Ca²⁺-dependent changes in the mitochondrial energetics in single dissociated mouse sensory neurons

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Depolarization of neurons promotes Ca²⁺ influx through voltage-activated channels, raising the intracellular Ca²⁺ concentration ([Ca²⁺]_i). The consequences of such changes in [Ca²⁺]_i for mitochondrial function were assessed in single, freshly dissociated mammalian neurons. Microfluorimetric techniques were used to measure [Ca²⁺]_i, mitochondrial membrane potential [$\Delta\psi_m$, Rhodamine 123 (Rh 123) fluorescence], NAD(P)H/NAD(P)⁺ autofluorescence and flavoprotein autofluorescence combined with whole-cell voltage-clamp techniques. Brief (100–500 ms) depolarization of the cell membrane by high K⁺ or by voltage commands raised [Ca²⁺]_i and depolarized $\Delta\psi_m$. The change in $\Delta\psi_m$ was dependent on extracellular Ca²⁺. Under voltage-clamp control of the cell membrane, the voltage-dependence of the change in Rh 123 fluorescence reflected that of the Ca²⁺ current. The response was reduced by Ca²⁺ buffers introduced into the cell. The behaviour of this signal is thus consistent with a mitochondrial response to raised [Ca²⁺]_i and does not reflect the change in cell membrane potential *per se*. Similar stimuli caused a rapid decrease of NAD(P)H autofluorescence, followed by an increase which could last several minutes. Flavoprotein fluorescence increased transiently, followed by a decrease lasting for several minutes. These signals indicate an initial oxidation of NAD(P)H and FADH, followed by a prolonged increase in the reduced state of both coenzymes. All these changes were dependent on extracellular [Ca²⁺]_i. Raising [Ca²⁺]_i again during the period of NAD⁺ reduction caused an oxidizing response. Ruthenium Red applied to the cells (i) reduced both the Ca²⁺ current and the depolarization-induced [Ca²⁺]_i transient and (ii) directly quenched Rh 123 fluorescence. When introduced into the cells with patch pipettes, it prevented the changes in autofluorescence without interfering with the Ca²⁺ conductance. Oligomycin blocked neither the response of $\Delta\psi_m$ nor of NADH autofluorescence, suggesting that the signals do not reflect a response to falling ATP/ADP·P_i ratios as a consequence of the high [Ca²⁺]_i. The changes in NADH autofluorescence were sustained in the presence of iodoacetic acid with pyruvate as substrate. Thus brief physiological elevations of [Ca²⁺]_i depolarize $\Delta\psi_m$, probably through Ca²⁺ cycling across the mitochondrial inner membrane. The changes in autofluorescence are consistent with (i) increased respiration which could result from the depolarization of $\Delta\psi_m$, followed rapidly by (ii) increased activity of the Ca²⁺-dependent intramitochondrial enzymes. Changes in [Ca²⁺]_i within a physiological range may thus promote significant and long-lasting changes in mitochondrial energy production.

INTRODUCTION

Concepts about the role of mitochondria in intracellular Ca²⁺ balance have been gradually changing over recent years. The view that mitochondria play an important role in buffering intracellular Ca²⁺ has shifted in the literature towards an emphasis on the principle that mitochondrial Ca²⁺ uptake has evolved as a mechanism to regulate intramitochondrial Ca²⁺ concentration ([Ca²⁺]_m), which in turn governs the activity of three rate-limiting mitochondrial dehydrogenases [for reviews, see Hansford (1985), Carafoli (1987), Crompton (1990) and McCormack *et al.* (1990)]. This mechanism establishes a potentially crucial role for [Ca²⁺]_m as a determinant of mitochondrial energy production. Although it may be the case that mitochondrial Ca²⁺ regulation is not central in setting resting intracellular Ca²⁺ concentrations ([Ca²⁺]_i) in most cells, it is nevertheless clear that the import of Ca²⁺ into mitochondria when [Ca²⁺]_i is raised can play a role in shaping the [Ca²⁺]_i transients that occur, for example, in response to the depolarization of neuronal and some other excitable cells. Thus Thayer & Miller (1990) and Duchon *et al.* (1990a,b) showed that the rate of clearance of a [Ca²⁺]_i load with depolarization of sensory neurons was significantly altered by inhibitors of mitochondrial electron transport or by mitochondrial depolarization with uncouplers. These findings also suggest that significant

mitochondrial Ca²⁺ influx must accompany the [Ca²⁺]_i transients generated by these stimuli, and so prompted the present study, in which evidence for functional consequences of increased [Ca²⁺]_i and, consequently, of [Ca²⁺]_m, has been sought. Evidence for a direct role of Ca²⁺ in the regulation of mitochondrial function is derived from studies of isolated mitochondria and isolated mitochondrial enzymes (Hansford, 1985; Denton & McCormack, 1990), supported by measurements of enzyme activity, metabolite concentrations, n.m.r. and NADH autofluorescence from a variety of intact tissues, most notably hepatocytes and cardiomyocytes [for reviews, see Hansford (1985) and McCormack *et al.* (1990)]. Given the importance of mitochondrial function in the control of the cellular economy, it seems important to document the behaviour of these organelles in their normal physiological environment within intact cells in response to physiological stimuli. The modulation of mitochondrial Ca²⁺ uptake by variable cytosolic factors such as the polyamine, spermine (Pegg & McCann, 1982; Lenzen *et al.*, 1986), the dependence of mitochondrial Na⁺/Ca²⁺ exchange on [Na⁺]_i, and the dependence of the K_D for the intramitochondrial enzymes for Ca²⁺ on Mg²⁺, ATP and ADP (Rutter & Denton, 1988) all suggest that the behaviour of these systems in intact cells may differ significantly from their behaviour in isolation. Studies of brain (Rosenthal & Jöbsis, 1971; Lothman *et al.*,

Abbreviations used: [Ca²⁺]_i, intracellular Ca²⁺ concentration; [Ca²⁺]_m, intramitochondrial Ca²⁺ concentration; $\Delta\psi_m$, mitochondrial membrane potential; Rh 123, Rhodamine 123; IAC, iodoacetic acid; TEA, tetraethylammonium chloride; AM, acetomethoxy; BAPTA, 1,2-bis-(*o*-amino-5,5'-difluorophenoxy)ethane-*NNN'*-tetra-acetic acid; RuR, Ruthenium Red; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide; [Ca²⁺]_o, extracellular Ca²⁺ concentration.

1975; Moffett & LaManna, 1978), brain slices (Segal *et al.*, 1980; Lipton, 1973) and synaptosomes (Scott & Nicholls, 1980; Hansford & Castro, 1985; Patel *et al.*, 1988; Erecińska & Dagani, 1990), and of isolated hepatocytes and cardiomyocytes (Hansford, 1985; Balaban & Blum, 1982; Eng *et al.*, 1989; see also Heineman & Balaban, 1990), all point to changes in mitochondrial activity in response to significant physiological stimulation. However, the complexities of intact neural tissues make interpretation of the data obtained from brain and brain slices difficult, and there is still little detailed information available about the behaviour of mitochondria in single neurons.

For the present study, neurons have been isolated from sensory ganglia, and a range of microfluorimetric techniques have been applied to identify changes in mitochondrial function in response to physiological stimuli. The use of microfluorimetry to monitor signals from single identified cells has several merits compared with the use of cell suspensions or synaptosomes. Most particularly, fast changes of a signal in a population may be lost unless the changes are synchronized between cells or subcellular fractions. It is also possible (i) to identify cells under phase-contrast illumination, to select healthy cells and reject cells that appear damaged from study and (ii) to watch changes in the cell during the manipulations, so that it is immediately apparent if a cell becomes damaged. The techniques have been supplemented by whole-cell patch-clamp electrophysiological recordings that serve both to establish the viability of the cell preparations and to allow measurements of cell resting potentials, of voltage and transmitter-gated currents and precise determination of the relative time courses of the changes in mitochondrial function with the change in membrane potential or current. The technique also allows direct manipulation of $[Ca^{2+}]_i$ buffering, and allows introduction of membrane-impermeant drugs directly into the cytosol.

Some of these data have already been published in a preliminary form (Duchen, 1991a).

METHODS

Cells were isolated as previously described (Duchen, 1990). Mice, 12–16 days old, were killed by decapitation. The spinal cord was exposed by a ventral laminectomy and the dorsal root ganglia were removed. The cord was continuously superfused with an ice-cold solution [(solution A), pH 7.3, containing (mM): NaCl, 130; NaHCO₃, 26; KCl, 3; KH₂PO₄, 1.25; MgSO₄, 2; CaCl₂, 2.0; D-glucose, 10] and equilibrated with O₂/CO₂ (19:1). The ganglia were then incubated in a similar, but Ca²⁺-free, saline, containing 0.4% collagenase (Sigma, Type II) incubated at 35 °C, and continuously bubbled with O₂/CO₂ (19:1). After 30 min the tissue was transferred to a solution containing 2 mM-CaCl₂, cysteine (175 µg/ml; Sigma) and papain (Worthington; 0.035%). The tissue was then transferred to an enzyme-free, Hepes-buffered saline solution [(solution B), pH 7.3, containing (mM): NaCl 156; KCl, 3; KH₂PO₄, 1.25; CaCl₂, 2; MgSO₄, 2; D-glucose, 10; Hepes, 5] and kept on ice. Cells were dispersed by repetitive trituration with Pasteur pipettes and suspended on a glass coverslip used as the base of a Perspex (Lucite) recording chamber.

Electrophysiological recordings were made using conventional patch-clamp recording techniques (Hamill *et al.*, 1981). Voltage commands and data acquisition were controlled using pClamp (Axon Instruments) and a microcomputer as previously described (Duchen, 1990). Patch pipettes had tip diameters of 1–2 µm and were filled with a solution containing (mM) either CsCl₂ (105) and tetraethylammonium chloride (TEA) (25) or KCl (130), MgCl₂ (2), NaCl (10), K-ATP (2), EGTA (1.0), Hepes (7.5), pH 7.2. The ATP/Mg combination was necessary to prevent run-down of

the Ca²⁺ current, and 1 mM-EGTA was found to be necessary to replace the intrinsic Ca²⁺ buffering capacity of the cells, which appeared to be impaired in whole-cell recordings without additional buffer (probably reflecting the wash-out of intrinsic Ca²⁺-binding compounds).

Cells were continuously superfused with solution B. This could be switched between each of four reservoirs through solenoid-operated valves. Ca²⁺-free saline contained no added Ca²⁺, which was replaced with 2 mM-MgCl₂, and 1 mM-EGTA. Drug solutions, including a saline containing 50 mM-KCl, iso-osmotically replacing NaCl, could be superfused on to the preparation. Alternatively, they could be ejected under pressure from micropipettes placed close to the cells. As this limits exposure to a drug to the cells studied and spares the rest of the preparation, this mode was generally preferred.

Fluorescence measurements were obtained as previously described in some detail (Biscoe & Duchen, 1990, 1991a,b; Duchen, 1991b). The system is based on an inverted microscope equipped with epifluorescence, an oil-immersion ×40 quartz objective (numerical aperture 1.3), and two photomultipliers used with home-made current/voltage converters. Data were stored on videotape (PCM 4/8; Medical Systems Corp.) and analysed off-line using a Labmaster interface with a microcomputer. For Indo-1 (see Grynkiewicz *et al.*, 1985), dual-emission microfluorimetry was used to obtain the ratio of fluorescence emitted at 405 nm to that at 488 nm, using an excitation wavelength of 340 nm. Calibration of the ratio to $[Ca^{2+}]_i$ requires application of the formula:

$$[Ca^{2+}]_i = K_D \cdot \beta \cdot (R_{max} - R) / (R - R_{min})$$

where R_{max} and R_{min} are the ratios at saturating and zero Ca²⁺ concentrations respectively, R is the experimentally determined value of the ratio, and K_D is the K_D of the Indo-1 for Ca²⁺, for which a value of 250 nM (Grynkiewicz *et al.*, 1985) was used. β is the ratio of maximal to minimal fluorescence (i.e. at minimal and maximal $[Ca^{2+}]_i$ respectively) measured at 488 nm. R_{max} was obtained by patch-clamping cells with pipettes containing no added Ca²⁺ buffer, and either simply pulling the pipette off the cell to maximize Ca²⁺ influx before the dye leaked away or putting the clamp into oscillation by overcompensating the capacitance, effectively electroporabilizing the cells. R_{min} was obtained by making whole-cell recordings from cells using 10 mM-1,2-bis-(*o*-amino-5,5'-difluorophenoxy)ethane-*NNN'*-acetic acid (BAPTA) in the pipette in a Ca²⁺-free saline (containing 1 mM-EGTA). Cells were loaded with Indo-1 as the acetomethoxy (AM) ester (5 µM) by incubating them at room temperature for 30–45 min. Most of the experiments described below were performed in unpatched cells, as the whole-cell configuration of the patch-clamp technique undoubtedly disturbs numerous cytosolic functions, including endogenous $[Ca^{2+}]_i$ buffering mechanisms.

Rhodamine 123 (Rh 123) was dissolved in aqueous solution and cells were loaded by incubation with 10 µg of the dye/ml for 10 min at room temperature. The dye was well retained after washing the cells. Rh 123 fluorescence was excited at 488 nm and measured at 530 nm. The fluorescence signals routinely changed in response to a wide range of metabolic reagents, as expected theoretically from chemiosmotic predictions for $\Delta\psi_m$ (see Duchen & Biscoe, 1990; Duchen *et al.*, 1990b; Biscoe & Duchen, 1991a,b). Some spectrophotometric measurements of Rh 123 fluorescence were made to test for direct interactions of agents used with the dye, and no significant change in the signal from the dye was seen on addition of Ca²⁺ (final concn. 1 µM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (1 µM), CN⁻ (1 mM) or oligomycin (2 µg/ml). Ca²⁺ at higher concentrations appeared to quench the signal.

Fig. 1. Changes in Indo-1 fluorescence and $[Ca^{2+}]_i$ in a single neuron with depolarization

An Indo-1-loaded sensory neuron was depolarized by a 100 ms pulse of 50 mM- K^+ (arrowheads). Fluorescence (F) was excited at 340 nm, and the traces in (a) show the raw fluorescence signals measured at 405 and 488 nm. (b) Shows the ratio of signals at 405 nm to the signal at 488 nm (after subtraction of background fluorescence), whereas (c) shows the computed change in $[Ca^{2+}]_i$.

Autofluorescence of NAD(P)H was monitored by exciting either at 340 or 360 nm and measuring light excited between 400 and 540 nm using a band-pass filter combination (Chance *et al.*, 1979). Flavoprotein autofluorescence was excited at 450 nm and measured with a band-pass filter between 510 and 590 nm. The former gave a signal that increased with CN^- and decreased with FCCP, whereas the latter showed the inverse responses to these stimuli.

Other drugs used were iodoacetic acid (IAc; 200 μ M; Sigma), oligomycin (Sigma; 0.2–2 μ g/ml) and Ruthenium red (RuR; Sigma; 1 μ M). The temperature was varied between room temperature (18–25 $^{\circ}C$) and 35 $^{\circ}C$ and is indicated where appropriate. In the results given below, no qualitative differences were seen on warming the cells.

RESULTS

Changes in $[Ca^{2+}]_i$ with depolarization

In all excitable cells depolarization of the plasma membrane raises $[Ca^{2+}]_i$. In neurons this is predominantly the consequence of the opening of voltage-gated Ca^{2+} selective ion channels. Fig. 1 shows the changes in Indo-1 fluorescence and the computed change in $[Ca^{2+}]_i$ that results from depolarization evoked by brief exposure of a single mouse sensory neuron to 50 mM- K^+ (arrow),

Fig. 2. (a) Changes in Rh 123 fluorescence on depolarization and (b) the Ca^{2+} -dependence of the Rh 123 response to K^+ -induced depolarization

(a) A single neuron was exposed to pulses of 50 mM- K^+ (arrowheads) of increasing duration (indicated next to each response). (b) For the period indicated, the superfusate was switched to one in which Ca^{2+} was replaced with Mg^{2+} and which included 1 mM-EGTA. During this period, application of 50 mM- K^+ for 250 ms (made up with no added Ca^{2+} and 1 mM-EGTA) had no effect at all. With return to the control superfusate, the response returned.

applied by pressure ejection from a pipette placed close to the cell. The actual depolarization of the cell has effectively the same duration as the pressure pulse of K^+ as the K^+ is rapidly (in < 1 s) washed away by the superfusate. The rate at which the Ca^{2+} load is removed varies somewhat between cells. The secondary recovery phase seen in some, but not all, cells has been described in some depth by Thayer & Miller (1990) and attributed to the re-equilibration of Ca^{2+} taken up into mitochondria during the $[Ca^{2+}]_i$ peak. The duration of the responses to a standard pulse of high K^+ for 150 ms was variable [the mean time ($\pm$ s.d.) from the stimulus until complete recovery to baseline was 125 ± 88 s ($n = 21$)]. Some cells showed recovery as a simple exponential (see Duchene *et al.*, 1990a). The responses were abolished by removal of extracellular Ca^{2+} ($[Ca^{2+}]_o$) or by blockade of voltage-gated Ca^{2+} channels with Co^{2+} , Cd^{2+} or D-600 (methoxy-verapamil), confirming that it resulted from influx through voltage-activated Ca^{2+} channels (not shown here, but see Duchene *et al.*, 1990a).

On elevation of $[Ca^{2+}]_i$, the uptake of Ca^{2+} by the mitochondrial uniporter depends on the mitochondrial membrane potential ($\Delta\psi_m$) established by the electron-transport chain and on the relative amplitude of the rise of $[Ca^{2+}]_i$, compared with the affinity of the uniporter for Ca^{2+} . A rise in $[Ca^{2+}]_i$ and then in $[Ca^{2+}]_m$ may be expected to have several consequences: (i) to depolarize

Fig. 3. Changes in Rh 123 fluorescence in voltage-clamped cells

Whole-cell voltage-clamp recordings were made from Rh 123-loaded cells. In (a) and (b) three responses to depolarizing voltage steps from a holding potential of -70 mV are superimposed. The appropriate step potential is indicated next to each trace. The duration of the voltage step is also indicated below. In (c) a composite fluorescence-voltage plot is shown in which all responses are normalized to the maximal responses at 0 mV. The number of cells from which the data points were obtained is indicated next to each point. The current-voltage plot of the Ca^{2+} current is shown below for comparison, in which the currents have been normalized to the peak inward Ca^{2+} current at -10 to 0 mV.

Fig. 4. Effects of extracellular RuR on $[\text{Ca}^{2+}]_i$ transients

Ca^{2+} transients were evoked by depolarization of unclamped cells by 150 ms pulses of 50 mM- K^+ . RuR was applied at 1 μM for the period indicated, clearly almost completely abolishing the $[\text{Ca}^{2+}]_i$ transient.

$\Delta\psi_m$, either directly through promotion of Ca^{2+} cycling or by increasing ATP consumption by Ca^{2+} -dependent plasmalemmal ATPases, decreasing the ATP/ADP·P_i ratio and (ii) to increase the activation of the Ca^{2+} -dependent mitochondrial dehydrogenases [for reviews, see Hansford (1985), Denton & McCormack (1990) and McCormack *et al.* (1990)]. It is also possible, of course, that depolarization will also raise $[\text{Na}^+]_i$, increasing the ATP consumption of the Na^+/K^+ ATPase such that the increased ADP depolarizes $\Delta\psi_m$ and increases the oxygen consumption (see Erecińska & Dagani, 1990; but also see below).

Changes in $\Delta\psi_m$ monitored with Rh 123

The lipophilic cation, Rhodamine 123 (Rh 123) is accumulated by mitochondria in response to the large negative membrane potential established by the electron-transport chain (Johnson *et al.*, 1980; Emaus *et al.*, 1986; Mokhova & Rozovskaya, 1986; Chen, 1988; Duchen & Biscoe, 1990; Duchen *et al.*, 1990b). Once accumulated, the dye appears to bind within the mitochondrial matrix, and its fluorescence is then quenched, possibly by the concentration of the dye within the organelles. Manipulations expected to depolarize $\Delta\psi_m$ (uncouplers, blockade of the respiratory chain) increase fluorescence intensity. The mechanism for this is uncertain, but may be due to redistribution into the cytosol, with consequent loss of quenching. All the cells that we have tested take up and retain the dye, which exhibits a bright green fluorescence when illuminated with light at 488 nm. The emission peak is at 530 nm (see Emaus *et al.*, 1986, and Fig. 6 below), so that fluorescence monitored at this wavelength gives a continual index of $\Delta\psi_m$. In response to brief plasmalemmal depolarizations in the presence of $[\text{Ca}^{2+}]_o$, Rh 123 fluorescence invariably increased in healthy cells (Fig. 2; $n \geq 30$). Similar changes have been seen in many other excitable cells, including rat chromaffin cells, sympathetic neurons from the superior cervical ganglion and Type I cells from the rabbit carotid body (see Biscoe & Duchen, 1991b, and M. R. Duchen, unpublished work). The change in fluorescence signal with increasing intensity of stimulus (increasing the duration of the depolarizing K^+ pulse, for example) showed a saturating relationship, similar to the change in $[\text{Ca}^{2+}]_i$ (see Silver *et al.*, 1990; Thayer & Miller, 1990). The response was completely abolished by removal of $[\text{Ca}^{2+}]_o$ (Fig. 2b; $n = 5$).

The Rh 123 signal in response to high K^+ could reflect plasmalemmal depolarization rather than mitochondrial depolarization. Too little is understood about the precise nature of the mechanisms that underlie the changes in Rh 123 fluorescence to be certain. The loss of the response in the absence of $[\text{Ca}^{2+}]_o$ strongly suggests that at least the bulk of the signal arises from mitochondrial depolarization, as the response of the cell membrane potential to K^+ -induced depolarization should be independent of $[\text{Ca}^{2+}]_o$. Simultaneous patch-clamp and fluorescence recordings were made to establish this relationship. Fig. 3 shows the change in the Rh 123 signal with depolarization of the cell membrane under voltage-clamp control. The signal was exactly parallel with the voltage-dependence of the Ca^{2+} current, which is shown below. Thus no change in Rh 123 fluorescence was seen at membrane potentials negative to the threshold for the activation of voltage-gated Ca^{2+} channels, around -40 mV, and minimal changes were seen at potentials positive to about $+60$ mV, the effective reversal potential for the Ca^{2+} current, while the response was maximal at 0 mV, the potential at which the Ca^{2+} current is maximal. A notable feature of these recordings was that rupture of the cell membrane to establish the whole-cell recording configuration did not lead to loss of Rh 123 fluorescence, as might be expected if some of the dye were cytosolic. Further, the Ca^{2+} buffering power of the pipette-filling solutions

Fig. 5. Effects of RuR on the high-threshold Ca^{2+} current

In (a) successive records from a cell under whole-cell voltage clamp are superimposed. The plasmalemmal potential was clamped at -70 mV, and depolarizing steps applied for the indicated period to 0 mV, initiating an inward Ca^{2+} current. Application of $1 \mu M$ -RuR (arrow) clearly reduced the current. In (b) the component blocked by RuR is shown, in which the response obtained after RuR was digitally subtracted from the control. In (c) the current-voltage relationships of the response before ($\circ$) and after ($\bullet$) are shown. The Ca^{2+} current evoked by a depolarizing step to 0 mV was clearly much reduced by RuR.

were critical in obtaining these responses. If no Ca^{2+} buffer was added to the solution, the signal increased with depolarization, but often failed to recover, whereas responses were almost undetectable when Ca^{2+} buffering power was increased with increased concentrations of EGTA or BAPTA.

Effects of RuR on Ca^{2+} current and Rh 123 fluorescence

The data shown above strongly suggest that the change in Rh 123 fluorescence reflects a direct response to mitochondrial Ca^{2+} influx. As RuR is often described as a selective and powerful inhibitor of the mitochondrial Ca^{2+} uniporter (Vasington *et al.*, 1972; Rottenberg & Scarpa, 1974), the effect of RuR on the response was examined. The extracellular application of RuR had rather complex effects. Most notably it blocked the $[Ca^{2+}]_i$ transient in response to depolarization (Fig. 4) (Gupta *et al.*, 1988; Taipale *et al.*, 1989).

This effect was due to blockade of the voltage-gated Ca^{2+} current, as illustrated in Fig. 5, which shows responses to a depolarizing step from a holding potential of -70 mV to 0 mV for about 50 ms. Responses before and after application (arrow) of RuR ($1 \mu M$) are shown and include an inactivating Na^+ current which gives way to a sustained Ca^{2+} current. The selective effect of the RuR on the Ca^{2+} current is illustrated in Fig. 6(b), which shows the component of the current blocked by RuR (a

Fig. 6. Effects of RuR on Rh 123 fluorescence

The effect of extracellular application of RuR ($1 \mu M$) on the Rh 123 fluorescence signal in a single sensory neuron is shown in (a). RuR decreased the baseline fluorescence signal and reduced the response to K^+ -induced depolarization immediately after application of the RuR. The response to K^+ then recovered, but with slowed kinetics. In (b) the excitation and emission spectra of Rh 123 in solution in a cuvette are shown before and after (---,) addition of $1 \mu M$ -RuR.

subtraction of the response after RuR from the control). The effect is not due to a shift in the voltage-sensitivity of the current as shown by the current-voltage (I/V) plots shown in Fig. 5(c), which shows the I/V relation of the sustained current before ($\circ$) and after ($\bullet$) $1 \mu M$ -RuR. This action alone reduced the usefulness of RuR as a tool in the present investigation. However, intracellular dialysis of the neurons with RuR did not reduce the Ca^{2+} current, which was stable for up to 30 min with RuR in the patch pipette ($n = 7$). Thayer & Miller (1990) have also shown that intracellular dialysis of dorsal-root-ganglia neurons does not reduce the $[Ca^{2+}]_i$ transient in response to depolarization.

Application of RuR also reduced the Rh 123 fluorescence signal (Fig. 6a; $n = 14$). This could be interpreted as a hyperpolarization of $\Delta\psi_m$. However, it turned out that RuR also reduced the fluorescence of a solution of Rh 123 in a cuvette, apparently quenching the fluorescence signal over its whole spectrum (Fig. 6b). The response to high K^+ immediately after the application of RuR was reduced, presumably reflecting the reduction in the $[Ca^{2+}]_i$ transient, and supporting the Ca^{2+} -dependence of the response. Together these data suggest that interpretation of data obtained using extracellular RuR must be regarded with great caution, and that RuR cannot usefully be used with Rh 123. A further phenomenon is noteworthy. After apparent quenching of the Rh 123 fluorescence by RuR, the time course of the Rh 123 fluorescence response to high K^+ was markedly slowed (Fig. 6a).

Fig. 7. Changes in NAD(P)H autofluorescence with depolarization

(a) An unclamped cell was depolarized with a 150 ms pulse of 50 mM- K^+ . The immediate decrease in fluorescence (oxidation) is shown on a faster time base in (b). Once the delayed increase in fluorescence was established, further depolarization of the cell elicited a further decrease in fluorescence, (c), showing that the mechanisms increasing fluorescence (reduction) were saturated.

As the fluorescence signal is presumably derived primarily from Rh 123 accumulated within mitochondria, the quenching implies that the RuR does penetrate the cell membrane into the mitochondria, but it is hard to determine whether the change in kinetics of the responses to high K^+ reflects a genuine change in the behaviour of $\Delta\psi_m$ or whether it is due to some interaction with the dye.

Consequences of elevated $[Ca^{2+}]_i$ for autofluorescence

Significant depolarization of $\Delta\psi_m$ and an increase in intramitochondrial Ca^{2+} might be reflected in alterations in the mitochondrial redox state, either with increased activation of the Ca^{2+} -dependent mitochondrial enzymes or altered respiration. These variables can be monitored by following changes in mitochondrial NAD(P)H autofluorescence. Fig. 7 shows a characteristic sequence of changes in NAD(P)H/NAD(P) $^+$ autofluorescence that followed the brief exposure of sensory neurons to high K^+ ($n \geq 24$). Typically the response consisted of a brief decrease in fluorescence, followed by a sustained increase that could last several minutes (mean $\pm$ S.D.: 160 ± 84 s to recover to baseline) after a pulse of only 100–200 ms. The time course of the decrease in fluorescence (Fig. 7b) correlated well in time with the rapid rise in $[Ca^{2+}]_i$ and with the depolarization of $\Delta\psi_m$ and could reflect a stimulation of the respiratory chain as a direct response to the mitochondrial depolarization. The prolonged increase in fluorescence is consistent with increased activation of the mitochondrial Ca^{2+} -dependent dehydrogenases (Moreno-Sanchez & Hansford, 1988), increasing the net reduced state of the pyridine nucleotide pool. If the high K^+ was applied during the prolonged increase, only the transient decrease in fluorescence was seen (Fig. 7c). The relative balance between these two components of the response varied between cells. In some cells, for example, the decrease in fluorescence was the predominant response, possibly suggesting that $[Ca^{2+}]_m$ was already high.

Fig. 8. Changes in NADH autofluorescence were abolished in Ca^{2+} -free superfusates

A response was elicited by a 150 ms pulse of 50 mM- K^+ that lasted for about 5 min and could not be obtained at all during superfusion with a saline solution in which Ca^{2+} was replaced with Mg^{2+} and to which 1 mM-EGTA was added. The response was retrieved with a return to the control superfusate.

The whole response was dependent on $[Ca^{2+}]_o$ (Fig. 8) and was maintained unchanged despite the presence of IAc and pyruvate ($n = 4$), so did not reflect an increased supply of glycolytic substrate in response to the high $[Ca^{2+}]_i$.

The mitochondrial origin of these signals was further supported by measurements of flavoprotein fluorescence (Fig. 9), which showed a brief initial increase in signal (an increased oxidation),

Fig. 9. Changes in flavoprotein autofluorescence with depolarization

A record of the fluorescence excited at 450 nm and emitted between 510 and 590 nm is shown from an unclamped cell depolarized with a 150 ms pulse of 50 mM- K^+ . The responses are exactly equivalent to the changes in NADH, consisting of an immediate, but brief, increase (an oxidation), followed by a prolonged decrease (reduction).

followed by a prolonged decrease, as expected from an increased reduction of the mitochondrial coenzymes.

These findings taken together are consistent with the hypothesis that the rise in $[Ca^{2+}]_i$ initially depolarizes mitochondria, so stimulating an increased rate of oxidation of the intra-mitochondrial coenzymes. The time course of the rise in $[Ca^{2+}]_i$, the depolarization of $\Delta\psi_m$, the decreased NAD(P)H fluorescence and the increased flavoprotein fluorescence are all consistent with a direct interrelationship.

Effects of oligomycin

The early effects of depolarization could represent direct reactions to an increased Ca^{2+} influx into the mitochondrial matrix. This would depolarize $\Delta\psi_m$ directly and stimulate respiration. Similar effects would result from a stimulation of Ca^{2+} -dependent ATPases after a rise in $[Ca^{2+}]_i$, so reducing the ATP/ADP- P_i ratio. This would in turn stimulate proton flux through the F_1F_0 -ATP synthase, depolarizing $\Delta\psi_m$ and stimulating respiration.

Both the depolarization of $\Delta\psi_m$ (Fig. 10a) and the increased oxidation of NAD(P)H (Fig. 10b) after plasmalemmal depolarization were maintained after blockade of the F_1F_0 -ATP synthase by oligomycin (2 μ g/ml). This suggests that these changes cannot reflect stimulation of respiration by increased ADP. The depolarization of $\Delta\psi_m$ was occasionally reduced in amplitude after oligomycin. This may simply reflect a reduction in the amplitude of the $[Ca^{2+}]_i$ transient produced by the high K^+ stimulus. The $[Ca^{2+}]_i$ transient depends on the activation of the Ca^{2+} current, which is itself both ATP-dependent and blocked by elevated $[Ca^{2+}]_i$ and which may therefore also be reduced by either a fall in ATP or a rise in $[Ca^{2+}]_i$ in response to oligomycin.

That oligomycin was effectively blocking the ATP synthase at the concentrations used was confirmed as (i) $\Delta\psi_m$ hyperpolarized slightly but irreversibly after exposure to oligomycin, and (ii) the depolarization of $\Delta\psi_m$ in response to inhibitors of mitochondrial electron transport (CN^- , rotenone) or to anoxia was much faster after exposure to oligomycin, as expected if reversal of the ATP synthase, slowing the rate of depolarization, is prevented by the oligomycin. These observations also serve to define the role of

Fig. 10. Effects of oligomycin on the responses to depolarization

Both the changes in $\Delta\psi_m$ (a) and the oxidizing change in NADH autofluorescence (b) persisted after blockade of the F_1F_0 -ATPase with oligomycin, applied for the periods indicated. The oligomycin hyperpolarized $\Delta\psi_m$ and increased the autofluorescence signal irreversibly.

the F_1F_0 -ATPase in slowing the rate of depolarization of $\Delta\psi_m$ with blockade of electron transport.

Effects of intracellular RuR on autofluorescence

The effects of RuR on the Rh 123 fluorescence were discussed above. For the reasons detailed above, the extracellular application of RuR was not felt to be instructive. However, intracellular dialysis of cells by inclusion of RuR in patch pipettes had no effect on resting autofluorescence signals, and yet appeared to prevent any response to depolarization. As the intracellular RuR did not alter the Ca^{2+} current, and did not prevent the $[Ca^{2+}]_i$ transient (see also Thayer & Miller, 1990), this provides yet further evidence that the entire response is directly promoted by the movement of Ca^{2+} into the mitochondria. Fig. 11 shows the changes in autofluorescence seen in response to depolarizing voltage-clamp commands in cells under whole-cell patch-clamp conditions, with (b) and without (a) the inclusion of 1 μ M-RuR in the pipette.

As it is not currently feasible for us to measure all these variables simultaneously, the relative rates of change of each variable compiled from different experiments are shown in Fig. 12. The rates of change of $[Ca^{2+}]_i$, $\Delta\psi_m$ and of increased oxidation of NAD(P)H could all be readily and well fitted as single exponential processes (few measurements of flavoprotein fluorescence were made in this way, as the signals were relatively small, and the long wavelength used meant that the measurements

Fig. 11. Effect of intracellular RuR on changes in autofluorescence

In whole-cell patch-clamp experiments a depolarizing command to 0 mV for 1 s from a holding potential of -70 mV (a) initiated the characteristic changes in autofluorescence described above. Inclusion of RuR ($1 \mu\text{M}$) in the pipette did not impair the Ca^{2+} current, which is shown below, but blocked the autofluorescence response, despite the higher gain used and the longer (4 s) depolarizing command.

were made in the dark). With K^{+} -induced depolarization the mean time constant for the rise of $[\text{Ca}^{2+}]_i$ was 215 ± 18 ms ($n = 8$); for Rh 123 fluorescence, it was $1028 \text{ ms} \pm 213$ ($n = 8$), for NADH oxidation, $687 \text{ ms} \pm 202$ ($n = 10$) and three measurements for flavoprotein oxidation gave time constants of 1020, 1290 and 1390 ms. Time constants for the onset of each response in voltage-clamped cells were significantly more variable and generally slowed, even with just 1 mM-EGTA as the added Ca^{2+} buffer. For example, using Rh 123, the time constant for the onset of the response to a depolarizing step to 0 mV was 2239 ± 1326 ms ($n = 5$), suggesting that the whole-cell recording significantly altered the kinetics of Ca^{2+} buffering or of the mitochondrial response.

DISCUSSION

These data show that changes in $[\text{Ca}^{2+}]_i$ within a range that might be expected to occur in response to physiological excitation in this population of neurons have significant and long-lasting consequences for the mitochondrial energetics and, therefore, for the cellular economy of these cells. Interestingly, several publications have previously described significant changes in cellular energetics as a result of changes in excitability in preparations of mammalian central nervous system, but the interpretation of those data was hampered particularly by the complexity of the intact tissue (Rosenthal & Jöbsis, 1971; Lipton, 1973; Lothman *et al.*, 1975; Moffett & LaManna, 1978; Segal *et al.*, 1980). The reductionist approach adopted here makes it possible to dissect the various possible explanations for the signals obtained to provide a unifying description of the underlying processes. That the changes both of autofluorescence and Rh 123 fluorescence persist during whole-cell patch-clamp experiments supports the contention that signals are mitochondrial rather than cytosolic, as cytosolic enzyme systems are quite rapidly disrupted by the whole-cell patch technique. The responses persist in patch-clamped cells despite the inclusion of MgATP in the pipette filling solution, providing some further evidence that the change

in fluorescence signals do not represent responses to changing [ATP], and the blockade of the autofluorescence changes by intracellular application of RuR with the patch pipette all support the simple explanation proposed and a mitochondrial origin for the responses described. The relative time courses of the changes in each variable measured further support the interpretation of the sequence of events following the rise in $[\text{Ca}^{2+}]_i$, although the rate of change of the depolarization of $\Delta\psi_m$ signalled by Rh 123 seems a little low. This might well reflect the mechanism underlying the change in Rh 123 fluorescence with depolarization, as redistribution of the dye will inevitably be slower than the actual change in $\Delta\psi_m$. The rates of change of the Rh 123 signal can thus probably only define a lower limit for the rate of change of $\Delta\psi_m$.

A related question is the extent to which changes in $[\text{Ca}^{2+}]_i$ brought about through other mechanisms might have the same metabolic consequences. There seems no reason to suppose that the cells distinguish one source of Ca^{2+} from another. The sensory neurons have very small or no response to caffeine, but chromaffin cells show significant biphasic changes in autofluorescence very similar to those shown above, as a consequence of Ca^{2+} release from intracellular pools produced by caffeine (M. R. Duchen, unpublished work). This is also consistent with results showing significant mitochondrial Ca^{2+} uptake in response to inositol trisphosphate-generating signals in other cell types [GH3 cells (Biden *et al.*, 1986); sea-urchin eggs (Eisen & Reynolds, 1985)], and changes in NADH autofluorescence, pyruvate dehydrogenase activity and related variables in hepatocytes in response to Ca^{2+} -mobilizing hormones (Balaban & Blum, 1982; see also McCormack *et al.*, 1990), suggesting that alterations in mitochondrial function may be a general concomitant of any Ca^{2+} -mobilizing stimulus in many cell types. The interesting rider to this suggestion is whether this response can be modulated by other cytosolic factors.

The principal observations here, namely that (i) a rise in $[\text{Ca}^{2+}]_i$ depolarizes $\Delta\psi_m$ and causes increased oxidation of mitochondrial NADH and FADH, suggesting that there must be a concomitant

Fig. 12. Relative time courses of responses to depolarization

This Figure shows a composite of records, each obtained from a different cell, to show the relative rates of onset of the changes in $[Ca^{2+}]_i$, NADH, FAD and Rh 123 signals. Each trace was well-fitted (correlation: $r^2 \geq 0.98$) by a single exponential function with time constants of 212 ms ($[Ca^{2+}]_i$), 520 ms (NADH), 1020 ms (FAD) and 850 ms (Rh 123).

increase in oxygen consumption, and (ii) a prolonged activation of intramitochondrial enzymes follows, have several implications. Firstly, they clearly show that these changes in cytosolic $[Ca^{2+}]_i$ are sufficient to promote substantial Ca^{2+} influx into the mitochondria, and are consistent with a substantive role for mitochondrial uptake in $[Ca^{2+}]_i$ buffering, as suggested previously by Duchen *et al.* (1990a) and by Thayer & Miller (1990) from the effects of mitochondrial depolarization on shaping of $[Ca^{2+}]_i$ transients. These responses can also be used as indicators that substantial Ca^{2+} movement into mitochondria has occurred. Secondly, the increase in oxygen consumption (inferred from the increased oxidation of NADH) following the rise in $[Ca^{2+}]_i$ may be profound, approaching the degree of stimulation of respiration seen with uncoupler. Changes in mitochondrial oxygen consumption in response to a rise in $[Ca^{2+}]_i$ have also been documented in the *Limulus* (horseshoe crab) photoreceptor (Fein & Tsacopoulos, 1988), suggesting that such functional response to elevated $[Ca^{2+}]_i$ may be quite widely conserved. In the intact nervous system, where oxygen diffusion may be limiting, any change in the oxygen consumption of a component of the tissue may decrease local oxygen potential, pO_2 , so conceivably affecting the microenvironment of adjacent cells. This may be especially significant under pathological conditions. For example, the increased oxygen consumption of neurons involved in epileptiform discharges may cause a fall in local tissue pO_2 sufficient to cause long-lasting hypoxic injury. In conditions in which the microcirculation is impaired by disease, even normal neuronal

activity may conceivably be sufficient to lower local pO_2 and impair local function.

The major response to raised intramitochondrial Ca^{2+} documented in other tissues is an increase of the NADH/NAD⁺ ratio, which would stimulate the activity of the respiratory chain and therefore increase oxygen consumption (Hansford, 1985; Brown *et al.*, 1990). One might predict that any increase in oxygen consumption will be maintained throughout the duration of both phases of the change in autofluorescence. Such an increase in oxygen consumption might be expected to increase (hyperpolarize) $\Delta\psi_m$. It is possible that the increase in NADH plays a role in accelerating the repolarization of $\Delta\psi_m$, but only simultaneous measurements of both variables would help resolve this issue. The issue is complicated by the involvement of Ca^{2+} in the regulation of an ATPase-inhibitory protein (Yamada *et al.*, 1980; Yamada & Huzel, 1988; Das & Harris, 1990). The elevated cytosolic $[Ca^{2+}]_i$ may thus effectively stimulate ATP synthase through the displacement of this ATPase-inhibitory protein. This would increase proton flux through the F_0 channel and perhaps offset any tendency for $\Delta\psi_m$ to hyperpolarize in response to the increased activity of the respiratory chain.

It is interesting that elevations in $[Ca^{2+}]_i$ have not previously been shown to cause depolarization of $\Delta\psi_m$ in other cell types. It seems possible that this reflects the higher activity of the Ca^{2+} uniporter in neural tissue (compared with liver and heart, for example) (Nicholls, 1978) and therefore greater Ca^{2+} uptake in response to $[Ca^{2+}]_i$ transients. The functional consequence of this apparent difference is obvious from the data reported here, although the basis for any differences between tissues is less clear, as most cell types undergo substantial $[Ca^{2+}]_i$ elevations in response to a wide variety of stimuli. It is not clear whether the prolonged change in NADH fluorescence that follows a brief stimulus reflects the time for which intramitochondrial $[Ca^{2+}]$ is elevated, or the long half-life of the activated form of pyruvate dehydrogenase phosphate phosphatase. In these neurons, the brief $[Ca^{2+}]_i$ transient is often followed by a long 'hump' (see Fig. 1), which can last for 5 or 6 min and which has been associated by Thayer & Miller (1990) with increased FCCP-releasable Ca^{2+} and probably reflects mitochondrial Ca^{2+} cycling, maintaining a $[Ca^{2+}]_i$ at 300–400 nM as mitochondrial and cytosolic Ca^{2+} re-equilibrate. This hump is not generally seen in other cell types (see, for example, Biscoe & Duchen, 1990a), and yet the prolonged increase in autofluorescence is also seen in some other excitable cells. The time courses of the $[Ca^{2+}]_i$ 'hump' and of the increase in autofluorescence were very similar (time from stimulus to complete recovery 125 ± 88 s and 160 ± 84 s respectively), suggesting that the duration of the autofluorescence change could be a direct index of the time taken for re-equilibration of intramitochondrial Ca^{2+} . An alternative explanation could be that the hump is in fact seen when Indo-1 has accumulated within the mitochondria and is a more direct reflection of intramitochondrial $[Ca^{2+}]$. It would clearly be desirable to measure $[Ca^{2+}]_m$ directly, or at least to be able to monitor $[Ca^{2+}]_i$ and autofluorescence simultaneously, and with the new generation of long-wavelength Ca^{2+} indicators, this may well prove feasible.

The results presented here suggest that Ca^{2+} can act as a primary regulator of mitochondrial oxidative phosphorylation in response to increased neuronal activity in these cells. This might seem to contrast somewhat with work in other preparations or using different protocols, in which a rise in $[Na^+]_i$, activation of the Na^+/K^+ -ATPase and a subsequent rise in ADP have been shown to increase oxygen consumption and oxidative phosphorylation. An alternative mechanism for such Na^+ -dependent effects is the activation of Na^+/Ca^{2+} exchange following the accumulation of intracellular Na^+ , although the exchanger is rather weak in many neurons (e.g. see Duchen *et al.*, 1990a), and

such responses would still be dependent on extracellular Ca^{2+} . For such experiments, veratridine has often been used to depolarize synaptosomes. Veratridine opens an Na^+ conductance and so will raise $[\text{Na}^+]_i$. However, in many neurons, changes in $[\text{Na}^+]_i$ with more physiological depolarizations are likely to be very small, as the Na^+ currently rapidly inactivates. The actual changes of $[\text{Na}^+]_i$ following stimulation of neurons may vary considerably among cell types and among modes of excitation (e.g. with transmitters that open cation-selective channels) and will also depend on the ability of the cell to sustain rapid trains of action potentials with sustained depolarization. The sensory neurons used for these experiments typically show relatively limited action potential activity on depolarization, as a large Ca^{2+} -dependent K^+ conductance tends to repolarize and shunt the membrane after a few action potentials. Furthermore, the sensory neurons have large Ca^{2+} currents compared with many other neuronal types. It should be clear, then, that any difference is only apparent, and that different mechanisms may be functionally important in different cells or under different conditions, depending on the relative $[\text{Ca}^{2+}]_i$ or $[\text{Na}^+]_i$ load imposed on the cell by its activity.

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