions very similar to those produced by anoxia, metabolic poisoning and possibly ischaemia (Cobbold & Bourne, 1984; Tani & Neely, 1990; Bowers *et al.* 1992). Irreversible damage to the myocyte may therefore involve activation of similar processes.

We have concentrated attention on myocytes that maintain some function after Ca^{2+} overload induced by Ca^{2+} repletion to the bathing fluid, although this seems to vary with the severity of the Ca^{2+} overload. Most myocytes developed a hypercontracture and some could regain control of $[Ca^{2+}]_i$, re-establish a normal mitochondrial membrane potential and recover mitochondrial NADH production. In others a rise in mitochondrial [NADH] only occurred if respiration was inhibited. The long-term failure of Ψ_m to recover could result from the activation of the cyslosporin-dependent pore (Crompton, Costi & Hayer, 1987), while the final disruption of the cell membrane could involve activation of phospholipases (Suleiman, Minezaki & Chapman, 1991).

Comparison with work on the whole heart

There are many points of agreement between the effects of Ca^{2+} overload on the perfused rat heart (Schaffer & Tan, 1985) and the present work on isolated guinea-pig myocytes. These are (i) the fall of O_2 consumption on Ca^{2+} depletion and evidence of some recovery on Ca^{2+} repletion, (ii) the inability of oxidative phosphorylation to provide sufficient ATP on Ca^{2+} repletion, (iii) the importance of the impairment of mitochondrial function in the changes that occur on Ca^{2+} repletion, and (iv) a fall in pH_i within 1 min of Ca^{2+} repletion. The one important disagreement concerns the changes in the $NADH/NAD^+$ ratio. This could easily result from the different techniques used. There are a

number of reasons why the calculation of the NADH/NAD^+ ratio may be in error: (i) the measurement of NH_3 is unlikely to be accurate; (ii) the assumption that the glutamate dehydrogenase reaction is near to equilibrium may be incorrect (Hassinen, 1986); and (iii) the rise in $[Na^+]_i$ during Ca^{2+} deprivation provokes an efflux of the very amino acids used in the calculation (Suleiman & Chapman, 1993). The reasons why the resting NADH/NAD^+ ratio is different when determined by autofluorescence as compared with biochemical measurement and calculation is likely to be related to the fact that autofluorescence measures primarily NADH bound to the mitochondrial membrane while chemical methods estimate free NADH and NAD^+ (Nuutinen, 1984). Furthermore, Eng *et al.* (1989) have drawn attention the fact that mitochondrial NADH autofluorescence is greatly enhanced by protein binding so that a change in autofluorescence could be due either to a changed redox state of bound NAD^+ or to a change in the binding or physical environment of each NADH molecule.

REFERENCES

Allen, D. G., Morris, P. G., Orchard, C. H. & Pirolo, J. S. (1985). A nuclear magnetic resonance study of metabolism in ferret heart during hypoxia and inhibition of glycolysis. *Journal of Physiology* **361**, 185–204.

Balaban, R. S. (1992). Regulation of oxidative phosphorylation in the mammalian cell. *American Journal of Physiology* **258**, C377–389.

Boink, A. B. T., Ruigrok, T. J. C., Maas, A. H. J. & Zimmerman, A. N. E. (1976). Changes in high energy phosphate compounds of isolated rat hearts during Ca^{2+}-free perfusion and reperfusion with Ca^{2+}. *Journal of Molecular and Cellular Cardiology* **8**, 973–979.

Bowers, K. C., Allshire, A. P. & Cobbold, P. H. (1992). Bioluminescent measurement in single cardiomyocytes of sudden cytosolic ATP depletion coincident with rigor. *Journal of Molecular and Cellular Cardiology* **24**, 213–218.

Bulkley, B. H., Nunnally, R. L. & Hollis, D. P. (1978). "Calcium paradox" and the effect of varied temperature on its development. A phosphorus nuclear magnetic resonance and morphologic study. *Laboratory Investigation* **39**, 133–140.

Chance, B. (1976). Pyridine nucleotide as an indicator of the oxygen requirements for energy-linked functions of mitochondria. *Circulation Research* **38**, 131–138.

Chance, B. & Baltscheffsky, H. (1958). Respiratory enzymes in oxidative phosphorylation VII. Binding of intramitochondrial reduced pyridine nucleotide. *Journal of Biological Chemistry* **233**, 736–739.

Chance, B., Schoener, B., Oshino, R., Itshak, F. & Nahase, Y. (1979). Oxidation-reduction ratio studies of mitochondria in freeze-trapped samples. *Journal of Biological Chemistry* **254**, 4764–4771.

Chapman, R. A. (1993). The effects of oximes on the dihydropyridine-sensitive Ca current in isolated guinea-pig ventricular myocytes. *Pflügers Archiv* **422**, 325–331.

Chapman, R. A., Chatamra, K. R. & Little, C. L. (1991). The increased resistance of guinea-pig ventricular myocytes to calcium repletion and depletion that is produced by enzymatic isolation in taurine-containing media, is antagonized by the presence of either taurine, β-alanine or haloperidol during the Ca-free period. *Journal of Physiology* **438**, 317*P*.

Chapman, R. A., Coray, A. & McGuigan, J. A. S. (1983). Sodium-calcium exchange in mammalian heart: the maintenance of low intracellular calcium concentration. In *Cardiac Metabolism*, ed. Drake, A. & Noble, M. I. M., pp. 117–149, John Wiley & Sons, London.

Chapman, R. A., Fozzard, H. A., Friederlander, I. R. & January, C. T. (1986). Effects of Ca^{2+}/Mg^{2+} removal on a^i_{Na}, a^i_K and tension in cardiac Purkinje fibers. *American Journal of Physiology* **251**, C920–927.

Chapman, R. A. & Suleman, I. H. (1986). Change in a^i_{Na} and pH_i in isolated ferret ventricular muscle on repletion of the bathing Ca following a period of Ca-deprivation. *Journal of Physiology* **381**, 120*P*.

Chapman, R. A. & Tunstall, J. (1987). The calcium paradox of the heart. *Progress in Biophysics and Molecular Biology* **50**, 67–96.

Cobbold, P. H. & Bourne, P. K. (1984). Aequorin measurements of free calcium in single heart cells. *Nature* **312**, 444–446.

Cox, D. A. & Matlib, M. A. (1993). A role for the mitochondrial Na^+–Ca^{2+} exchanger in the regulation of oxidative phosphorylation in isolated heart mitochondria. *Journal of Biological Chemistry* **268**, 938–947.

Crompton, M., Capano, M. & Carafoli, E. (1976). The sodium-induced efflux of calcium from heart mitochondria. *European Journal of Biochemistry* **69**, 453–462.

Crompton, M., Costi, A. & Hayer, L. M. (1987). Evidence for the presence of a reversible Ca^{2+}-dependent pore activated by oxidative stress in heart mitochondria. *Biochemical Journal* **245**, 752–754.

Das, A. M. & Harris, D. A. (1990). Defects in regulation of mitochondrial ATP synthetase in cardiomyocytes from spontaneously hypertensive rats. *American Journal of Physiology* **259**, H1264–1269.

Denton, R. M. & McCormack, J. G. (1980). On the role of the calcium transport cycle in heart and other mammalian mitochondria. *FEBS Letters* **199**, 1–8.

Duchen, M. R. & Biscoe, T. J. (1992*a*). Mitochondrial function in type I cells isolated from rabbit arterial chemoreceptors. *Journal of Physiology* **450**, 13–32.

Duchen, M. R. & Biscoe, T. J. (1992*b*). Relative mitochondrial membrane potential and $[Ca^{2+}]_i$ in type I cells isolated from the rabbit carotid body. *Journal of Physiology* **450**, 33–62.

Eng, J., Lynch, R. M. & Balaban, R. S. (1989). Nicotinamide adenine dinucleotide fluorescence spectroscopy and imaging in isolated cardiac myocytes. *Biophysical Journal* **55**, 621–630.

Hansford, R. G. (1985). Relation between calcium transport and control of energy metabolism. *Reviews of Physiology, Biochemistry and Pharmacology* **102**, 1–75.

Hassinen, I. E. (1986). Mitochondrial respiratory control in the myocardium. *Biochimica et Biophysica Acta* **853**, 135–151.

Lambert, M. R., Johnson, J. D., Lamka, K. G., Brierley, G. P. & Altschuld, R. A. (1986). Intracellular free Ca and the hypercontracture of adult rat heart myocytes. *Archives of Biochemistry and Biophysics* **245**, 426–435.

Lemasters, J. J., DiGuiseppi, J., Nieminen, A.-L. & Herman, B. (1987). Blebbing, free Ca^{2+} and mitochondrial potential preceding cell death in hepatocytes. *Nature* **325**, 78–81.

McCormack, J. G., Halestrap, A. P. & Denton, R. M. (1990). Role of calcium ions in the regulation of mammalian intramitochondrial metabolism. *Physiological Reviews* **70**, 391–425.

Minezaki, K. K., Suleiman, M.-S. & Chapman, R. A. (1992). Metabolic changes in isolated guinea-pig ventricular myocytes during calcium depletion and repletion as detected with a fluorescent technique. *Journal of Physiology* **452**, 164*P*.

Minezaki, K. K., Suleiman, M.-S. & Chapman, R. A. (1993*a*). The change in mitochondrial membrane potential and NADH in isolated guinea-pig ventricular myocytes during the calcium paradox. *Journal of Physiology* **459**, 83*P*.

MINEZAKI, K. K., SULEIMAN, M.-S. & CHAPMAN, R. A. (1993*b*). Metabolic change in isolated guinea-pig ventricular myocytes during calcium loading via calcium ionophore or the calcium paradox. *Journal of Physiology* **459**, 510*P*.

NUUTINEN, E. M. (1984). Subcellular origin of the surface fluorescence of reduced nicotinamide nucleotides in the isolated perfused rat heart. *Basic Research in Cardiology* **79**, 49–58.

NUUTINEN, E. M. & HASSINEN, I. (1981). Plasma membrane phosphate transport and extracellular phosphate concentration in the regulation of cellular respiration in isolated perfused rat heart. *Biochimica et Biophysica Acta* **637**, 481–489.

PENPARGKUL, S. & SCHEUER, J. (1969). Metabolic comparisons between hearts arrested by calcium deprivation or potassium excess. *American Journal of Physiology* **217**, 1405–1412.

REERS, M., SMITH, T. W. & CHEN, L. B. (1991). J-aggregate formation of a carbocyanine as a qualitative fluorescent indicator of membrane potential. *Biochemistry* **30**, 4480–4486.

RODRIGO, G. C. & CHAPMAN, R. A. (1990). A sodium-activated potassium current in intact ventricular myocytes isolated from the guinea-pig heart. *Experimental Physiology* **75**, 839–842.

RODRIGO, G. C. & CHAPMAN, R. A. (1991). The calcium paradox in isolated guinea-pig ventricular myocytes: effects of membrane potential and intracellular sodium. *Journal of Physiology* **434**, 627–645.

SCHAFFER, S. W. & TAN, B. H. (1985). Effect of calcium depletion and calcium paradox on myocardial energy metabolism. *Canadian Journal of Physiology and Pharmacology* **63**, 1384–1391.

SMILEY, S. T., REERS, M., MOTTOLA-HARTSHORN, C., LIN, M., CHEN, A., SMITH, T. W., STEELE, G. D. & BO CHEN, L. (1991). Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proceedings of the National Academy of Sciences of the USA* **88**, 3671–3675.

SULEIMAN, M.-S. & CHAPMAN, R. A. (1993). Changes in the principal free intracellular amino acids in the Langendorff perfused guinea-pig heart during arrest with calcium free or high potassium media. *Cardiovascular Research* **27**, 1810–1814.

SULEIMAN, M.-S., MINEZAKI, K. K. & CHAPMAN, R. A. (1991). A phospholipase A_2 inhibitor (Ro 31-4493) prevents the protein loss associated with the Ca-paradox in isolated guinea-pig hearts and is without effect on the currents through L-type Ca channels of isolated myocytes. *Journal of Physiology* **446**, 439*P*.

TANI, M. & NEELEY, J. R. (1990). Intermittent perfusion of ischemic myocardium: possible mechanism of the protective effects of myocardial function in isolated rat heart. *Circulation* **82**, 536–548.

THOMAS, J. A., BUCHSBAUM, R. N., ZIMNIAK, A. & RACKER, E. (1979). Intracellular pH measurements in Ehrlich ascites tumour cells utilising spectroscopic probes generated in situ. *Biochemistry* **18**, 2210–2218.

WILLIAMS, D. A. & FAY, F. S. (1990). Intracellular calibration of the fluorescent calcium indicator Fura-2. *Cell Calcium* **11**, 75–83.

Acknowledgements

We wish to thank Mrs Dawn Wallace for excellent technical assistance and Dr John Tunstall (University of Leicester) for advice and criticism of various versions of the manuscript.

Author's present address

K. K. Minezaki: Department of Cardiology, Jichi Medical School, 3311-1 Yakushiji, Tochigi, 329-04, Japan.

Received 26 February 1993; accepted 28 September 1993.