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RELATIVE MITOCHONDRIAL MEMBRANE POTENTIAL AND $[Ca^{2+}]_i$ IN TYPE I CELLS ISOLATED FROM THE RABBIT CAROTID BODY

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SUMMARY

1. In the accompanying paper (Duchen & Biscoe, 1992) we have described graded changes in autofluorescence derived from mitochondrial NAD(P)H in type I cells of the carotid body in response to changes of P_{O_2} over a physiologically significant range. These observations suggest that mitochondrial function in these cells is unusually sensitive to oxygen and could play a role in oxygen sensing. We have now explored further the relationships between hypoxia, mitchondrial membrane potential ($\Delta\psi_m$) and $[Ca^{2+}]_i$.

2. The fluorescence of Rhodamine 123 (Rh 123) accumulated within mitochondria is quenched by $\Delta\psi_m$. Mitochondrial depolarization thus increases the fluorescence signal. Blockade of electron transport (CN^-, anoxia, rotenone) and uncoupling agents (e.g. carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; FCCP) increased fluorescence by up to 80–120%, while fluorescence was reduced by blockade of the F_0 proton channel of the mitochondrial ATP synthase complex (oligomycin).

3. $\Delta\psi_m$ depolarized rapidly with anoxia, and was usually completely dissipated within 1–2 min. The depolarization of $\Delta\psi_m$ with anoxia (or CN^-) and repolarization on reoxygenation both followed a time course well characterized as the sum of two exponential processes. Oligomycin (0·2–2 μg/ml) hyperpolarized $\Delta\psi_m$ and abolished the slower components of both the depolarization with anoxia and of the subsequent repolarization. These data (i) illustrate the role of the F_1–F_0 ATP synthetase in slowing the rate of dissipation of $\Delta\psi_m$ on cessation of electron transport, (ii) confirm blockade of the ATP synthetase by oligomycin at these concentrations, and (iii) indicate significant accumulation of intramitochondrial ADP during 1–2 min of anoxia.

4. Depolarization of $\Delta\psi_m$ was graded with graded changes in P_{O_2} below about 60 mmHg. The stimulus–response curves thus constructed strongly resemble those for $[Ca^{2+}]_i$ and NAD(P)H with P_{O_2}. The change in $\Delta\psi_m$ closely followed changes in P_{O_2} with time.

5. The rate of rise of $[Ca^{2+}]_i$ in response to anoxia is strongly temperature sensitive. The rate of depolarization of $\Delta\psi_m$ with anoxia similarly increased at least two- to fivefold on warming from 22 to 36 °C. The change with FCCP was not significantly altered by temperature.

6. These data show that the mitochondrial membrane potential changes over a

physiological range of P_{O_2} values in type I cells. This contrasts with the behaviour in dissociated chromaffin cells and sensory neurons, in which no change was measurable until the P_{O_2} fell close to zero.

7. Intracellular $[Ca^{2+}]_i$ shows a graded increase with hypoxia over a similar range of P_{O_2} values to the changes in $\Delta\psi_m$, and is released, at least in part, from an intracellular store. The source of that calcium remains uncertain. We have previously suggested it is derived from mitochondria, and have now examined this proposal further.

8. Rotenone, which blocks mitochondrial electron transport at complex I, depolarized $\Delta\psi_m$ and raised $[Ca^{2+}]_i$. Any further change in $[Ca^{2+}]_i$ in response to anoxia was occluded. Tetramethyl-*p*-phenylene diamine (TMPD) bypasses the rotenone-induced block, directly reducing cytochrome *c*. TMPD reversed both the depolarization of $\Delta\psi_m$ and the rise in $[Ca^{2+}]_i$. CN^- depolarized $\Delta\psi_m$ and raised $[Ca^{2+}]_i$ despite the presence of TMPD, confirming this mechanism.

9. The changes in mitochondrial function with hypoxia are not secondary to the changes in $[Ca^{2+}]_i$. K^+-induced depolarization of the cells raises $[Ca^{2+}]_i$ more than does anoxia, but produced only small changes in $\Delta\psi_m$ which were abolished by removal of extracellular Ca^{2+} while the response to hypoxia was unchanged.

10. The simplest mechanisms that link changes in $[Ca^{2+}]_i$ with $\Delta\psi_m$ are (i) direct release of mitochondrial Ca^{2+} and (ii) release from a pool sensitive to the ATP/ADP.P_i ratio. Oligomycin, which blocks the F_1–F_0 ATP synthetase while hyperpolarizing $\Delta\psi_m$, initiates a rapid and sustained rise of $[Ca^{2+}]_i$ to levels similar to those seen with anoxia, FCCP or CN^-. Subsequent responses to these stimuli were much reduced in amplitude. In the absence of external Ca^{2+}, responses to oligomycin were present but were transient rather than sustained.

11. These results together suggest that $[Ca^{2+}]_i$ is entrained to $\Delta\psi_m$ during hypoxia in these cells and that depolarization of $\Delta\psi_m$, graded with P_{O_2}, therefore appears central to transduction in the carotid body. The effect of oligomycin suggests that alterations in ATP/ADP.P_i ratio could provide a link between changes in $\Delta\psi_m$ and $[Ca^{2+}]_i$.

12. The increase in $[Ca^{2+}]_i$ is thus probably derived from at least two intracellular sources, mitochondrial and non-mitochondrial.

INTRODUCTION

It is widely held that chemotransduction in the mammalian carotid body in response to a fall in P_{O_2} is effected through the release of a chemical transmitter from a receptor cell, the type I cell, and that the transmitter then initiates a neural signal which is conveyed to the brain. The intervening steps that couple the detection of a falling P_{O_2} to secretion remain under some considerable debate (see for example Mills & Jöbsis, 1970; Delpiano & Hescheler, 1989; López-López, González, Ureña & López-Barneo, 1989; and the accompanying paper, Duchen & Biscoe, 1992).

Over the years, the biochemical pharmacology of the structure has led to repeated suggestions that mitochondria are somehow involved in transduction (see, for example, Mills & Jöbsis, 1970, 1972; Mills, 1975; Mulligan, Lahiri & Storey, 1981), but more direct evidence has been lacking. In this and the accompanying paper, we have asked whether changes in mitochondrial function with changing P_{O_2} are

consistent with a significant role for these organelles in the process, and have attempted to identify a mechanism whereby such changes could lead to transmitter release.

The mitochondrial electron transport chain develops and maintains a potential across the inner mitochondrial membrane ($\Delta\psi_m$) of the order of -150 to -200 mV by expulsion of protons from the matrix. This provides the energy which drives the diffusion of protons back into the matrix through the proton channel (the F_0 subunit) of the F_1–F_0 ATP synthetase, promoting ATP synthesis. In addition, the balance of Ca^{2+} between the cytosol and the mitochondrial matrix is determined by the electrochemical potential for Ca^{2+} across the inner mitochondrial membrane, and predominantly by $\Delta\psi_m$. The forward rate of the electron transport chain is dependent on a variety of factors, but particularly upon the supply of oxygen, which becomes critical when the P_{O_2} reaches the apparent K_D of cytochrome *c* oxidase for oxygen; in other words when the oxidation of cytochrome *c* oxidase becomes rate limiting. In the accompanying paper (Duchen & Biscoe, 1992) we have described changes in autofluorescence in the type I cells isolated from the rabbit carotid body. We have shown that autofluorescence derived predominantly from mitochondrial NAD(P)H shows graded increases (an increase in the NAD(P)H/NAD(P) ratio) with graded decreases in P_{O_2} that are most readily explained if the type I cells express a specialized cytochrome *c* oxidase with a low affinity for oxygen. This contrasts with similarly dissociated dorsal root ganglion and chromaffin cells which show changes only when the P_{O_2} falls near to zero. These data are thus consistent with those of Mills & Jöbsis (1970, 1972), who presented evidence for the presence of a specialized, low-affinity cytochrome *aa*3 (a component of the cytochrome *c* oxidase complex) in the carotid body.

We now describe experiments with a fluorescent probe for mitochondrial potential (Rhodamine 123), and with indicators for $[Ca^{2+}]_i$. The results show both that (i) $\Delta\psi_m$ is appropriately and unusually sensitive to P_{O_2}, and that (ii) $[Ca^{2+}]_i$ is closely entrained to $\Delta\psi_m$. We suggest that the changes in autofluorescence reflect changes in $\Delta\psi_m$, which may underpin the rise in $[Ca^{2+}]_i$ that promotes transmitter release from these cells in response to hypoxia (Biscoe & Duchen, 1990*a*, *b*).

Preliminary accounts of some of these data have been published (Biscoe & Duchen, 1990*a*; Duchen & Biscoe, 1990).

METHODS

Cells were prepared as previously described (Duchen, Caddy, Kirby, Patterson, Ponte & Biscoe, 1988; Biscoe & Duchen, 1990*b*). In brief, New Zealand White rabbits of 1·5–4 kg weight were anaesthetized with sodium pentobarbitone (30 mg/kg), and were later killed by an overdose of pentobarbitone. The carotid bifurcation was removed into ice-cold saline and dissociated by trituration after enzyme treatment. Following dissociation, cells were kept on ice in the HEPES-based saline described below, or loaded with fluorophore as appropriate. Cells were plated onto a glass coverslip that formed the base of a simple chamber and were allowed to settle. The continuous superfusate could be switched between four reservoirs using solenoid-operated hydraulic switches. The exchange time of the bath varied between about 10 and 20 s. The composition of the saline used for all experiments described here was (mM): NaCl, 156; KCl, 3·0; KH_2PO_4; 1·25; $CaCl_2$, 2; $MgSO_4$, 2; D-glucose, 10; HEPES, 5; pH 7·3 (unless stated otherwise).

Variable degrees of hypoxia could be achieved by equilibrating the superfusates with nitrogen. Addition of $Na_2S_2O_4$ (500–750 μM) brought the P_{O_2} to 0 mmHg. The P_{O_2} in the bath was continuously

recorded using a home-made miniature platinum oxygen electrode, and the temperature was monitored using a thermocouple.

Type I cells were identified by their characteristic morphological features (Duchen *et al.* 1988). Fluorescence signals were usually obtained from small clusters of four to ten cells, as we have previously found that these are most reliably identifiable as type I cells, but data obtained from single cells were the same as from small clusters. The fluorescence was measured as previously described (Biscoe & Duchen, 1990*b*), using a Nikon ×40 oil immersion lens, an adjustable rectangular window on the output arm of the fluorescence microscope, a home-made beam splitter, and current-to-voltage converters for the two photomultiplier tubes. For all measurements the background signal potentially derived from a number of sources including the coverslip was subtracted from the total signal to leave only that recorded from the cells.

Rhodamine. Cells were loaded with Rhodamine 123 (Rh 123, Molecular Probes) by incubation with 10 μg/ml for about 10–15 mins, and with Indo-1 and Fluo-3 by incubation in suspension with the acetoxymethyl esters (2–5 μM; Molecular Probes) for 30–45 min, all at room temperature.

Rh 123 fluorescence was excited at 488 nm and measured at 530 nm (Emaus, Grunwald & Lemasters, 1986; Duchen, Pearce & Biscoe, 1990). We have not attempted to calibrate these signals in terms of $\Delta\psi_m$ at present. In order to introduce some measure for comparison between responses, we have presented all data as a percentage change in fluorescence from the baseline level, and found that this gave remarkably consistent responses.

Indo-1. For Indo-1, fluorescence was excited initially at 365 nm or in later experiments at 340 nm. Emission was continuously monitored at both 405 and 488 nm and the ratio of F_{405}/F_{488} used to obtain a signal dependent solely on $[Ca^{2+}]_i$. These records were calibrated later. When light at 365 nm was used for excitation, changes in $[Ca^{2+}]_i$ produced only a small increase in the signal at 405 nm, while a larger decrease was seen at 488 nm. When the 340 nm excitation was used, much larger changes were seen at 405 nm, and slightly smaller changes were seen at 488 nm. The clearer divergence of signals helps to sustain confidence that the changes really are due to changes in $[Ca^{2+}]_i$.

A number of problems arose when Indo-1 was used to measure $[Ca^{2+}]_i$. Most particularly, the excitation and emission wavelengths at which Indo-1 is used overlap considerably with those of NAD(P)H. The autofluorescence became a progressively more significant fraction of the total signal collected when larger clusters of cells were studied, probably because the loading with Indo-1 became less efficient in cells within such clusters (very approximately > than ten to fifteen cells). This has two consequences.

Firstly, it means that the effective background signal subtracted from the Indo-1 signals was underestimated, as the real background included the NAD(P)H fluorescence. This leads to an underestimate of the true 405 nm/488 nm ratio when $[Ca^{2+}]_i$ is high. Most of the manipulations performed in these experiments also change autofluorescence as well as $[Ca^{2+}]_i$. Thus errors are introduced into the calibration. For this reason, only small clusters of cells or single cells were used, for which the relative size of the signal for NADH fluorescence under these recording conditions is very small (usually < 5–10%). The observations that cyanide (which increases it) and FCCP (which decreases it) cause similar apparent changes in $[Ca^{2+}]_i$ indicate that the error cannot be large (see also Biscoe & Duchen, 1990*b*, fig. 1).

Secondly, a further problem that arose with Indo-1 was that its relatively high affinity (K_D about 120 nM) impairs resolution at higher levels of $[Ca^{2+}]_i$, as the signal at 488 nm becomes small and the ratio therefore becomes noisy. For some of the experiments described below, it was hard to be certain whether or not $[Ca^{2+}]_i$ was changing further after an initial rise.

Fluo-3. To solve these problems, we have also used the dye Fluo-3 which has a lower affinity for Ca^{2+} (K_D about 450 nM). The longer wavelengths used for Fluo-3 (excitation at 488 nm, emission at 530 nm) have the advantage that there is no significant recordable autofluorescence under these conditions. They were also useful for other reasons as described in the Results. The disadvantage is that artifactual changes in fluorophore concentration cannot be eliminated by taking the ratio at two wavelengths as for Indo-1. Furthermore, the single-wavelength emission makes calibration difficult. Calibration requires establishing minimum and maximum levels of $[Ca^{2+}]_i$ (i.e. of fluorescence) for each cell studied, which would involve exposing the whole bath to Ca^{2+}-free salines, a manoevure that tends to damage the remaining cells (see Biscoe & Duchen, 1990*b*). As the yield of cells from the carotid body is small, we have not attempted to calibrate these signals, and present the raw fluorescence traces.

Some experiments were performed to examine fluorescence spectra in cell-free solutions. For these, a Perkin–Elmer fluorimeter was used.

Fluorescence signals. These were digitized and stored on videotape (PCM-8, Medical Instruments). They were then re-sampled using a Labmaster A–D interface with a microcomputer and percentages, calibrations etc. calculated using spreadsheets. Curve-fitting routines supplied in pClamp (Axon Instruments) were used to fit exponentials to traces.

Drugs. These were occasionally applied in the superfusate, but more usually by local application on ejection under pressure from a patch pipette (diameter 2–4 μm) placed nearby in the continuously flowing superfusate. This prevented exposure of other cells in the bath to high concentrations. Drugs used included: NaCN (2 mM); carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 1–5 μM); oligomycin (0·25–2·5 μg/ml mixed A, B and C); rotenone (1 μM); tetramethyl-*p*-phenylene diamine (TMPD, 20–40 μg/ml) with 1–5 mM-sodium ascorbate; iodoacetic acid, 1 mM; atractyloside, 100 μM (all from Sigma). Bongkrekate was a generous gift from Dr Martin Crompton, and was used at a final concentration 10 μM.

All other details are found in previous papers (Biscoe & Duchen, 1990*b*; Duchen & Biscoe, 1992).

RESULTS

The results shown are based upon experiments carried out on 148 cells or groups of cells derived from fourteen rabbits.

Monitoring $\Delta\psi_m$ with Rhodamine 123

Rhodamine 123 (Rh 123) is taken up selectively by mitochondria (Johnson, Walsh & Chen, 1980; Johnson, Walsh, Bockus & Chen, 1981; Chen, 1988), and the uptake is dependent on the mitochondrial membrane potential ($\Delta\psi_m$). Its fluorescence is then

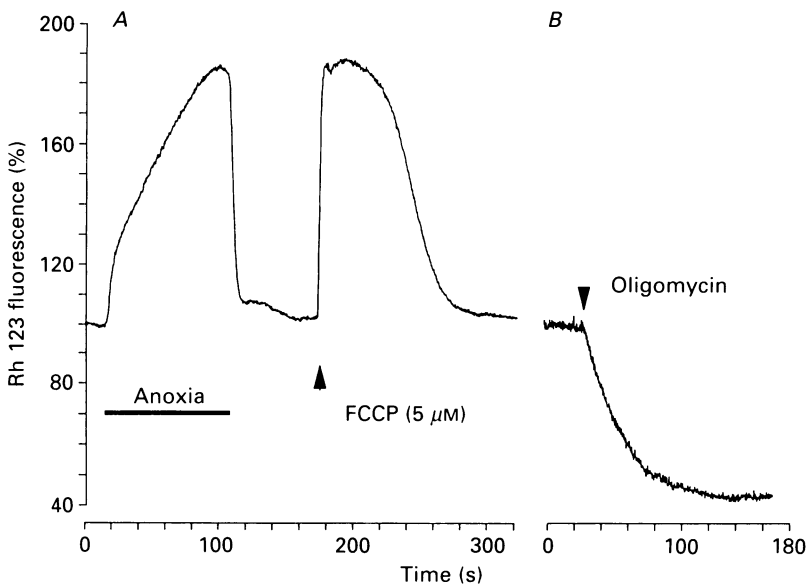

Fig. 1. Rh 123 fluorescence recorded as a percentage change from the resting level and plotted against time in seconds. *A*, the superfusate was switched from the control to an anoxic saline, as indicated. The fluorescence signal showed a biphasic increase (see text). The signal recovered rapidly on reoxygenation, showing a secondary slow component (see text). Brief application of 5 μM-FCCP then produced a rapid increase in signal, followed by a slow recovery. *B*, another cell, in which brief application of oligomycin led to a decrease in signal of about 40% from control. This response was irreversible.

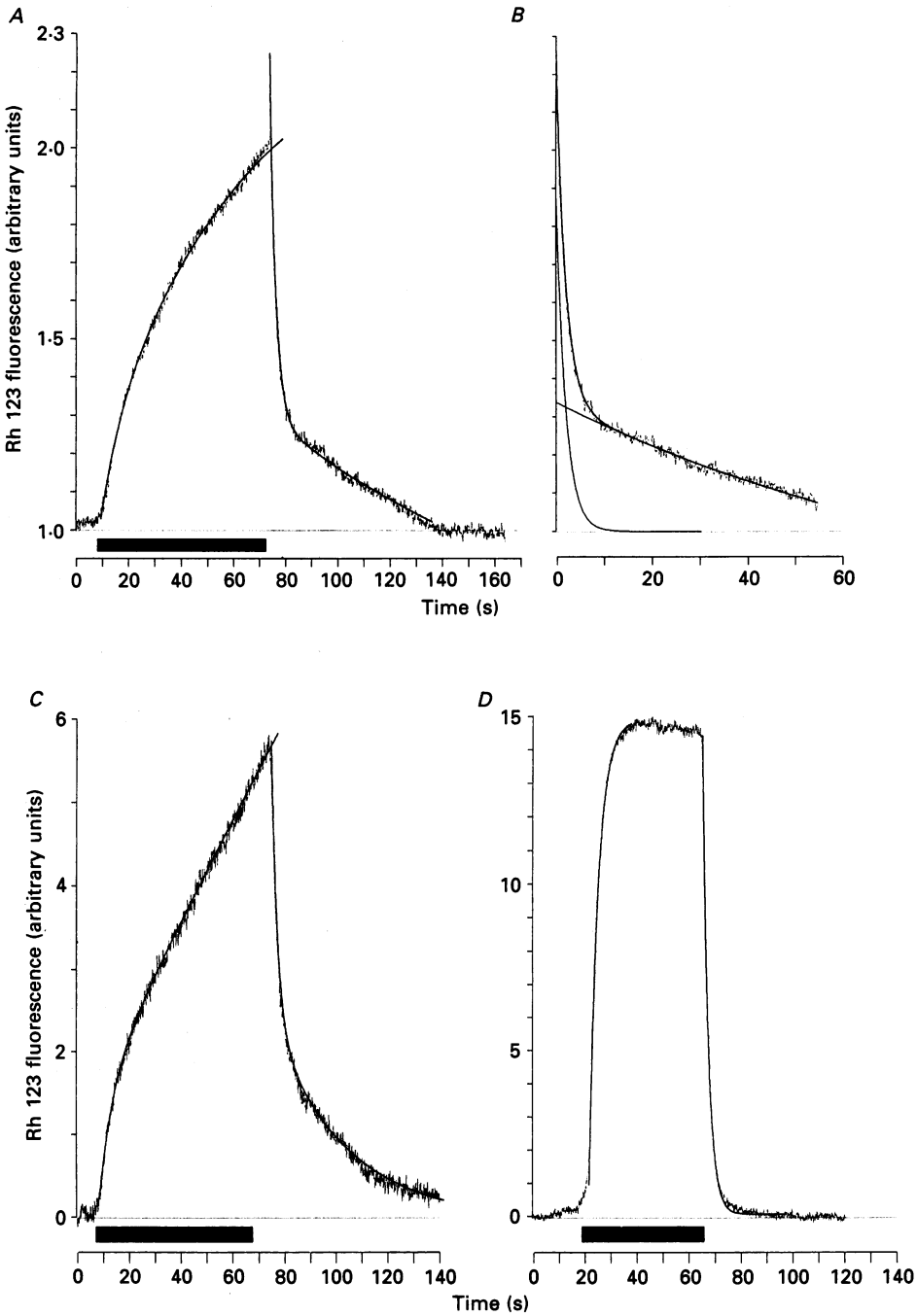

Fig. 2. Rh 123 fluorescence is plotted against time. Changes are shown in response to a period of anoxia as indicated by the bar. Both the onset and offset of the responses were fitted by the sum of two exponentials (continuous traces). The time constants for the rising phase of the response in *A* were 2·7 and 55·5 s, and for the recovery phase 1·98 and 99·56 s. *B* shows the graphical analysis of the relative contributions of the two components for the falling phase. The records in *C* and *D* show data from another cell before (*C*) and after (*D*) application of 2·5 μg/ml oligomycin. The time constants for the

quenched by $\Delta\psi_m$ (Emaus *et al.* 1986; Mokhova & Rozovskaya, 1986; Duchen *et al.* 1990) and so increases with mitochondrial depolarization. In isolated mitochondria, the relationship between fluorescence and $\Delta\psi_m$ is linear (Emaus *et al.* 1986). After the cells were exposed to Rh 123 and then washed, punctate fluorescence excluded from the nucleus could be clearly seen. Rh 123 has not been used very widely for continuous assessment of $\Delta\psi_m$; however, the responses of the fluorescence signal have invariably conformed to those expected on theoretical grounds and data included here further substantiate the use of Rh 123 for this purpose.

Blockade of electron transport by anoxia, CN^- or rotenone, and shunting of the mitochondrial membrane by the proton ionophore uncoupler carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), are all expected to depolarize $\Delta\psi_m$. Oligomycin blocks mitochondrial ATP synthesis by blocking the proton conductance of the F_0 subunit of the F_1–F_0 ATP synthetase in the inner mitochondrial membrane. It may therefore hyperpolarize $\Delta\psi_m$ when the F_0 proton channel represents a significant conductance dissipating $\Delta\psi_m$ at rest.

Fluorescence intensity monitored at 530 nm changed according to these predictions. There was an increase in response to an anoxic superfusate (Figs 1, 2*A* & *C*, 3, 7, 8 and 10), to CN^- (Fig. 10), to rotenone (Fig. 9) and to application of FCCP (Figs 1 and 7*B*), and a decrease in response to oligomycin (Figs 1 and 6). FCCP caused the signal to increase about twofold, while as expected responses to anoxia and to CN^- were rather more variable in amplitude, and rate of onset and offset. The effects of most agents were reversible, as the cells were continuously washed, but the effects of oligomycin on $\Delta\psi_m$, autofluorescence and $[Ca^{2+}]_i$ all proved irreversible.

Anoxia and $\Delta\psi_m$

In response to anoxia, Rh 123 fluorescence increased in two phases (Figs 1 and 2): an initial rapid phase, followed by a slower increase to a peak. Repolarization on reoxygenation was also biphasic, consisting of a rapid recovery followed by a slower decline to control levels as seen in Figs 1, 2, 3, 7 and 10. This feature of the response was seen also with CN^- (Fig. 10) but not obviously with *hypoxia* (Fig. 3) and was quantitatively variable amongst the population of cells studied ($n = 67$).

Both the onset and offset of the responses to anoxia were well fitted (correlation coefficient $r > 9{\cdot}98$) by the sum of two exponential processes, as shown in Fig. 2. The time constants for the offset of the response to anoxia were $2{\cdot}37 \pm 0{\cdot}4$ and $49{\cdot}4 \pm 26$ s (mean $\pm$ S.D., $n = 6$) (the rates for the onset were more variable). Most strikingly, prior exposure of the cells to oligomycin invariably abolished the slower components of the response (see Fig. 2*C* and *D*), apparently leaving the faster components unchanged (mean time constant for offset of $2{\cdot}27 \pm 0{\cdot}13$ s, $n = 5$).

Even at the lowest concentration that we tested (0·25 μg/ml) oligomycin caused an irreversible decrease in resting Rh 123 fluorescence of 20–40% (see Fig. 1), representing a hyperpolarization of $\Delta\psi_m$, and changed the rates of onset and offset of the response to anoxia ($n = 15$).

These effects of oligomycin by itself, and on the shape of the response to anoxia,

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onset in *C* were 5·28 and 73·7 s, and for the offset they were 2·2 and 25·4 s. After oligomycin (*D*) both the onset and offset were well fitted by *single* exponentials with time constants of 3·4 s (onset) and 2·24 s (offset). These signals are all shown in arbitrary fluorescence scales.

suggest that (i) under resting, normoxic conditions, a proton current through the F_0 channel dissipates $\Delta\psi_m$ significantly, hence the hyperpolarization; and that (ii) activity of the ATP synthetase limits the rate of mitochondrial depolarization with anoxia and contributes to the slower phases of both the depolarizing and repolarizing responses. Thus, during anoxia, the ATP synthetase is driven backwards, consuming

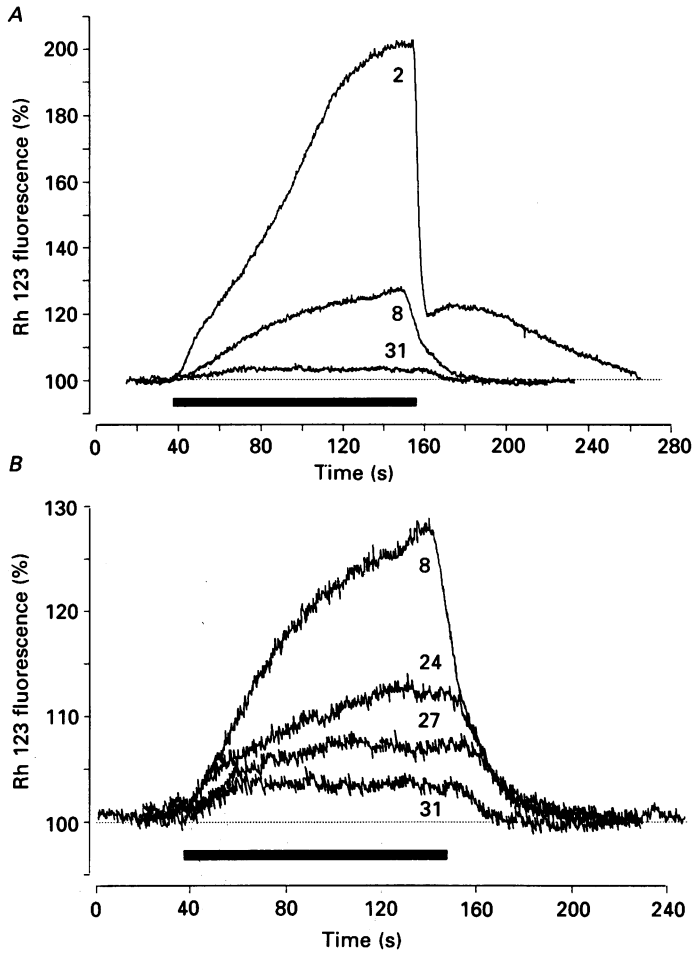

Fig. 3. A family of responses of Rh 123 fluorescence to superfusion with a range of P_{O_2} values (*A*) shown at a higher gain in *B*. The minimum P_{O_2} (in mmHg) achieved during the superfusion for the indicated period is shown next to the appropriate trace.

(glycolytic) ATP to extrude protons and maintain $\Delta\psi_m$. On reoxygenation, intramitochondrial ADP which accumulated during the anoxic period would increase the forward rate of the synthetase, depolarizing $\Delta\psi_m$.

The particular significance of these observations in the present context is (i) that we can be reasonably confident that these concentrations of oligomycin block the activity of the F_0–F_1 complex in both directions, i.e. ATP synthesis as well as ATP consumption; (ii) that they suggest that anoxia for these periods of time is accompanied by a significant change in the intramitochondrial phosphorylation

potential; and (iii) that they show the Rh 123 signal behaves as expected on theoretical grounds.

Hypoxia and graded changes in P_{O_2}

The major feature of the transducer is its ability to respond to graded changes in P_{O_2} with graded changes in output to yield an essentially hyperbolic, and monotonic,

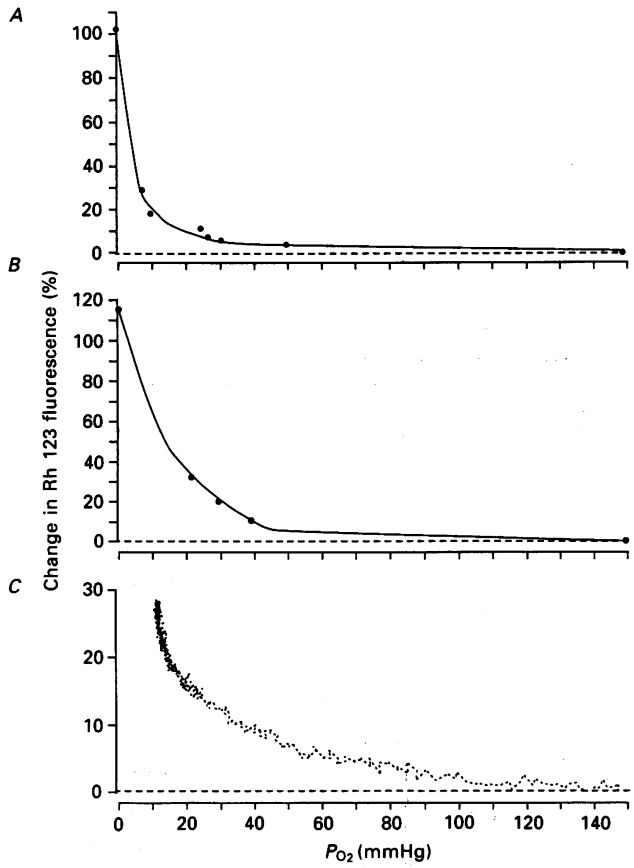

Fig. 4. Stimulus–response curves for three different cell groups. In *A* and *B*, the steady-state maximal increase in Rh 123 fluorescence is plotted against the minimum P_{O_2} achieved during superfusion. The trace in *C* shows a continuous stimulus–response curve, as the Rh 123 fluorescence is plotted directly against the trace from a P_{O_2} electrode placed close to the cells.

stimulus–response curve. We have shown that graded increases in $[Ca^{2+}]_i$ and NAD(P)H follow systematically graded decreases in P_{O_2} (Biscoe & Duchen, 1990*b*; Duchen & Biscoe, 1992) in the physiologically important range. Similarly Rh 123 fluorescence (and thus $\Delta\psi_m$) was also graded with P_{O_2}, as shown in Figs 3–5. For one experiment the traces obtained in response to superfusion with salines variably equilibrated with N_2 are superimposed in Fig. 3. The increase in fluorescence is progressive through the period of exposure as the fluid around the cells equilibrates to the new P_{O_2}.

The maximal fluorescence at a steady state was plotted against the minimum P_{O_2} achieved during a period of 60–100 s superfusion with a hypoxic superfusate ($n = 21$ groups of cells). Stimulus–response curves such as those shown in Fig. 4*A* and *B* were obtained. Given the difficulty in obtaining absolutely reproducible values for the

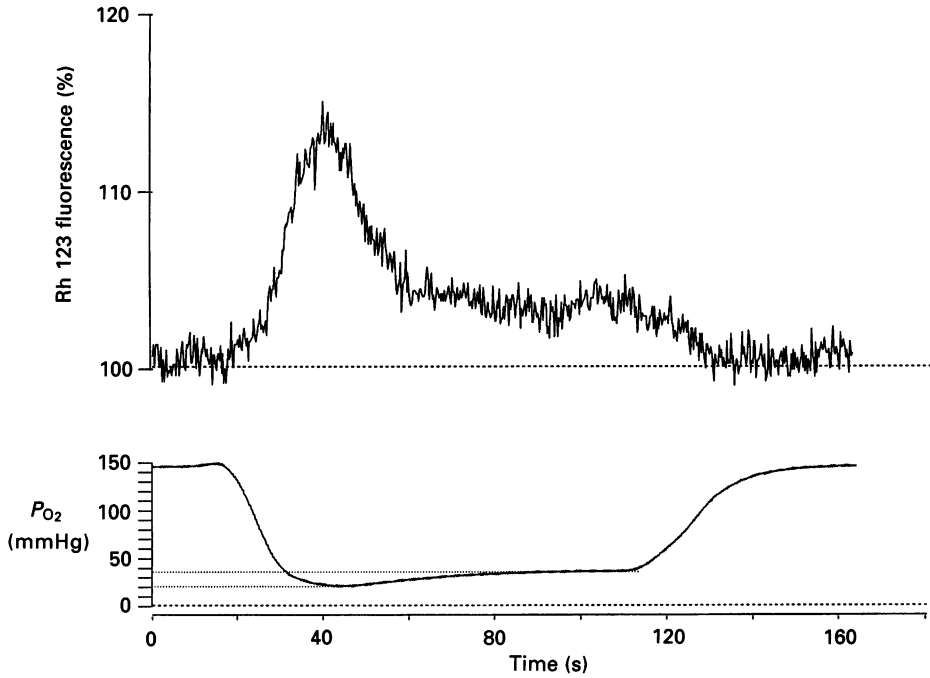

Fig. 5. The temporal sensitivity of the Rh 123 fluorescence with changing P_{O_2}. The P_{O_2} of the superfusate was varied by switching between reservoirs; lower trace from a P_{O_2} electrode in the bath. The P_{O_2} fell first to about 20 mmHg, and then rose steadily to about 40 mmHg. The electrode defines the limits of P_{O_2} and not the precise kinetics of the change (see text).

independent variable, P_{O_2}, it is not possible to plot a mean curve. Accordingly these curves are analogous to single-fibre recordings rather than the integrated whole-nerve response. The curve in Fig. 4*C* shows a continuous plot of Rh 123 fluorescence against the recording of P_{O_2} from an oxygen electrode (compare with Fig. 8, below). There is a striking parallel between these curves and the stimulus–response curves for $[Ca^{2+}]_i$ *vs.* P_{O_2} (Biscoe & Duchen, 1990*b*), NAD(P)H autofluorescence *vs.* P_{O_2} (Duchen & Biscoe, 1992), or of afferent action potential frequency *vs.* P_{O_2} (Biscoe, Purves & Sampson, 1970).

The rate at which $\Delta\psi_m$ can change with changing P_{O_2} is shown in part by the response seen in Fig. 5. In this experiment, the superfusate was switched between reservoirs equilibrated to different values of P_{O_2}, which fell initially from 150 to about 20 mmHg and then returned back to near 40 mmHg. Comparable changes were seen with NAD(P)H fluorescence using a similar protocol (Duchen & Biscoe, 1992, Fig. 3). The electrode serves essentially to define the limits of P_{O_2} and could not define the kinetics of the change precisely since it is usually too far away from the cells studied

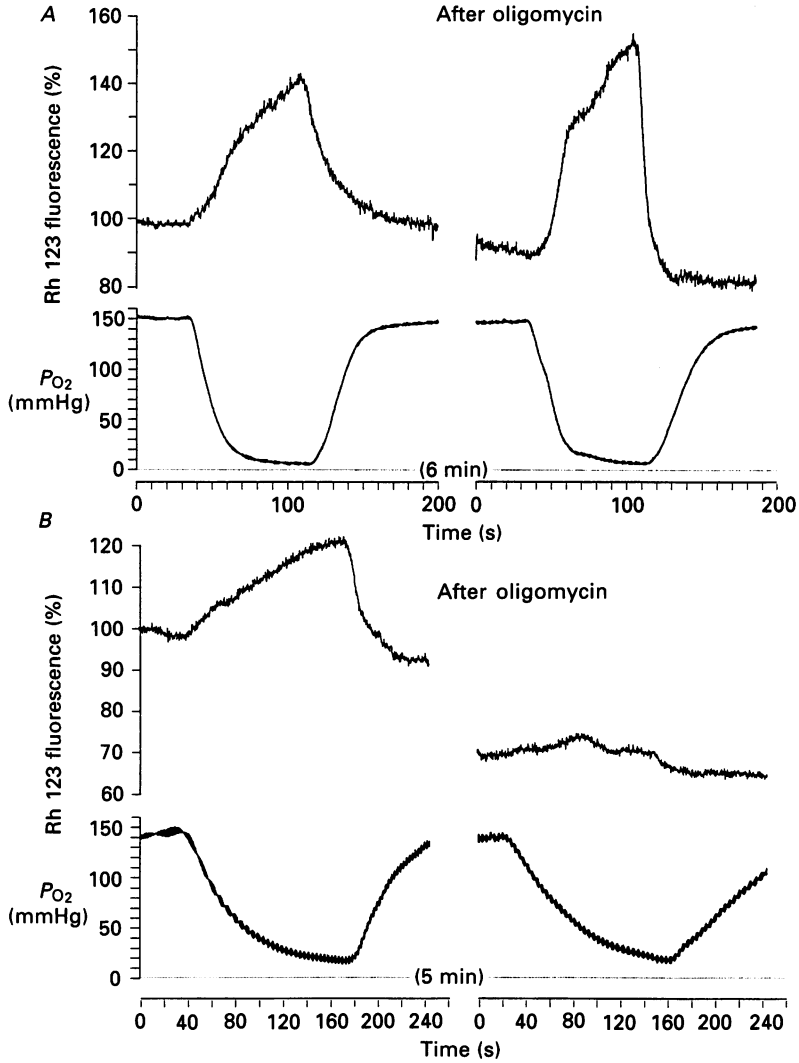

Fig. 6. Changes in Rh 123 fluorescence responses to a hypoxic superfusate (minimum P_{O_2} *ca* 8–10 mmHg) before and after oligomycin. The records in *A* and *B* show the contrasting effects of oligomycin on the response seen in two different clusters of cells. In those illustrated in *A*, the response became faster in onset and offset and was slightly exaggerated, while in *B* the response was apparently suppressed, and was hard to detect after oligomycin.

(> 200 μm). We often noted that the fluorescence signals from the cells acted as a more effective oxygen sensor than the oxygen electrode.

Hypoxia and the effect of oligomycin on $\Delta\psi_m$

As the rate of depolarization of $\Delta\psi_m$ with slowing of electron transport is limited by the reverse action of the ATP synthetase, we wondered whether graded changes to hypoxia might be more pronounced after exposure to oligomycin. An increase in

the rate of depolarization and therefore an increase in the size of responses to hypoxia was seen in two cells (Fig. 6*A*), but in most cells ($n = 9$), to our surprise, responses to hypoxia were significantly reduced, as though the sensitivity to oxygen was reduced (Fig. 6*B*), so apparently giving rise to a much steeper stimulus–response curve.

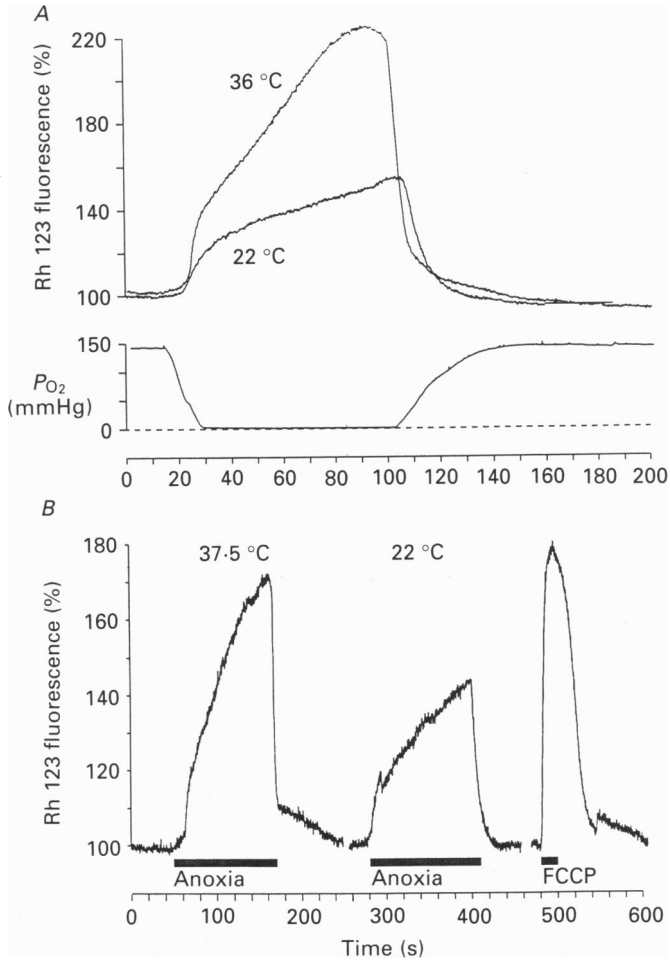

Fig. 7. The rate of change of the Rh 123 fluorescence with anoxia was temperature dependent. In *A*, the two records above at different temperatures are of responses to an anoxic superfusate shown by the P_{O_2} electrode trace below. All components of the response were quicker at 36 than at 22 °C. *B*, records from another cell along with a response to FCCP at 22 °C, confirming that the change in signal is not due to the temperature dependence of the Rh 123 fluorescence itself. Note also that the secondary, slower phase of recovery on reoxygenation is absent in the trace at 22 °C, compared to 37·5 °C.

Temperature sensitivity of the Rh 123 response to anoxia

We have previously shown that the rate of rise $[Ca^{2+}]_i$ with anoxia (and the apparent sensitivity to hypoxia) is very strongly temperature sensitive, increasing between four and eightfold with an increase in temperature from 25 to 35 °C (see

Biscoe & Duchen, 1990*b*, Figs 3 and 4). The rate of depolarization of $\Delta\psi_m$ with anoxia showed a similar temperature sensitivity ($n = 11$) as illustrated in Fig. 7, increasing up to fivefold over the temperature range 22 to 36 °C. That this was not due to a change in the behaviour of the Rh 123 with warming was confirmed by the

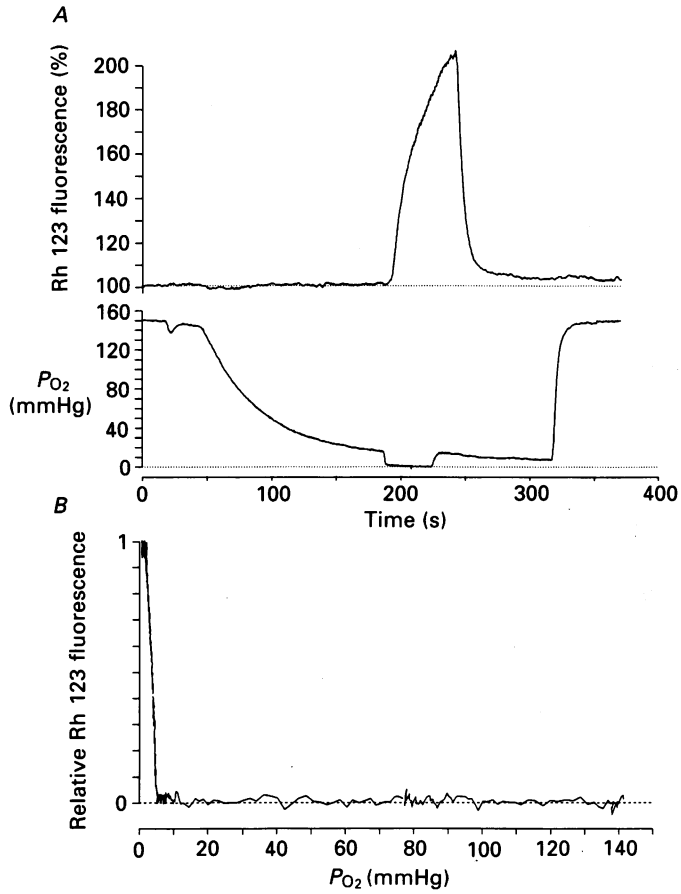

Fig. 8. *A*, Rh 123 fluorescence measured from a freshly isolated mouse sensory ganglion neuron and, below, tracing from a P_{O_2} electrode. The superfusate was switched first to a hypoxic saline, initiating a very slow fall to a P_{O_2} of about 17 mmHg with no change in Rh 123 fluorescence. Switching to an anoxic saline then initiated a typical response. The superfusate was then switched back to the hypoxic saline, and recovered, despite the continued fall of the P_{O_2} to about 6 mmHg. *B* shows a continuous plot of Rh 123 fluorescence as a function of P_{O_2} obtained from a freshly dissociated rat chromaffin cell. There was no measurable change in signal until the P_{O_2} fell below about 5 mmHg.

lack of a significant effect of a change in temperature on the response to FCCP. In response to FCCP, at a mean temperature of 21 °C, the signal changed by 79±16% (mean±S.D., $n = 6$); at 30 °C by 80±15% ($n = 6$); and at 36 °C by 90±16% ($n = 9$).

Comparison with other cell types

More generally, in adrenal chromaffin cells and in mouse sensory neurons $[Ca^{2+}]_i$, NAD fluorescence and $\Delta\psi_m$ all remain unaltered with hypoxia until the P_{O_2} is close

to zero. In Fig. 8, an example of the signal obtained from a sensory neuron is shown. As the P_{O_2} fell to about 17 mmHg, the signal remained flat, depolarizing only when the superfusate was switched to anoxia. The superfusate was switched back to a hypoxic saline, and the P_{O_2} continued to fall, but $\Delta\psi_m$ recovered even at this

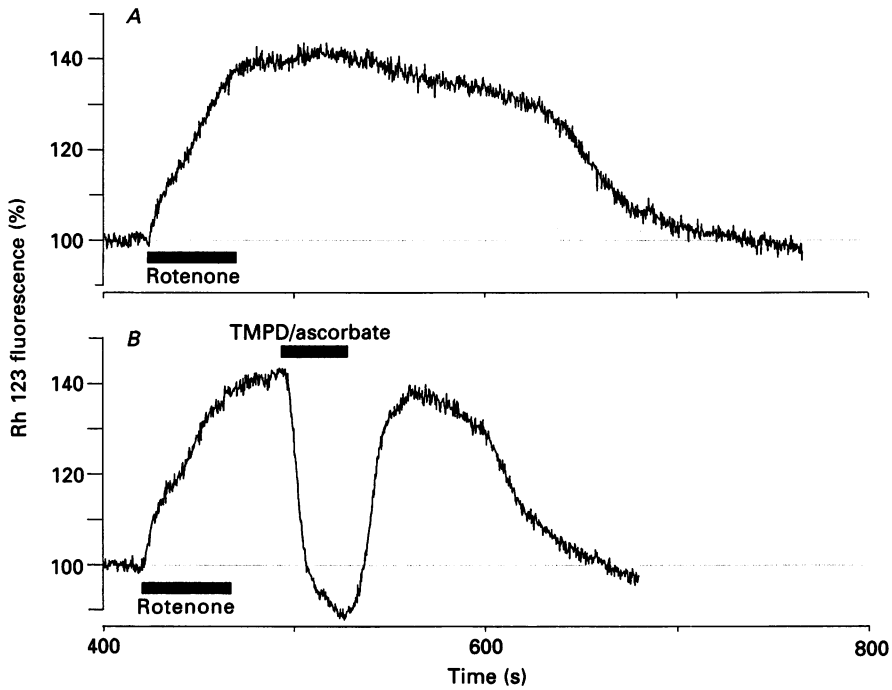

Fig. 9. Rh 123 fluorescence changes in response to rotenone, applied for the period indicated by the continuous bar. *A*, rotenone typically caused a prolonged depolarization of $\Delta\psi_m$, eventually recovering after 3–4 min. The upper and lower traces are a continuous record. A second application of rotenone (*B*) initiated a similar response. In this case, however, once the response was established, the superfusate was switched to a saline containing TMPD (20 μg/ml) and 1 mM-ascorbate, which rapidly reversed the effect of rotenone to a level about 10% below baseline. Wash-out of the TMPD allowed the rotenone effect to be restored until the response recovered.

sustained low P_{O_2}. In ten sensory neurons and twelve chromaffin cells, no change in Rh 123 fluorescence was measurable until the P_{O_2} fell below about 5 mmHg. This is further illustrated by Fig. 8*B*, which shows a continuous plot of Rh 123 fluorescence as a function of P_{O_2} from an adrenal chromaffin cell (compare with Fig. 4*C*).

Indeed, there is no reason to expect a response of mitochondrial electron transport (and therefore $\Delta\psi_m$) to hypoxia until the P_{O_2} reaches levels below the effective K_D for oxidation of cytochrome *aa*3, the terminal component of the electron transport sequence. In most tissues the value for this K_D is less than 1 mmHg (see Discussion).

$[Ca^{2+}]_i$ *and* $\Delta\psi_m$

We have previously suggested that depolarization of $\Delta\psi_m$ might promote Ca^{2+} efflux from mitochondria. A close relationship between $\Delta\psi_m$ and $[Ca^{2+}]_i$ is indicated by experiments in which $\Delta\psi_m$ has been manipulated by using the protocols

illustrated in Fig. 9. Rotenone, which induces a prolonged block of electron transport at complex I, depolarized $\Delta\psi_m$. The block could then be bypassed by using TMPD to reduce cytochrome *c* directly, making it available again for oxidation and supporting the activity of the electron transport chain, despite the continued block

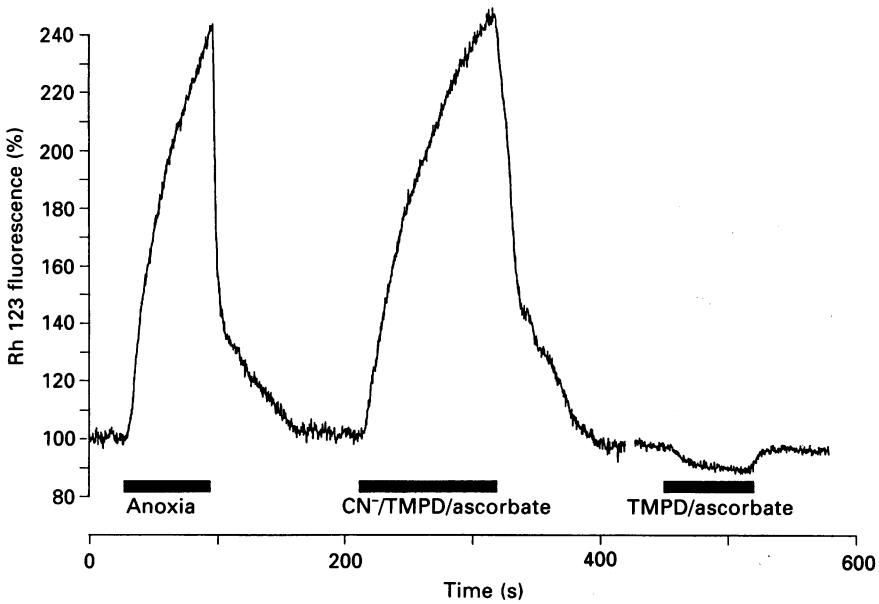

Fig. 10. These records show that the reduction of signal by TMPD is not due to quenching of fluorescence and that the effect is indeed consistent with the proposed mechanism, i.e. direct reduction of cytochrome *c* oxidase by the TMPD. There is a response to anoxia, followed by a response to 2 mM-CN^- in the presence of TMPD and ascorbate. Finally, TMPD and ascorbate applied alone produced a small reduction in the signal.

by rotenone (see Dutton, Wilson & Lee, 1970; Wilson, Rumsey, Green & Vanderkooi, 1983).

Rotenone, TMPD and $\Delta\psi_m$

Figure 9*A* shows the depolarization of $\Delta\psi_m$ in a cluster of type I cells after brief exposure to rotenone ($n = 7$). The response was usually prolonged taking several minutes before eventual recovery.

Depolarization of $\Delta\psi_m$ was reversed ($n = 7$), sometimes to a potential greater than the initial resting level, by exposure to 20 μg/ml TMPD with 1 mM-sodium ascorbate (required to re-reduce the TMPD) (Fig. 9*B*). On switching back to the control superfusate $\Delta\psi_m$ depolarized again while the response to rotenone persisted.

That the change in signal was not due to quenching of Rh 123 fluorescence by the TMPD was demonstrated separately in two experiments. Firstly, CN^- in the presence of TMPD and ascorbate together caused a depolarization of $\Delta\psi_m$ similar to the response to anoxia while TMPD and ascorbate alone caused only a smll hyperpolarization (Fig. 10).

Secondly, any possible interaction between the Rh 123 and TMPD was excluded

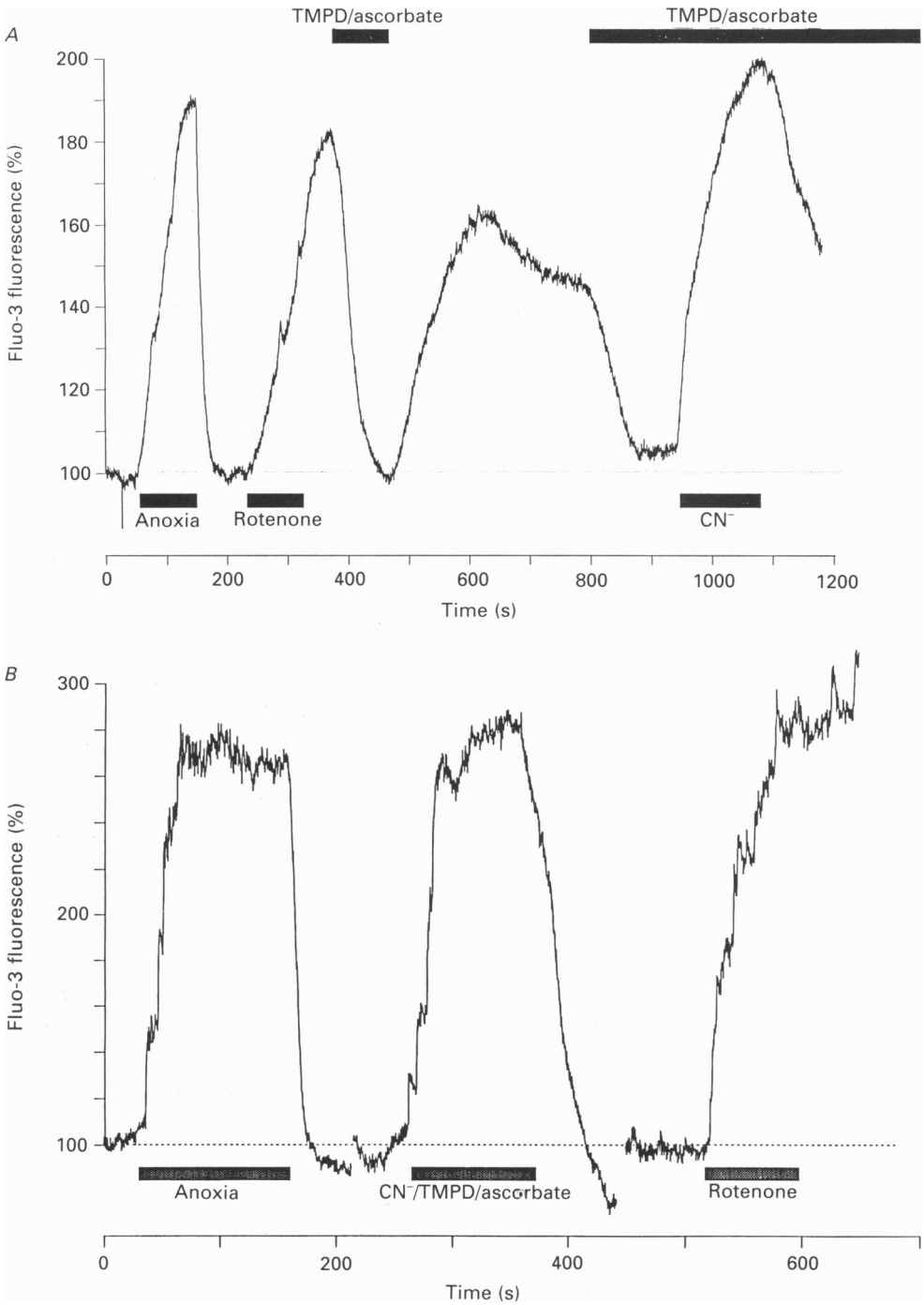

Fig. 11. $[Ca^{2+}]_i$ follows the changes expected of $\Delta\psi_m$. Fluo-3 was used as a $[Ca^{2+}]_i$ indicator, as TMPD alone initiated fluorescence changes at the near-UV wavelengths used for Indo-1. The signals have not been calibrated in terms of $[Ca^{2+}]_i$ but are shown simply as percentage changes in fluorescence. In *A*, the experiment was similar to that of Fig. 9 and shows first a response to anoxia which raised $[Ca^{2+}]_i$; rotenone raised $[Ca^{2+}]_i$ to a

by measuring Rh 123 spectra with and without addition of TMPD and ascorbate in a cell-free solution. These results are all appropriate to the known action of the TMPD, as CN^- blocks electron transfer beyond the site of action of the TMPD.

Rotenone and $[Ca^{2+}]_i$

Having established the efficacy of the manipulation of $\Delta\psi_m$ with these agents, we then examined the effects of rotenone and TMPD on $[Ca^{2+}]_i$. With the calcium fluorophore Indo-1, we found that TMPD induced artifactual changes in the fluorescence signal measured at 405 nm, without changing the signal at 488 nm significantly. These responses were unaltered by removing Ca^{2+} from the superfusate. The changes were not seen with TMPD and Indo-1 alone in a cuvette but were seen with cells not loaded with Indo-1 under equivalent conditions. So the changes appeared to reflect an interaction between the TMPD and some component of the cell. As the oxidized form of the TMPD is blue (Wurster's Blue), it is possible that under these conditions it interferes with the fluorescence signals through energy transfer or absorbance. These experiments were therefore repeated using Fluo-3 to monitor $[Ca^{2+}]_i$, in the hope that the longer wavelengths required for Fluo-3 excitation (see Methods) would avoid this artifact.

When Fluo-3 was used, $[Ca^{2+}]_i$ changed in response to this sequence of manipulations similarly to $\Delta\psi_m$. These responses are illustrated in Fig. 11, which shows the signals recorded with Fluo-3 in response to anoxia, rotenone and CN^- in the presence of TMPD and ascorbate.

Anoxia and rotenone both raised $[Ca^{2+}]_i$ ($n = 17$) as expected (Fig. 11*A* and *B*). The response to rotenone was typically long lasting, resembling the Rh 123 signal in response to this agent. Again resembling the Rh 123 signal, the increase in $[Ca^{2+}]_i$ following rotenone could be reversed by the concurrent administration of TMPD with ascorbate ($n = 5$) (Fig. 11*A*). The rise in $[Ca^{2+}]_i$ following CN^- was unaffected by the presence of TMPD/ascorbate, ($n = 4$) (Fig. 11*A* and *B*), confirming the mechanism of action of the TMPD and again demonstrating that the reduction in signal by TMPD is not due to quenching of fluorescence.

A further instructive comparison was found in the time course of recovery of $\Delta\psi_m$ and $[Ca^{2+}]_i$ from exposure to anoxia or CN^-. There was a striking similarity between the time course of recovery from each agent for each variable (Fig. 12). The recovery from CN^- was invariably slower than recovery from anoxia, presumably due to the slower diffusion rates across the cell membrane for CN^- than for oxygen.

These results together place a series of events that follow changes in P_{O_2} in the mitochondrion so that as P_{O_2} falls $\Delta\psi_m$ is depolarized, mitochondrial redox state becomes reduced, and somehow $[Ca^{2+}]_i$ rises.

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comparable degree. The response was reversed for the duration of the *ca* 100 s application of TMPD/ascorbate. Subsequent re-application of TMPD/ascorbate again lowered the $[Ca^{2+}]_i$ to the resting level. Concurrent application of CN^- raised $[Ca^{2+}]_i$ to a similar degree as anoxia, and the presence of TMPD made no difference to the response. In *B*, a response to anoxia was followed by application of cyanide (2 mM) mixed with TMPD and ascorbate. Both stimuli raised $[Ca^{2+}]_i$ to a similar level. Rotenone provoked a sustained rise in $[Ca^{2+}]_i$ which recovered after 5 min.

Consequences of a rise in $[Ca^{2+}]_i$

These data suggest that depolarization of mitochondrial membrane potential may be a crucial event that triggers all subsequent changes in cell biology when the P_{O_2} falls. To ensure that the changes we have documented are primary responses to

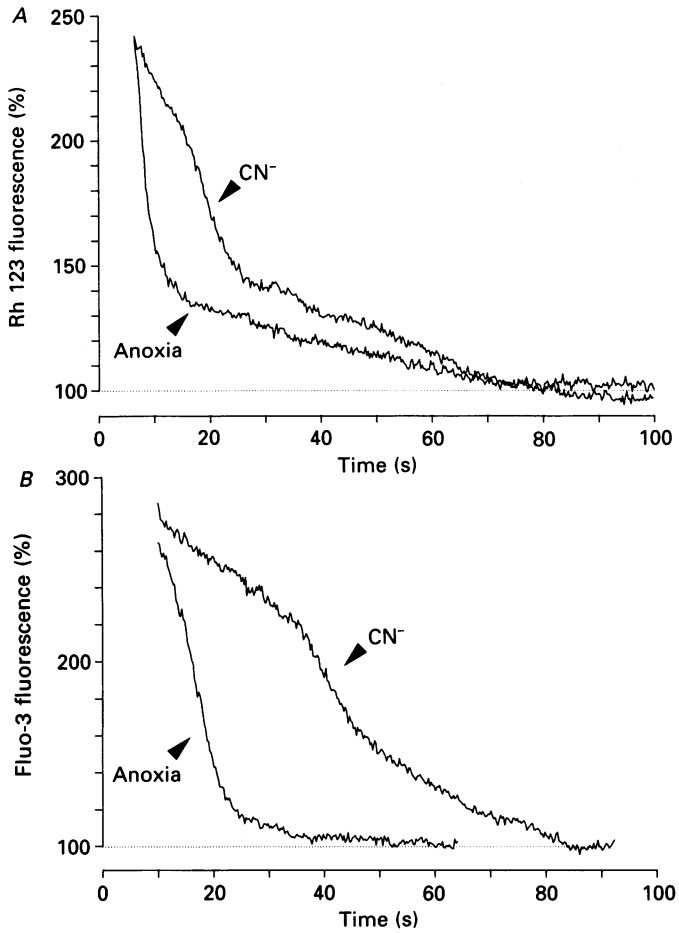

Fig. 12. The time course of recovery from anoxia and cyanide (CN^-) of $\Delta\psi_m$ shown by Rh 123 fluorescence (*A*) with that of $[Ca^{2+}]_i$ as indicated by Fluo-3 fluorescence (*B*). Note that recovery of both variables from CN^- was always slower than recovery from anoxia, presumably because of the slower diffusion of CN^- out of the cell compared to that of O_2 into the cell.

hypoxia, and do not reflect a secondary response to a rise in $[Ca^{2+}]_i$ originating through some other mechanism, we have examined the consequence of raised $[Ca^{2+}]_i$ on the Rh 123 signal. This is particularly pertinent as others have suggested alternative mechanisms for the rise in $[Ca^{2+}]_i$, proposing that hypoxia depolarizes the type I cells and raises $[Ca^{2+}]_i$ by promoting voltage-gated Ca^{2+} influx (López-López *et al.* 1989; Delpiano & Hescheler, 1989).

A rise in $[Ca^{2+}]_i$, where the calcium originates from a non-mitochondrial source, may have various effects on $\Delta\psi_m$. If such a rise is sufficient to change matrix calcium, this could increase the activities of NAD-linked dehydrogenases that are key regulatory enzymes in oxidative metabolism (McCormack & Denton, 1984; see review by Hansford, 1985). This would increase the net reduction of NADH, possibly promoting the activity of the electron transport chain leading to a rise in (hyperpolarization of) $\Delta\psi_m$.

However, a rise in $[Ca^{2+}]_i$ may have at least two other effects: (i) to promote the activity of Ca^{2+}-dependent ATPases, reducing the cytoplasmic ATP/ADP.P_i ratio and thus to depolarize $\Delta\psi_m$, promoting ATP synthesis; and (ii) to depolarize $\Delta\psi_m$ more directly by increasing Ca^{2+} cycling across the inner mitochondrial membrane, which dissipates $\Delta\psi_m$.

Because the effect on $\Delta\psi_m$ is difficult to predict we investigated the consequence for $\Delta\psi_m$ of the rise in $[Ca^{2+}]_i$ following K^+-induced depolarization. This raises $[Ca^{2+}]_i$ through voltage-gated calcium entry as much or more than does anoxia ($n = 14$), and certainly more than intermediate levels of hypoxia (Biscoe & Duchen, 1990*b*).

Following plasma membrane depolarization by brief (150–200 ms) application of 50 mM-K^+ (replaced isotonically for Na^+), Rh 123 fluorescence increased by about 8%. Figure 13*A* shows responses from a small cluster of cells first to K^+-induced depolarization and then to hypoxia (P_{O_2} of about 8 mmHg). The response from another cell illustrated in Fig. 13*B* shows, on an expanded time base, the largest response to K^+ that we have seen.

The change in Rh 123 fluorescence in response to K^+-induced depolarization was completely abolished if Ca^{2+} was excluded from the superfusate, confirming that this response results from $[Ca^{2+}]_i$ influx, while the response to hypoxia was largely independent of $[Ca^{2+}]_o$.

These responses are a feature of other excitable cells where the signals may be larger and are therefore easier to study systematically (Duchen, 1991), and such data will be discussed elsewhere.

Mechanisms for the rise in $[Ca^{2+}]_i$

The data presented above and in Duchen & Biscoe (1992) suggest strongly that mitochondria of type I cells are specially sensitive to changes in P_{O_2}, and that they have properties which could underpin transduction. Together with our previous data, the sequence of events following a fall in P_{O_2} would involve a change in mitochondrial electron transport. This would then initiate a rise in $[Ca^{2+}]_i$, which in turn would promote secretion of a neurotransmitter excitatory to the nerve terminals that synapse with the type I cells. The question then arises as to how the connection between depolarization of $\Delta\psi_m$ and a rise in $[Ca^{2+}]_i$ is established.

There are two obvious mechanisms that would raise $[Ca^{2+}]_i$ with a fall in $\Delta\psi_m$.

First, mitochondrial depolarization would inevitably lead directly to the release of mitochondrial Ca^{2+} since Ca^{2+} is maintained within the mitochondrial matrix by the large electrochemical potential (Rottenberg & Scarpa, 1974; see Discussion, Biscoe & Duchen, 1990*a*, *b*). For this mechanism to be effective requires that concentrations of Ca^{2+} within the matrix are sufficient to raise $[Ca^{2+}]_i$ in the cytosol following a fall in $\Delta\psi_m$ (see Discussion below).

Second, ATP synthesis has a very steep dependence on $\Delta\psi_m$ (see for example Pietrobon, Azzone & Walz, 1981; Pietrobon & Azzone, 1982). Thus, the loss of proton motive force needed for the synthesis of ATP will lead to a decline in the rate of oxidative phosphorylation, without altering ATP utilization. The consequences for

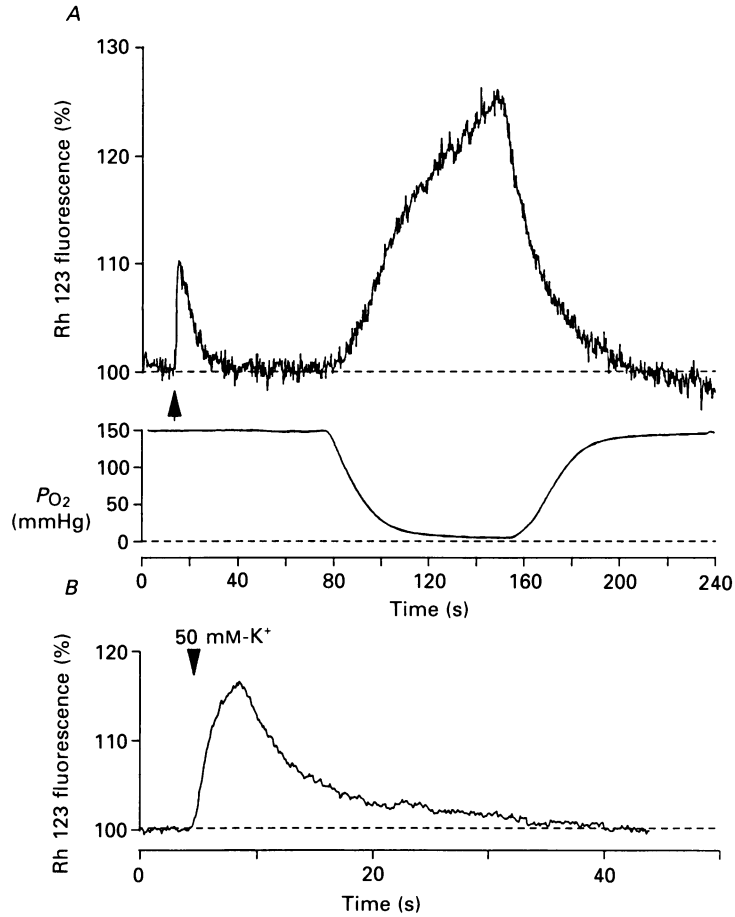

Fig. 13. Rh 123 responses to application of 50 mM-K^+ and hypoxia. A brief application of high K^+ (arrow) increased Rh 123 fluorescence about 10%. Superfusion with a saline at a P_{O_2} of about 8 mmHg produced the response shown, with an increase in fluorescence of about 26%. The trace in *B* shows, at an expanded time base, the largest response to high K^+ that we have seen.

the cytosolic phosphorylation potential will depend on the rate of ATP hydrolysis and the rate of additional supply of ATP from glycolysis. A significant fall in the ATP/ADP.P_i ratio might release Ca^{2+} from a store highly sensitive to that ratio.

All our data show that $\Delta\psi_m$ depolarizes with hypoxia, and so are fully consistent with the first mechanism, but do not exclude the second. If the second process operates, then a rise in $[Ca^{2+}]_i$ should be provoked by agents which lower the phosphorylation potential by mechanisms independent of mitochondrial depolarization.

We have therefore examined the effects of oligomycin (see above), atractyloside and bongkrekate (which prevent the export of mitochondrial ATP so depleting cytosolic ATP), and of iodoacetic acid which blocks glyceraldehyde-3-phosphate dehydrogenase preventing production of glycolytic ATP.

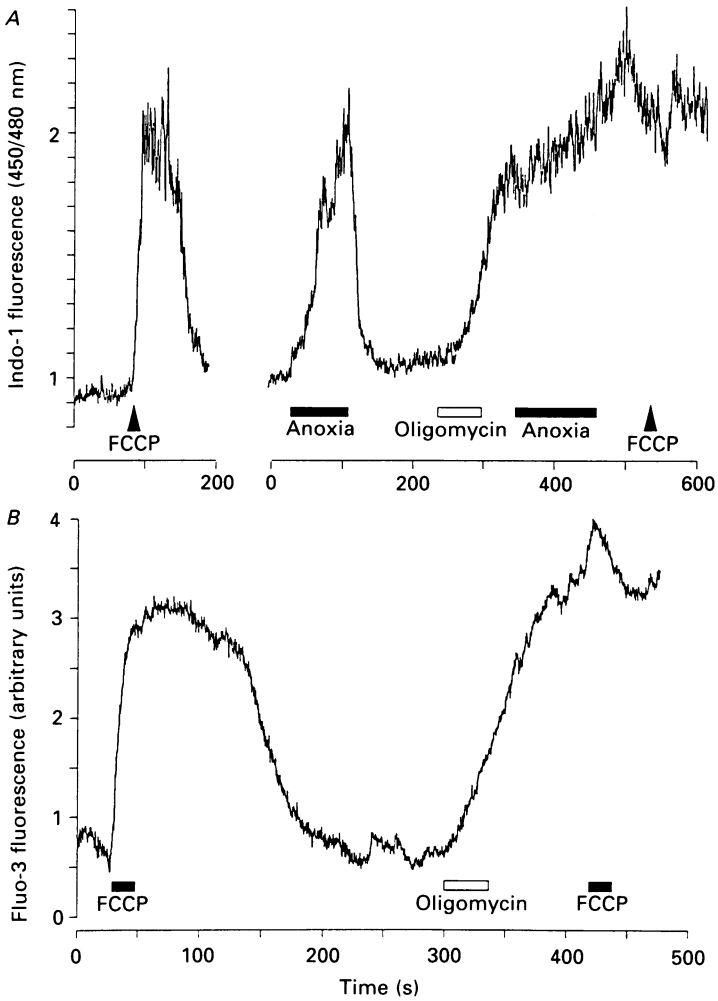

Fig. 14. Interactions of oligomycin with the $[Ca^{2+}]_i$ responses to anoxia and FCCP. The record in *A* was obtained from type I cells loaded with Indo-1, and the traces show the ratio of fluorescence measured at 405 to that at 488 nm, an index of $[Ca^{2+}]_i$. Application of FCCP and a period of anoxia led to similar increases in $[Ca^{2+}]_i$. Brief exposure to oligomycin raised $[Ca^{2+}]_i$ again to a plateau from which cells rarely recovered. A further period of anoxia, as indicated by the filled bar, may have raised $[Ca^{2+}]_i$ slightly. No significant response could be seen in this record to FCCP. In *B*, a similar experiment is shown in which Fluo-3 was used as the $[Ca^{2+}]_i$ indicator. An initial response to FCCP is shown. Oligomycin again raised $[Ca^{2+}]_i$ to similar levels. A further application of FCCP caused only the slightest of further responses.

The action of oligomycin on $[Ca^{2+}]_i$

In Indo-1-loaded cells, oligomycin caused an irreversible rise in $[Ca^{2+}]_i$ to a plateau, sometimes comparable in magnitude and speed of onset with the change that follows anoxia (Fig. 14*A*), though occasionally much less. The rate of change of

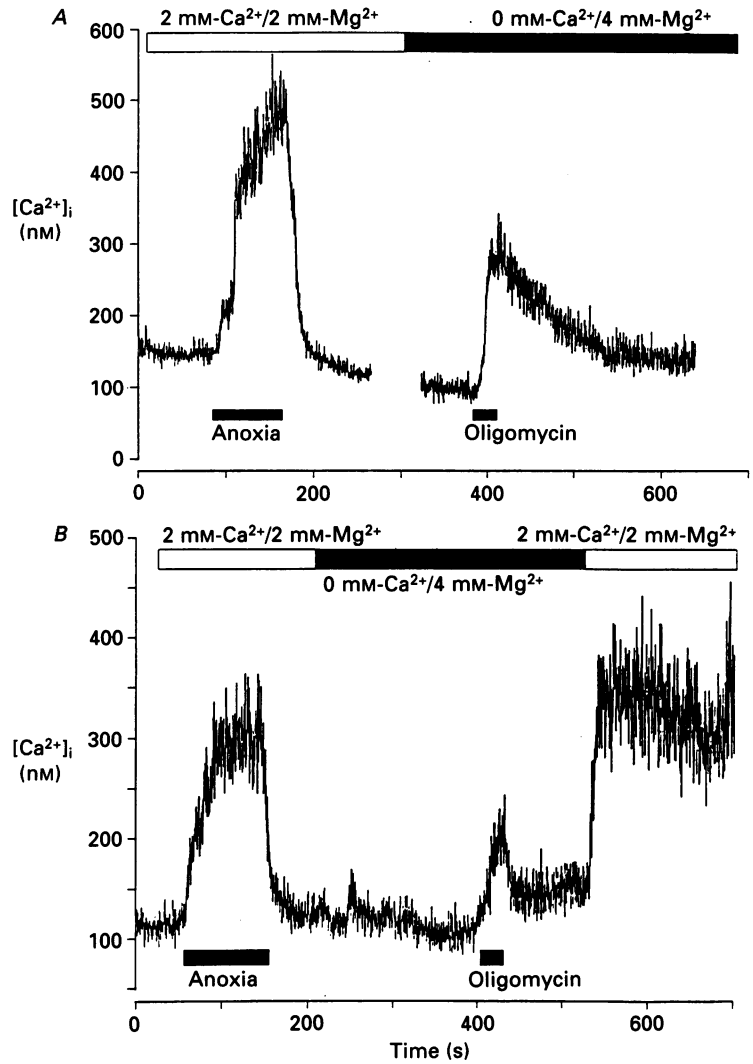

Fig. 15. Oligomycin appears to release Ca^{2+} from an internal pool. As the effects of oligomycin tend to be irreversible, and as oligomycin raises $[Ca^{2+}]_i$ to levels similar to those seen with anoxia, responses to anoxia are used as controls. The superfusate was switched as shown by the filled bar to one containing no added Ca^{2+}, which was replaced with Mg^{2+}. After about 2 min, to ensure complete wash-out of any Ca^{2+} from the bath, oligomycin was applied locally. The traces shown in *A* and *B* were obtained from two separate cell groups. In each, oligomycin raised $[Ca^{2+}]_i$, but the response was transient in contrast to the usual response in the presence of $[Ca^{2+}]_o$. When the superfusate was switched back to the control, $[Ca^{2+}]_i$ rapidly rose and remained at a plateau level.

$[Ca^{2+}]_i$ was temperature dependent, and at 35 °C was similar to the rate of rise in response to anoxia.

When oligomycin was applied at 35 °C, and the rise in $[Ca^{2+}]_i$ allowed to reach its plateau level, subsequent exposures to anoxia caused variable increases in $[Ca^{2+}]_i$ ($n = 19$) (Fig. 14*A*). Likewise the response to FCCP was always attenuated though not always abolished ($n = 15$) (Fig. 14*A* and *B*). There was not in all cases clear evidence of occlusion by oligomycin of the responses to the second stimulus ($n = 7$).

At lower temperatures, the rise in $[Ca^{2+}]_i$ with oligomycin was slowed, and then exposure to anoxia precipitated a rapid increase in $[Ca^{2+}]_i$. Similarly, if FCCP was applied at 30 °C, a rapid rise in $[Ca^{2+}]_i$ was superimposed on the slower rise initiated by the oligomycin.

When the extracellular calcium was reduced to zero an increase in $[Ca^{2+}]_i$ was still evoked by oligomycin though the increase was not sustained ($n = 6$) (Fig. 15*A* and *B*).

On returning to normal levels of $[Ca^{2+}]_o$ the intracellular calcium rose to the peak obtained with for example an anoxic stimulus (Fig. 15*B*) and remained at a plateau (see also Biscoe & Buchen, 1990*b*).

Oligomycin is not as selective for the ATP synthetase as one might wish. Most particularly, it is known to block the plasmalemmal Na^+–K^+ pump (Fah, Koval & Albers, 1966). This alone could raise $[Ca^{2+}]_i$ (through intracellular accumulation of Na^+ and stimulation of Na^+–Ca^{2+} exchange); we tested the effect of ouabain (1 mM), but this caused only a very slow rise in $[Ca^{2+}]_i$ ($n = 5$). It seems unlikely that block of the Na^+–K^+ pump could account for the effect of oligomycin.

Atractyloside, bongkrekate and iodoacetic acid

Atractyloside and bongkrekate had no observable effects on the variables measured here after applications of several minutes perhaps suggesting that they did not penetrate the cell membranes at the concentrations we used. Neither could we find any effects on the dorsal root ganglion cells both acutely dissociated and cultured. Iodoacetate had such a long and slow time course of action that we were again unable to demonstrate any consistent reproducible effects on a variety of cells.

DISCUSSION

Rhodamine 123 has not been much used in the way described here. The relatively standard biochemical manipulations carried out here serve, in part, to confirm that the signal behaves as predicted by chemiosmotic theory.

Taken together our evidence here and elsewhere (Biscoe & Duchen, 1990*a*, *b*; Duchen & Biscoe, 1992) leads us to conclude that a crucial step in the transduction of a change in P_{O_2} in the carotid body is a rise in $[Ca^{2+}]_i$ where the calcium is released predominantly from an intracellular store.

The first obvious candidate for a mechanism promoting this release is the mitochondrial electron transport chain which has an intrinsic O_2 sensor in complex IV, cytochrome oxidase (or cytochrome *aa*3). The large negative mitochondrial membrane potential ($\Delta\psi_m$) is generated by the translation of electrons and the

expulsion of protons by the redox couples of the electron transport chain. Intramitochondrial Ca^{2+} is retained and influx promoted by this electrochemical potential.

The results with Rh 123 demonstrate that $\Delta\psi_m$ of type I cells is related to the oxygen partial pressure of the superfusing solution over a range of values of P_{O_2} that do not appear to alter mitochondrial function in most other cell types. Furthermore this range of P_{O_2} values is compatible with that found within the structure of the carotid body tissue by direct experiment (cf. Nair, Buerk & Whalen, 1986). The stimulus–response relationship for $\Delta\psi_m$ *vs.* P_{O_2} shows a hyperbolic relationship strikingly similar to that for $[Ca^{2+}]_i$, for NADH, and for action potential frequency.

In hepatocytes and cardiac myocytes, cytochrome *aa*3 is 80–90% reduced before pyridine nucleotide reduction or $\Delta\psi_m$ have changed half-maximally (Chance, 1976). We do not know the exact relationship between the state of cytochrome *aa*3 reduction and the rate of electron transport, and so it does not seem sensible for us to speculate about a possible P_{50} for the cytochrome in these cells.

We have not attempted to calibrate the Rh 123 signal directly in terms of a potential difference. Expression of the change in fluorescence as a percentage change in signal yielded remarkably consistent data. In addition, a relative calibration can be achieved with the uncoupler FCCP, which acts as a proton ionophore and collapses $\Delta\psi_m$. A striking feature of the responses to anoxia in the type I cells was the relatively rapid rate at which the Rh 123 signal increased towards the minimum $\Delta\psi_m$ indicated by the response to FCCP.

The response of $\Delta\psi_m$ to hypoxia was very temperature dependent, just as was the increase in $[Ca^{2+}]_i$. The parallel movement of $[Ca^{2+}]_i$ with $\Delta\psi_m$ as the latter was manipulated with combinations of rotenone and TMPD/ascorbate and the similarity of the time courses of changes in $\Delta\psi_m$ with changes in $[Ca^{2+}]_i$ in response to a whole series of manipulations provides yet further indication for a direct involvement of mitochondrial electron transport in the control of $[Ca^{2+}]_i$.

Mechanisms to couple mitochondrial depolarization to raised $[Ca^{2+}]_i$

Attempts to identify the intracellular mechanisms that couple the rise in $[Ca^{2+}]_i$ with the altered mitochondrial status suggest that our previous emphasis (Biscoe, Duchen, Eisner, O'Neil & Valdeolmillos, 1989; Biscoe & Duchen, 1990*a*, *b*) on a mitochondrial source represented a simplified view. The answer may be more complex and our data cannot at present resolve the issue completely to our satisfaction.

Depolarization of the mitochondria could raise $[Ca^{2+}]_i$ in (at least) two ways.

First it would promote a net efflux of Ca^{2+} from the mitochondria and so raise $[Ca^{2+}]_i$. This rise would only assume physiological significance if the mitochondrial store is large enough. A simple calculation indicates that this would in principle be possible. Mitochondria make up slightly more than 10% of the volume of the cytoplasm of type I cells (McDonald, 1981). An increase of at least 100–200 nM in $[Ca^{2+}]_i$ would be a plausible expectation during hypoxia (Biscoe & Duchen, 1990*b*). If this were to occur in 90% of the total cell volume by release from a pool in the other 10%, the mitochondria, then this pool of releasable calcium would need to have a concentration of 1–2 μM, consistent with estimates for intramitochondrial $[Ca^{2+}]$ of 0·5–3 μM (Crompton, 1990). This assumes that the nucleus as well as the

cytoplasm is freely permeable to the calcium. As this is probably not the case then account would need to be taken of the approximately one-third of the cell volume occupied by the nucleus; so the figures for $[Ca^{2+}]_i$ might be still more favourable to this explanation.

Our experiments on the change in autofluorescence seen in response to elevation of $[Ca^{2+}]_i$ through stimulation of voltage-gated Ca^{2+} entry, described in the accompanying paper (Duchen & Biscoe, 1992), give some further clues to the status of intramitochondrial $[Ca^{2+}]$. The rise in NADH/NAD ratio in response to raised $[Ca^{2+}]_i$ induced by depolarization with K^+ suggests that mitochondrial dehydrogenases cannot be maximally active at rest. The $K_{0.5}$ for pyruvate dehydrogenase phosphatase *in vitro* is about 1 μM, with maximal activity seen at around 2-5 μM, although these measurements are critically dependent on other conditions of the assays. This therefore remains consistent with mitochondrial release as a mechanism, while suggesting that mitochondrial $[Ca^{2+}]$ is not peculiarly high in these cells.

The data obtained with oligomycin would nevertheless suggest that this mechanism cannot alone account for the rise in $[Ca^{2+}]_i$ with even complete anoxia. Oligomycin raises $[Ca^{2+}]_i$ rapidly to a new plateau level, even though $\Delta\psi_m$ is hyperpolarized. A component of this response is due to release from an intracellular pool, suggesting that Ca^{2+} may be released without mitochondrial depolarization.

Furthermore, while $[Ca^{2+}]_i$ is high, after exposure to oligomycin, mitochondrial depolarization should raise $[Ca^{2+}]_i$ even further than it does at rest, as a high cytosolic $[Ca^{2+}]_i$ coupled to mitochondrial hyperpolarization would be expected to raise intramitochondrial $[Ca^{2+}]$ and therefore to increase FCCP-releasable Ca^{2+}. This clearly did not occur, and suggests that mitochondrial Ca^{2+} release alone is unlikely to account for the rise in $[Ca^{2+}]_i$ with hypoxia.

A second mechanism that could raise $[Ca^{2+}]_i$ in response to a fall in $\Delta\psi_m$ could be a decline in the rate of ATP production. This would come about because of the reduction in the driving force for protons through the F_0–F_1 ATP synthetase complex. The rate of ATP synthesis shows a very steep dependence on $\Delta\psi_m$ (Maloney & Schattschneider, 1980; Pietrobon *et al.* 1981; Zoratti *et al.* 1982), so that relatively small depolarizations of $\Delta\psi_m$ could reduce ATP production significantly. As ATP utilization would be unaffected, a drop in the ATP/ADP.P_i ratio would result. This could possibly release Ca^{2+} from a non-mitochondrial, ATP-dependent store that is highly sensitive to the ATP/ADP.P_i ratio, presumably the mechanism involved in the oligomycin-induced rise in $[Ca^{2+}]_i$.

The data presented here would thus tend to favour Ca^{2+} release from a store sensitive to the ATP/ADP.P_i ratio, but the distinction between the two proposed mechanisms has proved difficult to clarify unequivocally, and both our own data and those of others remain conflicting. Indeed, it remains perfectly possible that some other intermediate reflecting the redox state of the cells is involved.

Evidence against a role for the ATP/ADP.P_i ratio comes from whole-cell patch clamp recordings, as we have shown previously that the electrophysiological correlate of the response to cyanide in these cells (Biscoe & Duchen, 1989) and also in DRG cells (Duchen, 1990), an increased Ca^{2+}-activated potassium conductance, is unaffected by the inclusion of ATP or an ATP-regenerating system in the patch pipette. However, it could still be argued that the rate of ATP delivery through the pipette may be inadequate to maintain a normal ATP/ADP.P_i ratio in the face of

blockade of electron transport. Indeed, some other evidence suggests exactly that. The regenerating system did not prevent the immediate effects of either CN^- or FCCP on a GABA-gated current in DRG cells, but did promote subsequent recovery of the response which was irreversibly lost if the ATP-regenerating system was omitted (Duchen, 1989 and M. R. Duchen, unpublished observations).

Other evidence against a central role for ATP comes from direct measurements of ATP made by Obeso, Almaraz & Gonzalez (1989). The time scale of their experiments was more prolonged than those described here, but they found that at concentrations of FCCP, CN^- and anoxia that induced equivalent levels of excitation of the intact structure *in vitro*, there was no consistent change in ATP. However, only ATP, and not the ATP/ADP.P_i ratio, were measured. Furthermore, while our data presented here might indicate that ADP rises during anoxia, leading to a rebound stimulation of respiration on reoxygenation, such changes were not clearly identified during hypoxia, during which we know that $[Ca^{2+}]_i$ rises. We cannot tell if this is simply an inadequacy of the level of resolution, if it really means that ADP rises only in response to *anoxia* and not to hypoxia, or, indeed whether there is some explanation other than a rise in [ADP] for the changes in signals we have described.

Our conclusions thus rest very heavily on data obtained with oligomycin. While this drug is undoubtedly acting as expected at the F_1–F_0 complex, it is also known to act as an inhibitor of the plasmalemma Na^+–K^+-ATPase (Fahn *et al.* 1966), and may even have effects elsewhere in the cell. The other agents we have used to lower ATP/ADP have had equivocal effects, and we have to be uncertain about their efficacy. Thus we feel that the issue of the intracellular source of the calcium is still unresolved, and it will only be clarified by further experimentation.

In this context it is interesting to note the parallel between the responses of isolated type I cells documented here, especially in terms of changes in $[Ca^{2+}]_i$, with the response properties of the intact structure. Thus, Mulligan *et al.* (1981) found that oligomycin induced a vigorous excitation of the whole carotid body *in vivo*, following which responses to FCCP, hypoxia, CN^- and antimycin A, all normally potent stimuli, were all reduced or abolished.

Changes in $\Delta\psi_m$ are not secondary to changes in $[Ca^{2+}]_i$

The depolarization of $\Delta\psi_m$ that follows a rise in $[Ca^{2+}]_i$ resulting from voltage-gated Ca^{2+} entry into the cell, is explicable in terms of known biochemistry and further illustrates the complexity of the observable responses and the nature of the possible interactions. It should be clear, however, from these data that secondary effects of a rise in $[Ca^{2+}]_i$ on $\Delta\psi_m$ cannot account for the changes in $\Delta\psi_m$ seen with hypoxia: the response to hypoxia is independent of $[Ca^{2+}]_o$ while the response to high K^+ is wholly dependent on $[Ca^{2+}]_o$ and the responses of $\Delta\psi_m$ to hypoxia are significantly larger than those seen to stimulation with high K^+, even though the rise in $[Ca^{2+}]_i$ is usually greater with high K^+ than it is even with anoxia.

Specialization of the type I cell

We have shown that the changes described here are not a certain nor an inevitable consequence of hypoxia for any cell, but in fact contrast to the responses of other cells that we have examined. The central question then is how can transduction work

through a mitochondrial mechanism when the levels of P_{O_2} detected by the cells are significantly higher than that required to saturate the cytochrome oxidase in most tissues (but see Wilson, Erecińska, Drown & Silver, 1979). Such comparisons suggest that the mitochondrial electron transport chain must be specialized in the type I cells, raising the sensitivity of the cells to moderate changes in P_{O_2}.

Mills & Jöbsis (1970, 1972) presented evidence for the presence of a cytochrome oxidase with a low apparent affinity for oxygen. The presence of such a cytochrome would certainly account most readily for our data, as mitochondrial electron transport would then change at relatively high P_{O_2} levels. As shown here there are changes in all variables studied that can only be attributed to alterations in mitochondrial electron transport at P_{O_2} values much higher than 1 mmHg. Obviously no direct measure of the status of the cytochrome has been attempted here, and no assessment of the effective P_{50} for the cytochrome can be made directly from our data, as the cytochrome is 80–90% reduced before $\Delta\psi_m$ or NAD reduction are 50% complete. Mills & Jöbsis (1972) estimated that the cytochrome must have a P_{50} of 90 mmHg. This was based on what at the time was a reasonable judgement of the likely tissue P_{O_2}, before direct measurements had been made. A P_{50} of the order of 40–60 mmHg seems more probable on a re-reading of their data.

The presence of a cytochrome of low apparent affinity could mean that the affinity of the cytochrome is altered by some feature of the intracellular environment of the type I cell cytosol, that the cytochrome has undergone a post-transcriptional modification, or that there is a specialized gene product. There is at present little to indicate which of these explanations may suffice.

One interpretation of the experiments with oligomycin is that the sensitivity of the cytochrome for oxygen is a function of an aspect of its local environment that may be modified by this drug, perhaps the intramitochondrial ATP/ADP ratio, rather than being an intrinsic property. We have yet to examine this issue further.

It is also possible that the oxygen sensitivity is in turn a function of the oxygen consumption of the cells, which would be reduced by oligomycin. This is consistent with previous studies that have demonstrated that the P_{50} for cytochrome *c* oxidase decreases with a decreasing ATP/ADP.P_i ratio (Wilson *et al.* 1988). Whatever causes the alteration in the cytochrome *aa*3 may account for the change in sensitivity of the chemoreceptors during the passage from fetal to infant life.

It also remains feasible that the cells actually possess two populations of mitochondria, some of which express the specialized cytochrome oxidase and play a role in transduction, whilst others play a more conventional role in ATP generation. Evidence for such a proposition was procured by Mills & Jöbsis (1970, 1972) who suggested two separate sites, receptor and non-receptor. Nair *et al.* (1986) concurred but had in mind two populations of receptor cells containing the two cytochromes. If the two types of mitochondria were present in the same receptor cell then maybe only one group would respond to *hypoxia*, while all mitochondria would respond to *anoxia*. A fall in the ATP/ADP ratio would only be significant with anoxia, as suggested by some of our data, and cellular function would not be generally compromised by the mitochondrial specialization required to detect hypoxia.

If this were to be the circumstance then the argument would have to be that in normal life the origin of calcium causing the rise in $[Ca^{2+}]_i$ was the population of

mitochondria expressing a low-affinity cytochrome. It could not be some other ATP-sensitive calcium store, as the cytoplasmic ATP levels would not normally fall. Given the additional presence of mitochondria with a normal high-affinity cytochrome *aa*3, ATP levels would only fall in the exceptional case of anoxia. In this way the type I cells would be able to conduct their normal cell economy whilst acting as transducers.

On balance, the data in this and the accompanying paper are fully consistent with a central role for a specialized mitochondrial electron transport chain in transduction, and we conclude that changes in $\Delta\psi_m$ are a key to transduction and to the rise in $[Ca^{2+}]_i$ that subsequently leads to transmitter release.

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