The effect of mitochondrial inhibitors on membrane currents in isolated neonatal rat carotid body type I cells

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Inhibitors of mitochondrial energy metabolism have long been known to be potent stimulants of the carotid body, yet their mechanism of action remains obscure. We have therefore investigated the effects of rotenone, myxothiazol, antimycin A, cyanide (CN^-) and oligomycin on isolated carotid body type I cells. All five compounds caused a rapid rise in intracellular Ca^{2+}, which was inhibited on removal of extracellular Ca^{2+}. Under current clamp conditions rotenone and CN^- caused a rapid membrane depolarization and elevation of $[Ca^{2+}]_i$. Voltage clamping cells to -70 mV substantially attenuated this rise in $[Ca^{2+}]_i$. Rotenone, cyanide, myxothiazol and oligomycin significantly inhibited resting background K^+ currents. Thus rotenone, myxothiazol, cyanide and oligomycin mimic the effects of hypoxia in that they all inhibit background K^+ current leading to membrane depolarization and voltage-gated calcium entry. Hypoxia, however, failed to have any additional effect upon membrane currents in the presence of CN^- or rotenone or the mitochondrial uncoupler *p*-trifluoromethoxyphenyl hydrazone (FCCP). Thus not only do mitochondrial inhibitors mimic the effects of hypoxia, but they also abolish oxygen sensitivity. These observations suggest that there is a close link between oxygen sensing and mitochondrial function in type I cells. Mechanisms that could account for this link and the actions of mitochondrial inhibitors are discussed.

The carotid body is the primary peripheral arterial chemoreceptor. Type I cells of the carotid body act as sensors for fluctuations in blood P_{O_2}, P_{CO_2} and pH. Hypoxia, hypercapnia or acidosis results in an elevation of activity in the carotid sinus nerve (CSN), which ultimately innervates the respiratory centres of the brain. Thus the carotid body can initiate corrective respiratory reflexes crucial for maintaining blood P_{O_2}, P_{CO_2} and pH within acceptable levels.

The mechanisms by which the carotid body transduces a hypoxic stimulus into elevated CSN activity remain controversial. There is a body of evidence which suggests that hypoxia leads to type I cell depolarization, calcium entry and neurosecretion (Buckler & Vaughan-Jones, 1994; Urena *et al.* 1994; Montoro *et al.* 1996). In neonatal rat type I cells this depolarization appears to be mediated, in part, via inhibition of a TASK-like background potassium channel which is active at the resting membrane potential of the type I cell (Buckler, 1997; Buckler *et al.* 2000). The biochemical pathways

leading to the modulation of these, or other, oxygen-sensitive ion channels, however, remain obscure.

It has been known for a long time that agents that inhibit mitochondrial energy metabolism are potent stimulants of the carotid body (Heymans *et al.* 1931; Anichkov & Belen'kii, 1963; Krylov & Anichkov, 1968; Mills & Jobsis. 1971, Duchen & Biscoe, 1992*a,b*). These observations originally led to the hypothesis that oxygen sensing by the carotid body may itself be mediated through some aspect of mitochondrial function. This hypothesis has fallen from favour in the past decade, principally because it is believed that the effects of hypoxia and mitochondrial inhibitors are mediated through different calcium signalling pathways, with hypoxia promoting voltage-gated calcium entry (Buckler & Vaughan-Jones, 1994) and mitochondrial inhibitors promoting calcium release from intracellular stores (Biscoe *et al.* 1989; Biscoe & Duchen, 1989, 1990). More recent studies, however, have challenged this view by demonstrating that the primary effect of mitochondrial uncouplers is to inhibit type I cell background K^+ channels

leading to membrane depolarization and voltage gated Ca^{2+} entry (Buckler & Vaughan-Jones, 1998). These latter observations have prompted us to reinvestigate the effects of other inhibitors of mitochondrial energy metabolism upon type I cell calcium signalling. In this paper we show that three structurally and functionally diverse inhibitors of mitochondrial electron transport (rotenone, myxothiazol and NaCN) and an inhibitor of ATP synthase (oligomycin) all mimic the effects of hypoxia upon intracellular calcium $[Ca^{2+}]_i$ and background K^+ currents in neonatal rat type I cells.

Methods

Cell isolation

Carotid bodies were excised from anaesthetized (4% halothane) Sprague-Dawley rat pups (10–15 days old) and enzymatically dispersed using collagenase (0.4 mg ml^{-1}, type I, Worthington) and trypsin (0.15–0.2 mg ml^{-1}, Sigma) as previously described (Buckler, 1997). The rats were then killed by decapitation whilst still anaesthetized. The isolated cells were maintained in Ham's F-12 medium (supplemented with: 10% heat-inactivated fetal calf serum, 100 i.u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 84 U l^{-1} insulin, Sigma) and plated out onto glass coverslips coated with poly-D-lysine (Sigma). Cells were kept at 37°C, 5% CO_2 in air until use (2–12 h).

Measurement of recording chamber oxygen levels (P_{O_2})

The P_{O_2} of the solutions was monitored using a needle electrode attached to a Strathkelvin instruments oxygen meter (model 781, Glasgow).

Measurement of intracellular calcium

Intracellular calcium was measured using indo-1 or fura-2. Cells were loaded with fura-2 or indo-1 by incubation in a solution of 5 μM fura-2 AM or 2.5 μM indo-1 AM in culture medium at room temperature (20–24°C) for 20–30 min. Indo-1 fluorescence was excited at 340 ± 5 nm and measured at 405 ± 16 and 495 ± 10 nm using photomultiplier tubes. Fura-2 fluorescence was excited alternately (2 Hz) at 340 nm and 380 nm using a Cairn monochromator (Faversham, Kent) and measured at 510 ± 20 nm using a photomultiplier tube. The fluorescence emission ratio 405 nm/495 nm for indo-1, and the fluorescence excitation ratio 340 nm/380 nm for

fura-2, were calibrated as previously described (Buckler & Vaughan-Jones, 1994).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was monitored using rhodamine 123 (Duchen & Biscoe, 1992*b*). Cells were incubated with 10 μg ml^{-1} rhodamine 123 in culture medium at room temperature for 30 min. Rhodamine 123 fluorescence was excited at 490 nm and measured at 525 ± 10 nm. Because rhodamine 123 slowly leaks out of cells, causing a gradual decline in signal intensity, selected regions of the data were scaled by subtracting the minimum (baseline) fluorescence intensity and then normalizing to the maximum fluorescence intensity obtained in the presence of FCCP.

Measurement of cyanide concentration

The concentrations of cyanide presented in Fig. 1 were measured colourimetrically using a Hanna instruments cyanide meter HI 93714 (http://www.hannainst.com).

All cyanide solutions were replaced every 30–60 min. Cyanide is extremely volatile in buffered solutions exposed to air, or actively aerated, as it is rapidly lost as HCN. This problem is exemplified in Fig. 1, which shows a series of successive recordings of mitochondrial membrane potential (Ψ_m) from type I cells using the same solution of NaCN over a period of 4 h. Initially this NaCN solution causes mitochondrial depolarization, as evidenced by a rise in rhodamine 123 fluorescence and fully inhibits mitochondrial repolarization following the brief application and removal of FCCP. However, it should be noted that upon removal of CN$^-$ the mitochondria fully repolarize indicating that the effects of CN$^-$ on mitochondrial function in type I cells are fully reversible (see also Duchen & Biscoe, 1992*b*). The concentration of NaCN in solution at this time point (time 0) was 1 mM. Within 1 h the CN$^-$ concentration in the solution had fallen to 300 μM and there was some evidence of slight repolarization of Ψ_m following removal of FCCP in the continuing presence of CN$^-$ (Fig. 1*B*, compare change in Ψ_m in second trace in the presence of CN *versus* anoxia). By 2 h the concentration of CN$^-$ had fallen to 60 μM and there was a prompt partial repolarization of Ψ_m following FCCP removal in the presence of this same NaCN solution (Fig. 1*C*). By 4 h the concentration of CN$^-$ in this solution was 2 μM. At this time point the NaCN solution had no detectable effect on resting Ψ_m or upon the rate of repolarization of Ψ_m following removal of FCCP (Fig. 1*E*). It is apparent from these studies that (a) complete
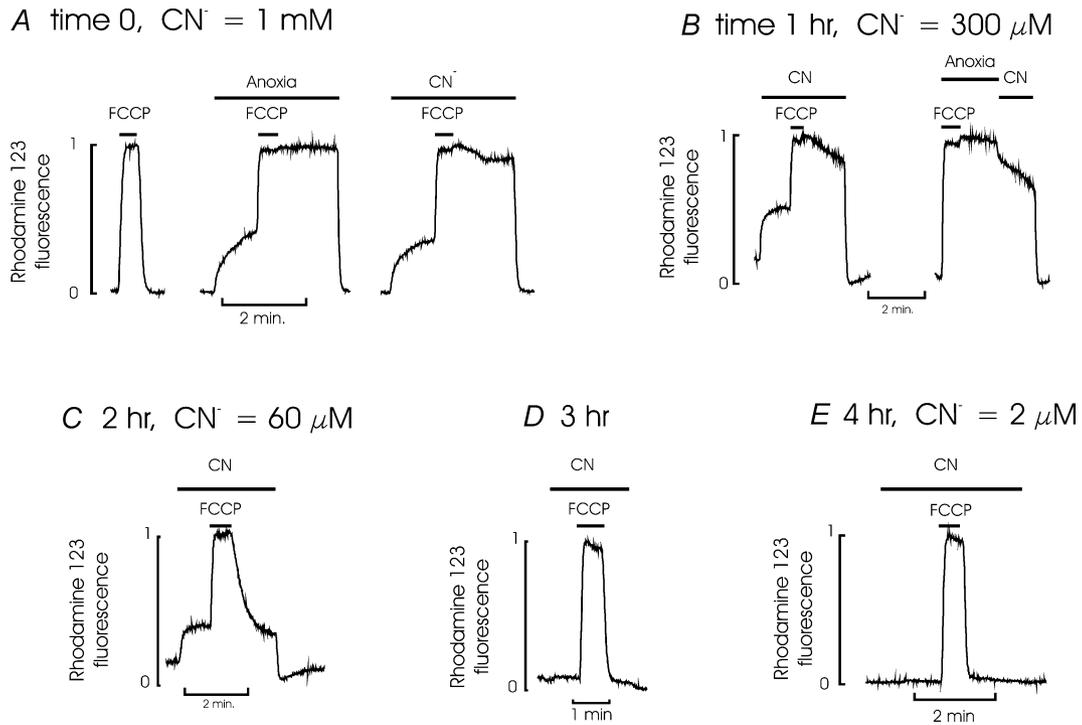
Figure 1. Effect of cyanide on mitochondrial membrane potential

This figure shows the results of 5 successive experiments recording the effects of NaCN on mitochondrial membrane potential (Ψ_m) carried out over a period of 4 h using the same solution of NaCN in bicarbonate buffered medium. This solution was maintained at 37°C and gassed with a 5% CO_2, 95% air mixture. Ψ_m was monitored using rhodamine 123 (see Methods). Note that an increase in fluorescence intensity reflects a decrease in Ψ_m. *A*, recordings obtained from a group of type I cells at time 0 (i.e. immediately after adding NaCN to the solution). The first trace shows a normal response to FCCP, a mitochondrial uncoupler, which causes a rapid mitochondrial depolarization. Upon removal of FCCP the mitochondria rapidly repolarize. The second trace, from the same group of cells, shows that anoxia (0.5 mM $Na_2S_2O_4$ added to the solution and gassed with 95% N_2, 5% CO_2) also depolarizes mitochondria. The further addition of FCCP rapidly completes mitochondrial depolarization. Upon removal of FCCP in the continued presence of anoxia, the mitochondria remain depolarized until oxygen is reintroduced. This confirms that the rhodamine 123 signal does indeed reflect oxygen-dependant mitochondrial electron transport and not the redistribution of the dye between other cellular compartments. The third trace (again from the same cells) shows that NaCN also causes a slow mitochondrial depolarization. The further subsequent addition of FCCP again fully depolarizes the mitochondria. Upon removal of FCCP in the continued presence of CN^- mitochondria remain fully depolarized indicating that the NaCN solution is completely blocking electron transport. Upon completion of this segment of the recording a sample of the NaCN solution was removed and assayed for CN^- (see methods). The CN^- concentration was 1.0 mM. *B*, 1 h later another group of cells were exposed to the same NaCN solution which caused a marked depolarization of Ψ_m. There was however, some evidence of a slow repolarization of Ψ_m following addition and removal of FCCP in the continued presence of CN^- suggesting that electron transport may not be fully inhibited. This repolarization is even more evident in the second trace (same cells) where, following FCCP addition and removal, the cells are first exposed to anoxia, which prevents any repolarization and then to CN^- solution in which there is a clear partial repolarization of Ψ_m. The concentration of CN^- at this point was 300 μM. *C*, recording of the effects of CN^- of Ψ_m 2 h after preparation of the NaCN solution. Note that the CN^- solution still produces some depolarization of the resting Ψ_m but only partially inhibits repolarization of Ψ_m following addition and removal of FCCP. The CN^- concentration at this time point was 60 μM which was clearly insufficient to fully block electron transport. *D*, recording of the effects of CN^- on Ψ_m 3 h after the preparation of the CN^- solution. Note that the CN^- solution produces an only just discernable effect on resting Ψ_m and has little effect on repolarization of Ψ_m following FCCP addition and removal, indicating that the CN^- solution was having only a minor effect on electron transport. *E*, recording of the effects of CN^- on Ψ_m 4 h after preparation of the CN^- solution. Note that the CN^- solution has no effect on either resting Ψ_m or upon repolarization of Ψm following FCCP addition and removal. The CN^- concentration at this time point was 2 μM.

inhibition of electron transport in type I cells requires quite high levels of CN^- and (b) that the actual levels of CN^- in a solution exposed to air for even relatively short periods of time can be substantially less than intended. It is therefore apparent that high (mM) levels of CN^- need to be added to solutions if full inhibition of electron transport is to be reliably achieved and that that such solutions can only be used for relatively short periods of time. In this study we have used CN^- solutions with a nominal concentration of 2 mM (i.e. 2 mmol l^{-1} CN^- added to the solution) which were replaced every 30–60 min. These studies also indicate that the use of low concentrations of CN^- in solutions exposed to, or equilibrated with, a gas phase could result in there being insufficient CN^- in solution to have any significant effect upon electron transport at all.

Electrophysiology

Prior to attempting electrophysiological recordings we first confirmed that selected, indo-1-loaded, cells responded to a hypoxic challenge (6 Torr) with a rapid and robust increase in $[Ca^{2+}]_i$. Perforated patch whole cell recordings were then performed with simultaneous measurement of intracellular calcium as previously described (Buckler, 1997). Experiments were conducted using an Axopatch-1D amplifier (Axon Instruments). Voltage clamp commands were generated from a program written by one of the authors (K.J.B.) using an analog to digital and digital to analog converter (CED1401; Cambridge Electronic Design, Cambridge, UK). Consequently all voltage ramps are staircases with step amplitude of 0.78 mV. Membrane potential and current were filtered at 1 kHz and digitized at 2–4 kHz. Cells were subjected to repetitive voltage ramps at 0.5 Hz and the resultant current records averaged. Each value of n is the average of 10 ramp currents under one experimental condition for one individual cell.

Solutions and reagents

Filling solution for perforated patch recordings contained (mM): K_2SO_4, 55; KCl, 30; $MgCl_2$, 5.0; EGTA, 1.0; Hepes, 20; glucose, 10; pH adjusted to 7.3 with NaOH at room temperature. Amphotericin B, 240 μg ml^{-1}, was added from a stock solution of 60 mg ml^{-1} in DMSO. Data are presented without correction for liquid junction potentials (approximately 3 mV).

The standard HCO_3^--buffered saline contained (mM): NaCl, 117; KCl, 4.5; $NaHCO_3$, 23; $MgCl_2$, 1.0; $CaCl_2$, 2.5; glucose, 11; pH 7.4–7.45. Elevated K^+ Tyrode solution contained 101.5 mM NaCl and 20 mM KCl but was

otherwise the same as the previous solution. Ca^{2+}-free solutions were made by not adding any Ca^{2+} to the solutions and including 0.25–1.0 mM EGTA. In order to make solutions hypoxic they were bubbled with 5% CO_2, 95% N_2 otherwise they were bubbled with 5% CO_2, 95% air. Salines containing H_2O_2 were prepared by direct addition of 30% w/w H_2O_2 (sigma) prior to use and were replaced every hour.

Solutions were perfused through a recording chamber with a volume of 80 μl at 2–3 ml min^{-1}. All experiments were conducted at 34–37°C.

Amphotericin, tetramethyl-*p*-phenylenediamine, methyl succinate, FCCP, rotenone, myxothiazol, oligomycin, NaCN and rhodamine 123 were obtained from Sigma. Indo-1 AM was obtained from Calbiochem and fura-2 AM was obtained from Cambridge Bioscience (Cambridge, UK).

Statistics

All data are presented as means $\pm$ standard error of the mean. Statistical significance of results was assessed using Student's paired or unpaired t test as appropriate.

Results

Effects of inhibitors of electron transport and ATP synthase on $[Ca^{2+}]_i$ in type I cells

We have investigated the effects of four different inhibitors of electron transport, each targeting a different component of the electron transport chain, and an inhibitor of ATP synthase upon intracellular calcium in isolated rat type I cells. All type I cells were first challenged with a hypoxic stimulus (6 Torr) to confirm their oxygen sensitivity. Only cells that responded to this hypoxic stimulus with a brisk rise in intracellular Ca^{2+} were included in this study.

Rotenone inhibits mitochondrial electron transport by acting at complex I (NADH CoQ1 reductase) of the respiratory chain and preventing the reduction of ubiquinone (Earley *et al.* 1987). Application of 1.0 μM rotenone caused a rapid rise in $[Ca^{2+}]_i$ in all cells studied ($\Delta[Ca^{2+}]_i = 869.5 \pm 91.8$ nM, $n = 6$, $P < 0.0003$). Following rotenone removal $[Ca^{2+}]_i$ recovery was slow and was often delayed by a period of several minutes (Fig. 2). Removal of extracellular Ca^{2+} (Ca-free + 1 mM EGTA) completely abolished the rise in $[Ca^{2+}]_i$ ($n = 6$, Fig. 2*A*).

As rotenone inhibits at a proximal location in the electron transport chain, it is possible to bypass the effects of rotenone on mitochondrial energy metabolism by providing an alternative source of electrons which can feed into the electron transport chain downstream of

complex I. Tetramethyl-*p*-phenylenediamine (TMPD) can donate electrons to cytochrome *c* which can then pass on electrons to complex IV which transports protons across the inner mitochondrial membrane thus maintaining mitochondrial membrane potential. The ability of TMPD (in the presence of ascorbate which maintains TMPD in the reduced state) to rapidly reverse the effects of rotenone upon mitochondrial membrane potential in type I cells has been previously demonstrated by Duchen & Biscoe (1992*b*). These researchers also demonstrated that TMPD could reverse the effects of rotenone upon intracellular calcium in type I cells. Figure 2*B* confirms this earlier result, showing that the rise in calcium in response to rotenone was reversed by TMPD ($40 \ \mu g \ ml^{-1}$) and ascorbate (5 mM). Note however, that calcium still increased in response to hypoxia despite the presence of TMPD, ascorbate and rotenone ($[Ca^{2+}]_i = 78 \pm 8$ nM in the presence of TMPD, ascorbate and rotenone *versus* 576 ± 121 nM in the presence of TMPD, ascorbate, rotenone and hypoxia; $n = 6$; see Discussion). We were not, however, able to antagonize the effects of rotenone upon intracellular $[Ca^{2+}]_i$ or prevent rotenone-induced

mitochondrial depolarization with exogenously applied methyl succinate (5 mM; data not shown). This probably reflects a failure of succinate to enter the cell sufficiently rapidly to provide an adequate alternative source of electrons via complex II.

Myxothiazol inhibits electron transport at complex III (ubiquinol–cytochrome *c* reductase) by binding to, or near, the low potential haem b (Thierbach & Michaelis, 1982). Application of 1 μM myxothiazol caused a rapid but irreversible rise in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i = 677.6 \pm 81.9$ nM, $n = 7 \ P < 0.0003$). It should be noted that myxothiazol often took approximately a 1-min perfusion before it elicited its effects. This rise in $[Ca^{2+}]_i$ was substantially inhibited by removal of extracellular calcium ($92.8 \pm 1.8\%$, $P < 0.0003$, $n = 7$, Fig. 3*A*). Antimycin A also inhibits electron transport at complex III but at a more distal point than myxothiazol. It prevents the conversion of ubisemiquinone to ubiquinone. Application of 0.5 μM antimycin A also caused a rapid and irreversible rise in $[Ca^{2+}]_i$ very similar to that observed with myxothiazol ($\Delta[Ca^{2+}]_i = 652.6 \pm 155.2$ nM, $P < 0.006$, $n = 7$, Fig. 3*B*). This rise in $[Ca^{2+}]_i$ was significantly inhibited by removal
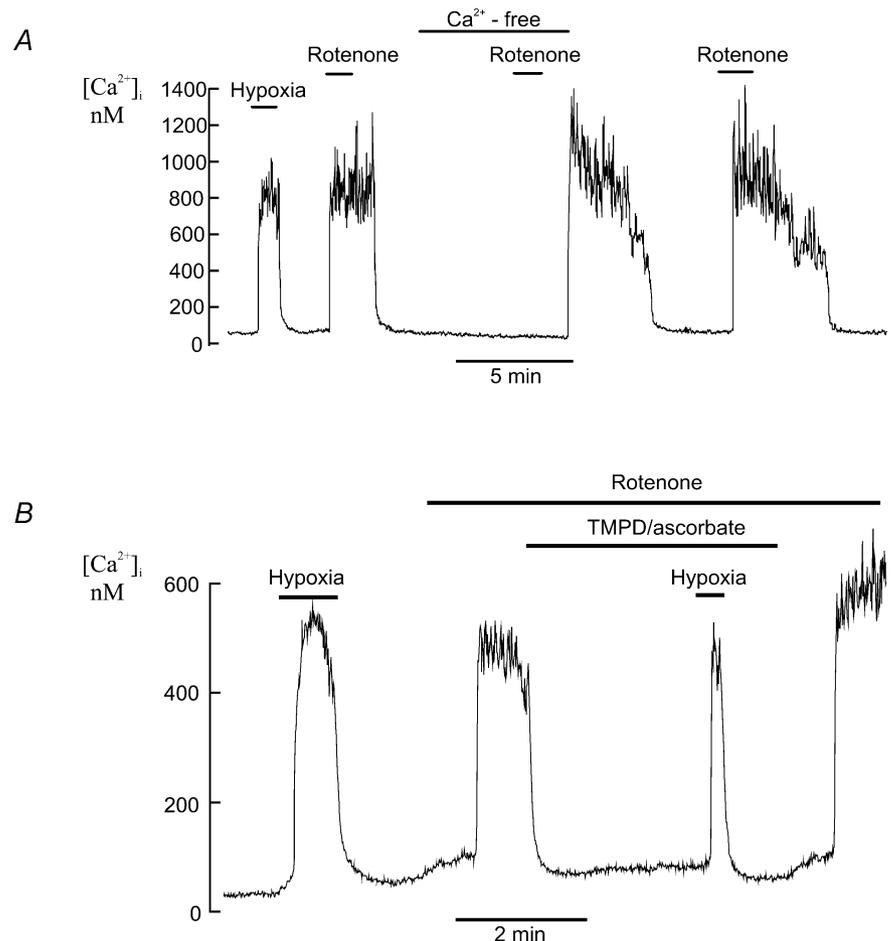
Figure 2. Effects of rotenone on $[Ca^{2+}]_i$ in type I cells

A, effects of hypoxia and rotenone (1 μM) on type I cell $[Ca^{2+}]_i$ (measured using Indo-1). Rotenone induces a rapid increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} but not in the absence of extracellular Ca^{2+}. The rotenone-induced rise in $[Ca^{2+}]_i$ was slowly reversed upon rotenone removal with a variable delay of several minutes. Note that following the second exposure to rotenone the effects of rotenone had not fully reversed before Ca^{2+} was reintroduced into the medium resulting in a transient rise in $[Ca^{2+}]_i$. *B*, the rotenone-induced rise in $[Ca^{2+}]_i$ was reversed by application of the electron donor TMPD + ascorbate (see Results). Note that in the presence of rotenone, TMPD and ascorbate, hypoxia induced an abrupt rise in $[Ca^{2+}]_i$ (see Discussion). Intracellular calcium was measured using fura-2.

of extracellular calcium ($89.2 \pm 3.4\%$, $n = 7$, $P < 0.005$, Fig. 3*B*).

Cyanide is the classical inhibitor of complex IV (cytochrome *c* oxidase), the final component of the electron transport chain. Complex IV catalyses the oxidation of cytochrome *c* and the reduction of oxygen. Cyanide causes the cytochrome oxidase to complex with cytochrome a_3 (van Gelder & Muijers, 1966). Application of cyanide (nominally 2 mM, but see Methods) produced a rapid and reversible rise in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i = 584.3 \pm 105.6$ nM, $n = 7$, $P < 0.003$). The calcium response to cyanide was significantly attenuated in the absence of extracellular Ca^{2+} ($90.6 \pm 2.3\%$, $P < 0.003$, $n = 7$, Fig. 3*C*).

Another mechanism by which oxidative phosphorylation can be arrested is by inhibiting the F_1–F_0 ATP synthase using the compound oligomycin. Similar effects to those observed with the electron transport inhibitors were seen with oligomycin. Oligomycin at

2.5 μg ml^{-1} caused a rapid, but irreversible rise in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i = 675.2 \pm 84.5$ nM, $n = 6$, $P < 0.001$) and either removal of extracellular Ca^{2+} during the oligomycin response (Fig. 4*A*) or application of oligomycin in the absence of extracellular Ca^{2+} (Fig. 4*B*) virtually abolished this rise in $[Ca^{2+}]_i$ ($95.0 \pm 1.2\%$, $P < 0.003$, $n = 6$).

These results indicate that inhibition of mitochondrial electron transport or inhibition of the mitochondrial ATP synthase causes a rapid rise in intracellular Ca^{2+} and that this rise in $[Ca^{2+}]_i$ is due predominantly to Ca^{2+} entry from the extracellular medium. The slow reversibility of the rotenone response may indicate that dissociation from complex I is slow, or that efflux from the cell is slow or that wash out of this drug from the experimental chamber is slow. The effects of oligomycin, antimycin A and myxothiazol were essentially irreversible. Moreover we found that extensive washing of the experimental chamber and perfusion lines with ethanol was necessary
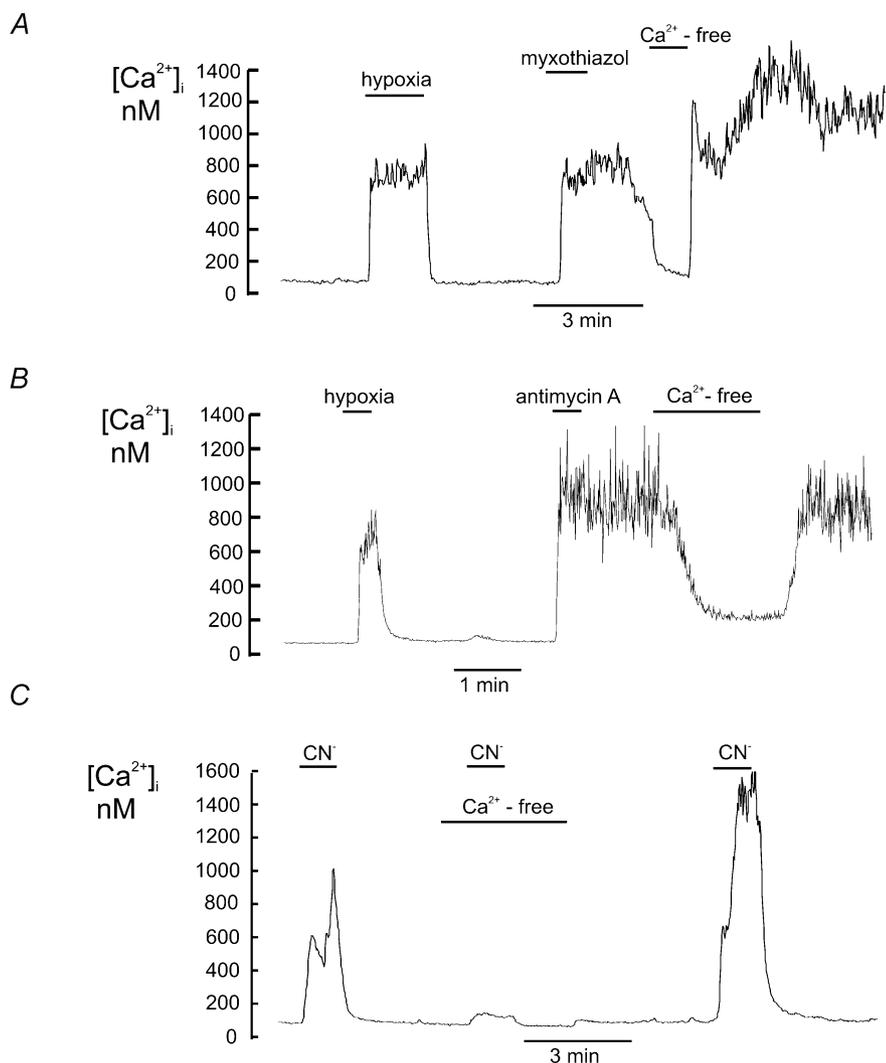
Figure 3. Effects of electron transport inhibitors on $[Ca^{2+}]_i$ in type I cells
Effects of electron transport inhibitors on intracellular calcium in type I cells (measured using indo-1) in the presence and absence of extracellular calcium. *A*, myxothiazol (1 μM) caused a large increase in intracellular calcium which was slow in onset, often with a delay of 30 s to 2 min, and which was maintained even after wash out of the drug. This sustained increase in $[Ca^{2+}]_i$ was substantially reduced by removal of extracellular Ca^{2+}. *B*, antimycin A (0.5 μM) caused a rapid increase in $[Ca^{2+}]_i$ that was also maintained following wash out of the drug. The antimycin A induced increase in $[Ca^{2+}]_i$ was largely reversed by removal of extracellular Ca^2. *C*, cyanide (nominally 2 mM; see methods) caused a rapid and reversible increase $[Ca^{2+}]_i$. The cyanide induced increase in $[Ca^{2+}]_i$ was greatly reduced in calcium free media.

after each experiment utilizing myxothiazol, oligomycin or antimycin A to prevent spontaneous increases in $[Ca^{2+}]_i$ when fresh cells were introduced into the recording chamber.

Effects of electron transport inhibitors on membrane potential and $[Ca^{2+}]_i$

It has been shown that hypoxia depolarizes type I cells leading to an influx of Ca^{2+} (Buckler, 1997) and that hypoxia and mitochondrial uncouplers inhibit a TASK-like background K^+ current (Buckler, 1997; Buckler & Vaughan-Jones, 1998; Buckler *et al.* 2000). We therefore investigated whether other mitochondrial inhibitors would also depolarize type I cells. To this end membrane potentials were recorded from cells in the current clamp configuration and $[Ca^{2+}]_i$ was simultaneously measured. NaCN at 2 mM depolarized cells from a resting membrane potential of -59.8 ± 1.8 mV to -41.7 ± 1.1 mV ($n = 10$, $P < 0.0001$, see Fig. 5*A* for an example); this depolarization was accompanied by a rapid and robust rise in intracellular Ca^{2+}. This NaCN-induced rise in $[Ca^{2+}]_i$ was substantially attenuated by voltage clamping the cells to -70 mV (rise in $[Ca^{2+}]_i$ in current clamp = 496 ± 39 nM; rise in $[Ca^{2+}]_i$

in voltage clamp = 79 ± 13 nM, $n = 5$, $P < 0.005$, see Fig. 5*A* and *B*). Rotenone at 1 μM also rapidly depolarized type I cells from -49.4 ± 2.9 mV to -26.4 ± 1.1 mV ($n = 9$, $P < 0.0002$, see Fig. 6*A*). Again this depolarization was accompanied by a sharp rise in $[Ca^{2+}]_i$ that was attenuated by $74.3 \pm 4.8\%$ ($n = 9$, $P < 0.005$, see Fig. 6) when the cells were clamped at -70 mV. The cells were switched to voltage clamp conditions during the depolarization rather than the method employed with the previous NaCN experiments due to the slow reversibility of the rotenone effect.

Effects of electron transport inhibitors and oligomycin on membrane currents

Under voltage clamp conditions voltage ramps from -100 to -40 mV were applied to cells at 2 Hz. Generation of current–voltage relationships from the applied voltage ramps (Fig. 7*A*) revealed that bath application of 1.0 μM rotenone significantly reduced type I cell membrane conductance. Membrane conductance, measured between -60 and -50 mV, was reduced from 285 ± 31 pS (control) to 108 ± 49 pS in the presence of rotenone ($n = 8$, $P < 0.002$). Similar results were also seen with the other two electron transport inhibitors and with oligomycin.
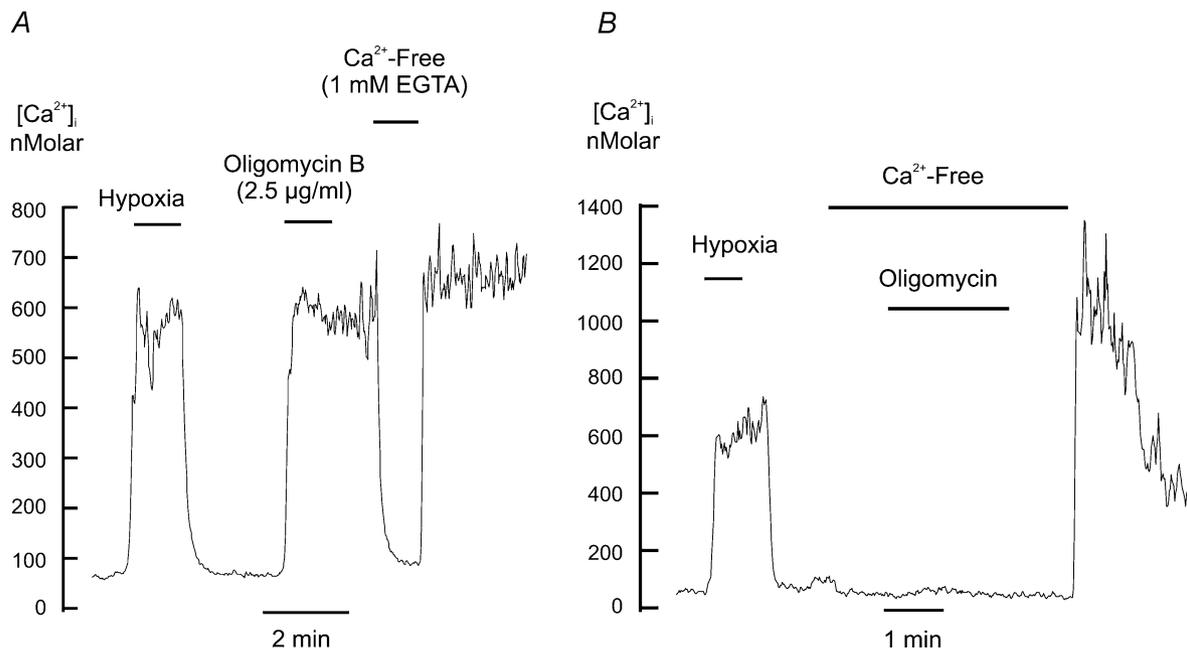

Figure 4. Effect of ATP synthase blockade on $[Ca^{2+}]_i$ in type I cells

Effects of hypoxia and oligomycin on $[Ca^{2+}]_i$ (measured using indo-1) in type I cells. *A*, effect of removing extracellular calcium after application of oligomycin. *B*, effect of removing extracellular calcium prior to and during the application of oligomycin. Note that in the presence of extracellular calcium oligomycin induced a large rise in $[Ca^{2+}]_i$ that was maintained following oligomycin removal. Removal of extracellular calcium prevented or reversed this oligomycin induced rise in $[Ca^{2+}]_i$.

Myxothiazol at 100 nM reduced cell conductance from 312 ± 43 pS to 30 ± 62 pS ($n = 6$, $P < 0.01$, Fig. 7B). NaCN at 2.0 mM reduced cell conductance from 359 ± 57 pS to 136 ± 34 pS ($n = 7$, $P < 0.005$, Fig. 7C). Oligomycin at 2.5 μg ml^{-1} reduced type I cell conductance from 324 ± 28 pS to 105 ± 24 pS ($n = 8$, $P < 0.0001$, Fig. 7D). In addition to the decrease in membrane conductance, application of mitochondrial inhibitors also resulted in the loss of any zero current potential over the voltage range tested indicating that all four mitochondrial inhibitors may be expected to depolarize type I cells (see Fig. 7).

In order to ascertain whether the reduction of membrane conductance caused by all four of these mitochondrial inhibitors was mediated via the inhibition of the TASK-like background K$^+$ current we carried out experiments at two different concentrations of extracellular $[K^+]_o$. Control I–V relationships were first determined in the presence of 4.5 mM $[K^+]_o$ and in the presence of 20 mM $[K^+]_o$. The mitochondrial inhibitor was then applied and I–V relationships were re-determined in the presence of both 4.5 mM and 20 mM $[K^+]_o$ (see Fig. 8A). Inhibitor-sensitive current was then calculated by

subtracting the I–V relationship observed in the presence of the mitochondrial inhibitor from the control I–V relationship at each level of extracellular K$^+$. All four inhibitor-sensitive currents (see Fig. 8B–E) were shifted to a more negative range in 20 mM K$^+$ compared to 4.5 mM K$^+$ and reversal potentials, where evident (Fig. 8C and D), were shifted in a positive direction along the voltage axis indicating that a proportion of the mitochondrial inhibitor-sensitive current is carried by K$^+$ ions. It was noted however, that the reversal potentials for a number of inhibitor-sensitive currents were negative to those expected for a pure K$^+$ current. A reversal potential for both oligomycin and rotenone was not obtained under control conditions over the voltage range tested in this series of experiments (but see Figs 7D and 9A) and in 20 mM K$^+$ were negative to the equilibrium potential for potassium ions ($E_K = -55$ mV; reversal potentials for rotenone $= -93.1$ mV, 3 currents failed to reverse, $n = 8$; reversal potential for oligomycin $= -77.0 \pm 6.6$ mV, $n = 8$). The reversal potential for the myxothiazol-sensitive current obtained under control conditions (-90.2 mV, $n = 6$, 4 currents failed to reverse) was close to the predicted
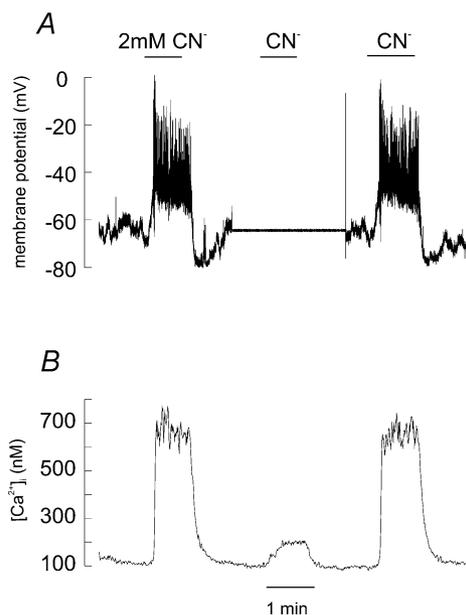
Figure 5. Effects of NaCN on resting membrane potential and [Ca^{2+}]$_i$ in a type I cell
A, perforated patch recording of membrane potential. Experiment starts and finishes in current clamp mode and shows a rapid membrane depolarization in response to NaCN application (nominally 2 mM). In the middle of the experiment (where the membrane potential trace is flat) the cell was voltage clamped at -70 mV and cyanide was reapplied. *B*, simultaneous recording of [Ca^{2+}]$_i$ from the same cell (using indo-1) showing that the rise in [Ca^{2+}]$_i$ in response to NaCN was greatly attenuated when the cell was voltage clamped at -70 mV.
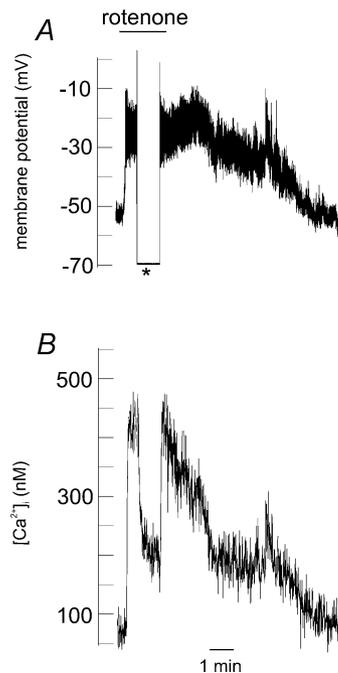
Figure 6. Effects of 1 μm rotenone on resting membrane potential and [Ca^{2+}]$_i$ in a type I cell
*A*, perforated patch recording of membrane potential in a type I cell. The asterisk denotes a switch from voltage recording (current clamp) mode to voltage clamping the membrane potential to -70 mV. Application of rotenone causes an abrupt membrane depolarization. *B*, simultaneous recording of [Ca^{2+}]$_i$ (using indo-1) from the same cell; note the rapid fall in [Ca^{2+}]$_i$ upon on voltage clamping the cell to -70 mV.

E_K of -96 mV, but that in 20 mM K$^+$ (-77.8 mV) was again negative to E_K. The control reversal potential for the NaCN-sensitive current was -78.7 mV ($n = 7$, 2 currents failed to reverse) and in 20 mM K$^+$ was -48.0 ± 2.2 mV ($n = 7$).

We also estimated potassium conductance at -70 mV from the change in holding current upon switching from 4.5 mM K$^+$ to 20 mM K$^+$, i.e. $g_K = (I_{4.5} - I_{20})/(E_{K20} - E_{K4.5})$, in the presence and absence of inhibitor, where $I_{4.5}$ = membrane current at -70 mV in 4.5 mM [K$^+$] Tyrode solution, I_{20} = membrane current at -70 mV in 20 mM [K$^+$] Tyrode solution, $E_{K4.5}$ = potassium equilibrium potential in 4.5 mM [K$^+$] Tyrode solution, and E_{K20} = potassium equilibrium potential in 20 mM [K$^+$] Tyrode

solution. (Note this calculation assumes that g_K remains constant with change in [K]$_o$. There is, however, evidence that background K$^+$ channels in type I cells display GHK-type rectification (Williams & Buckler, 2004). The values for g_K calculated here may therefore tend to overestimate the true g_K in 4.5 mM [K$^+$]$_o$ (and underestimate g_K in 20 mM [K$^+$]$_o$).)

From these values it was possible to calculate the percentage inhibition of resting K$^+$ conductance by each inhibitor. Rotenone (1.0 μM) reduced resting K$^+$ conductance by approximately 42% from 224 ± 22.9 pS to 129.5 ± 17.7 pS ($n = 8$, $P < 0.0003$). Myxothiazol (0.1 μM) reduced resting K$^+$ conductance by 31% from 225.5 ± 15.2 pS to 159.5 ± 18.3 pS ($n = 8$, $P < 0.02$). NaCN
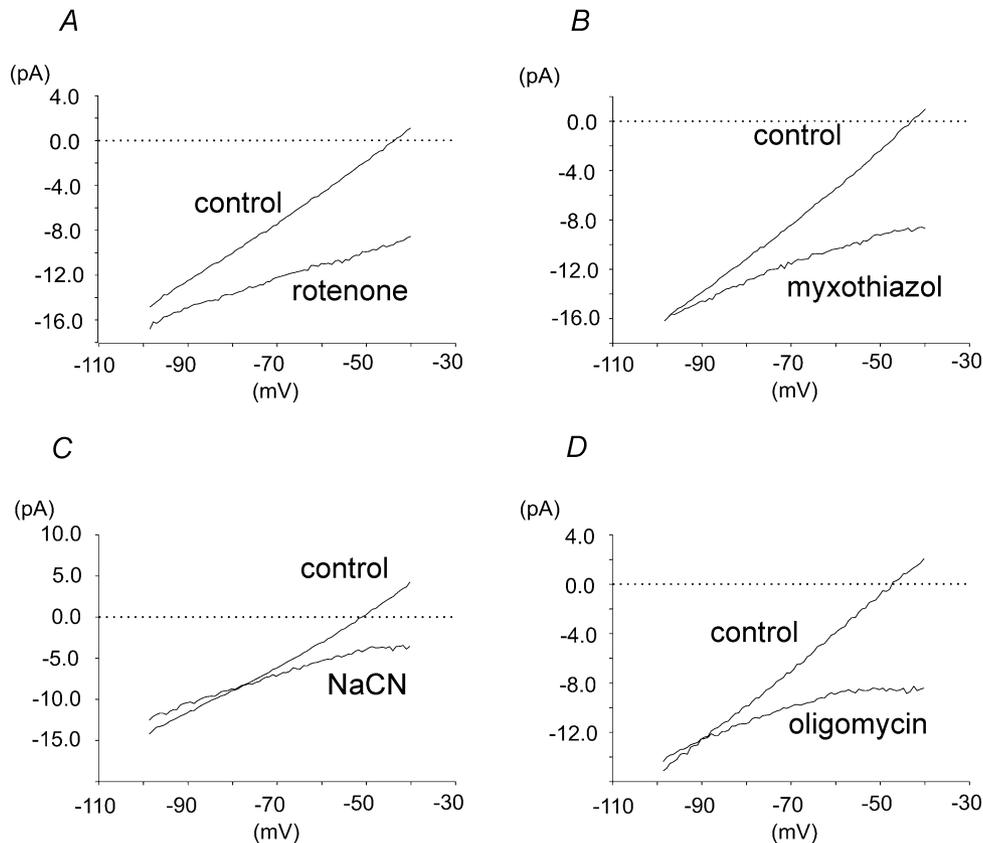

Figure 7. Effects of electron transport inhibitors and an ATP synthase inhibitor on membrane currents in type I cells
A, mean whole cell current–voltage (*I*–*V*) relationships for type I cells in the presence and absence of rotenone 1 μM ($n = 8$). *B*, mean whole cell *I*–*V* relationships in the presence and absence of myxothiazol 100 nM ($n = 6$). *C*, mean whole cell *I*–*V* relationships in the presence and absence of NaCN 2 mM ($n = 7$). *D*, mean whole cell *I*–*V* relationships in the presence and absence of oligomycin 2.5 μg ml^{-1} ($n = 8$). The slope of the above *I*–*V* plots give an indication of resting membrane conductance. The potential at which the *I*–*V* plots transect the zero current axis (the zero current potential) corresponds to the predicted resting membrane potential. Note that the *I*–*V* relationships obtained in the presence of all four mitochondrial inhibitors show not only a decrease in the resting membrane conductance but also the absence of a zero current potential within the voltage range tested (indicating that all four drugs will cause membrane depolarization).

(2 mM) caused a 63% reduction in resting K^+ conductance from 419.8 ± 57.6 pS to 166.9 ± 30.3 pS ($n = 7$, $P < 0.0003$) and finally oligomycin (2.5 μg ml^{-1}) caused a 39% reduction in K^+ conductance from 217.5

$\pm$ 36.9 pS to 118.6 ± 9.8 pS ($n = 8$, $P < 0.02$). Anoxia also inhibits resting membrane conductance to potassium ions at -70 mV by 55% (from 406 ± 66 pS to 181 ± 38 pS) as do mitochondrial uncouplers (by
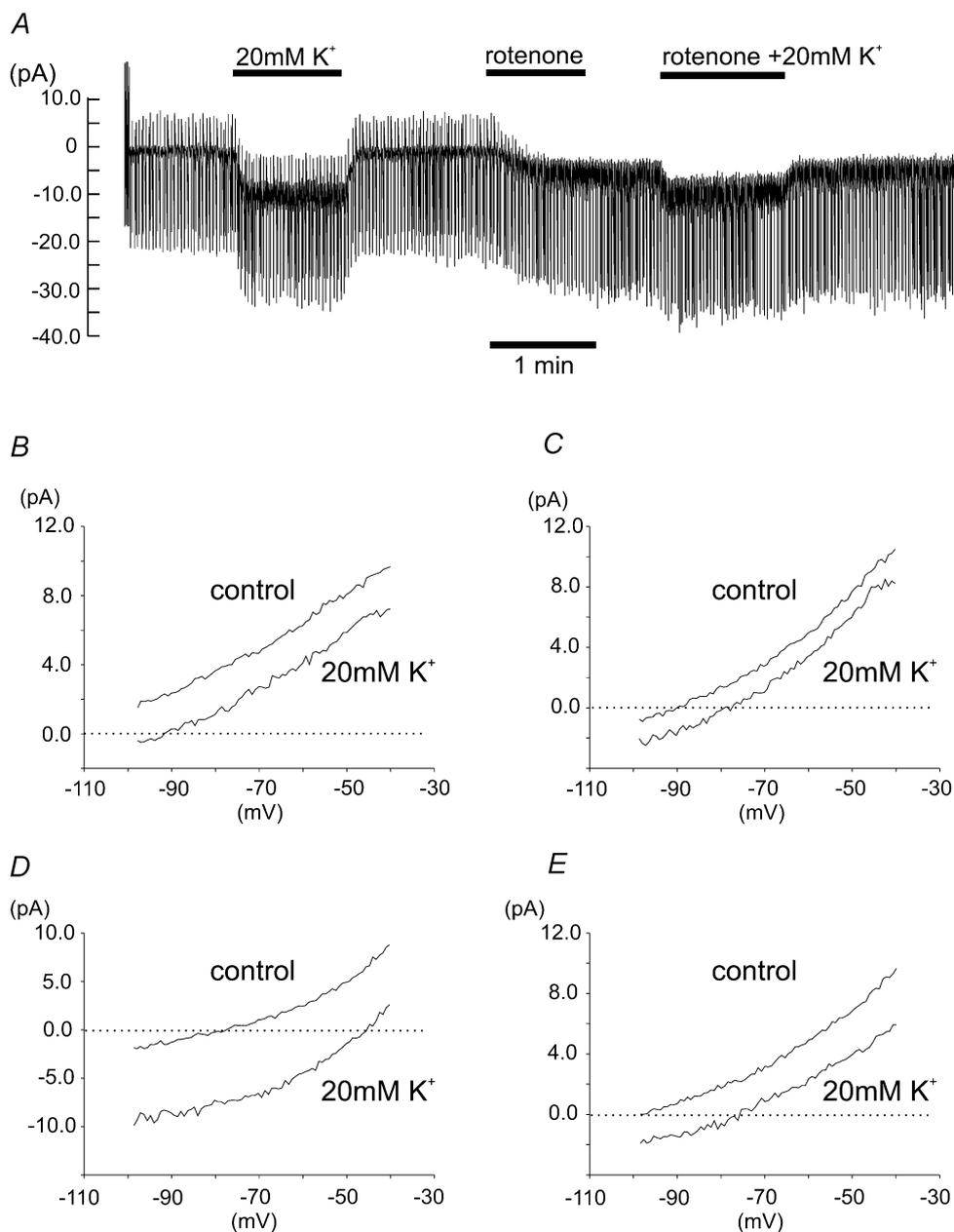
Figure 8. Current–voltage relationships of inhibitor-sensitive currents
A, an example of membrane current recorded from a type I cell voltage clamped at a holding potential of -70 mV and subjected to repetitive voltage ramps from -100 mV to -40 mV at 0.5 Hz. The effects of high K^+ (20 mM), rotenone and rotenone in the presence of high K^+ are shown. *B*, mean *I–V* relationships of rotenone-sensitive currents (1 μM, $n = 8$) obtained by subtracting the *I–V* relationship obtained in the presence of rotenone from the control *I–V* relationship in both normal extracellular K^+ (4.5 mM) and at elevated extracellular K^+ (20 mM). *C*, mean *I–V* relationships of myxothiazol-sensitive currents (100 nM, $n = 6$) at normal and elevated extracellular K^+. *D*, mean *I–V* relationships of NaCN-sensitive currents (2 mM, $n = 7$). *E*, mean *I–V* relationships of oligomycin-sensitive currents (2.5 μg ml^{-1}, $n = 8$). Note the depolarizing, inward, shift in all the inhibitor-sensitive currents when extracellular K^+ was elevated to 20 mM.

56%, see Buckler & Vaughan-Jones, 1998). Thus both mitochondrial inhibitors and anoxia cause a comparable reduction in the resting membrane conductance to potassium ions. It should be noted, however, that none of these compounds completely inhibit resting K^+ conductance. Taken together, these data show that a substantial proportion of the mitochondrial inhibitor-sensitive current is clearly carried by K^+ ions although other membrane currents may also be modulated.

It was also noted during the course of these voltage clamp experiments that application of rotenone, myxothiazol, CN^- or oligomycin caused only a very small rise in intracellular calcium (data not shown) compared to that seen in cells not subject to voltage-clamp control of membrane potential. This observation further supports the conclusion that the rise in $[Ca^{2+}]_i$ normally seen with these compounds is primarily mediated via membrane depolarization and voltage gated calcium entry (the origin of the residual small rise in $[Ca^{2+}]_i$ seen in voltage clamp will be discussed later).

Effect of hypoxia on membrane currents in combination with electron transport inhibitors and the mitochondrial uncoupler FCCP

The above, and previously published data (Buckler & Vaughan-Jones, 1998), indicate that inhibitors of oxidative phosphorylation have similar effects on type I cells to those of hypoxia. In order to determine whether metabolic inhibitors and hypoxia modulate membrane currents through a common mechanism we investigated the effects of hypoxia on membrane currents in the presence of
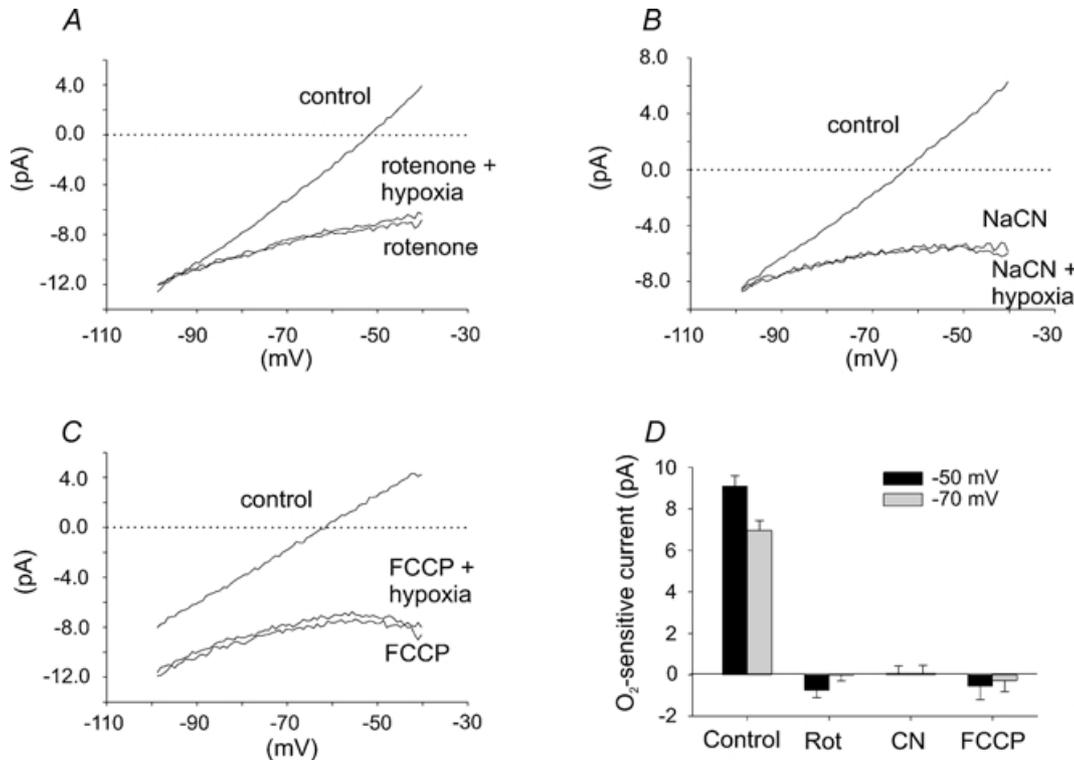
Figure 9. Effect of hypoxia, in combination with application of rotenone, NaCN and FCCP, on membrane currents in type I cells

A, mean *I–V* relationships (*n* = 6) in control conditions, in the presence of 1 µM rotenone and in the presence of 1 µM rotenone and hypoxia (6 Torr). *B*, mean *I–V* relationships (*n* = 6) in control conditions, in the presence of 2 mM NaCN and in the presence of 2 mM NaCN and hypoxia (6 Torr). *C*, mean *I–V* relationships (*n* = 4) in the presence of 1 µM FCCP and in the presence of 1 µM FCCP and hypoxia (6 Torr). Note the absence of any additional effect of hypoxia upon membrane currents in the presence of rotenone, cyanide or FCCP. *D*, comparison of mean (+ S.E.M.) oxygen-sensitive current measured at two different potentials −50 mV (black bars) and −70 mV (grey bars) under control conditions (control − hypoxia; *n* = 46) with that determined in the presence of rotenone (rotenone − hypoxia and rotenone; *n* = 6), cyanide (cyanide − hypoxia and cyanide; *n* = 6) and FCCP (FCCP − hypoxia and FCCP; *n* = 4). Note that the magnitude of the oxygen-sensitive current measured in the presence of mitochondrial inhibitors was negligible compared to that determined under control conditions.

rotenone, cyanide and FCCP. *I–V* relationships were constructed in control conditions, in the presence of 1 μM rotenone, 2 mM NaCN and 1 μM FCCP and in the presence of each compound in a hypoxic solution (6 Torr). As can be seen from the data presented in Fig. 9, hypoxia (6 Torr) had no significant effect on membrane currents in the presence of rotenone ($n = 6$, Fig. 9*A*), NaCN ($n = 6$, Fig. 9*B*) or FCCP ($n = 4$, Fig. 9*C*). In contrast, in the absence of these mitochondrial inhibitors, hypoxia substantially inhibits background K^+ currents (see, e.g. Buckler, 1997; Buckler *et al.* 2000); Fig. 9*D* shows a quantitative comparison of the relative magnitudes of the O_2-sensitive current recorded under control conditions with that seen in the presence of the above mitochondrial inhibitors at two potentials, -50 and -70 mV (at both potentials the difference between the control O_2-sensitive current, and that recorded in the presence of any of the three mitochondrial inhibitors, was significantly different $P < 0.0001$). Thus inhibitors of oxidative phosphorylation abolish hypoxic sensitivity of background K^+ current. It is important to note that these results contrast with the work of Searle *et al.* (2002) who suggested that rotenone may be a non-selective K^+ channel inhibitor. However, in the study by Searle *et al.* (2002), the effects of rotenone were reversed by hypoxia, an effect which we failed to observe in type I cells.

Discussion

The mechanism by which carotid body type I cells transduce a fall in oxygen tension to excitatory transmitter release is not yet fully understood, although a number of hypotheses have been advanced (see Prabhakar, 2000 for a review). The data presented here support the hypothesis that mitochondria play a critical role in oxygen sensing.

The idea that the mitochondria are involved in the sensing of oxygen changes in the carotid body is by no means new. Mills & Jobsis (1971) presented evidence for a specialized cytochrome aa_3 in carotid body mitochondria and subsequently elegant work by Duchen & Biscoe (1992*a*) demonstrated that mitochondrial function in type I cells was unusually sensitive to oxygen. Duchen & Biscoe (1992*b*) were also the first to demonstrate that interrupting electron transport, blocking the F_1–F_0-ATPase or chemically uncoupling the mitochondria resulted in a rise in intracellular Ca^{2+}. They concluded that the rise in intracellular Ca^{2+} in response to both hypoxia and inhibitors of mitochondrial metabolism was mediated by a depolarization of mitochondrial membrane potential and Ca^{2+} release from mitochondria and other intracellular sources (Duchen & Biscoe, 1992*b*). It was subsequently demonstrated that the Ca^{2+} response to

hypoxia was mediated primarily via voltage-gated calcium entry, rather than release from internal stores (Buckler & Vaughan-Jones, 1994). Consequently a common current view is that the mechanisms by which hypoxia and mitochondrial inhibitors excite the carotid body are different with hypoxia promoting voltage-gated calcium entry and metabolic inhibitors promoting calcium release (Lopez-Barneo *et al.* 2001). In contrast, the data we present here reveal that inhibition of mitochondrial function excites carotid body type I cells in a manner very similar to that seen with hypoxia.

Effects of Mitochondrial inhibitors on calcium signalling in type I cells

Pharmacological inhibition of mitochondrial electron transport at three different points in the electron transport chain using rotenone (complex I), myxothiazol, antimycin A (complex III) or cyanide (complex IV) caused a rapid rise in intracellular Ca^{2+}. With all four electron transport inhibitors the calcium response was primarily dependent upon extracellular calcium. Oligomycin, which is known to excite the carotid body *in vivo* (Mulligan *et al.* 1981), also evoked a rapid increase in type I cell $[Ca^{2+}]_i$ that was again dependent upon extracellular calcium. Thus inhibition of mitochondrial electron transport, or inhibition of the F_1–F_0-ATPase, or mitochondrial uncoupling (Buckler & Vaughan-Jones, 1998) have similar effects on type I cell $[Ca^{2+}]_i$ to those observed with hypoxia, i.e. a rapid rise in $[Ca^{2+}]_i$ that is largely mediated by calcium influx. The only significant differences we observed were in the degree of reversibility of some of these agents with the effects of uncouplers (Buckler & Vaughan-Jones, 1998) and CN^- being rapidly reversible, rotenone being slowly reversible and oligomycin, antimycin A and myxothiazol being essentially irreversible.

Hypoxia and mitochondrial uncouplers evoke a rise in intracellular calcium by inducing membrane depolarization and voltage-gated calcium entry (Buckler & Vaughan-Jones, 1994, 1998). We therefore investigated the effects of both cyanide and rotenone on membrane potential in type I cells using perforated patch current clamp. Both compounds caused a rapid cell depolarization and a rapid rise in $[Ca^{2+}]_i$. The rise in Ca^{2+} was significantly attenuated when the cells were voltage clamped at -70 mV. We also observed that both myxothiazol and oligomycin caused only a very small increase in $[Ca^{2+}]_i$ during other voltage-clamp experiments (i.e. during application of voltage ramps to determine the effects of these compounds upon membrane conductance). Thus the rise in $[Ca^{2+}]_i$ in response to

inhibition of electron transport or inhibition of the ATP synthase is also *primarily* mediated by voltage gated calcium entry. There may however, also be a small proportion of the rise in $[Ca^{2+}]_i$ that is not due to voltage-gated Ca^{2+} entry since a small rise in $[Ca^{2+}]_i$ persisted both in voltage clamped cells and in the absence of extracellular calcium (see Figs 3*C* and 5*B*). A similar small, voltage- and Ca^{2+}_o- independent rise in $[Ca^{2+}]_i$ has also been observed with both anoxia and mitochondrial inhibitors (Buckler & Vaughan-Jones, 1994, 1998). This component of the Ca^{2+} signal presumably results from Ca^{2+} release from internal stores, e.g. mitochondria or the endoplasmic reticulum. A recent study, however, clearly showed that agents that empty intracellular Ca^{2+} stores (caffeine, ryanodine, thapsigargin and cyclopiazonic acid) were unable to elicit changes in $[Ca^{2+}]_i$ of sufficient magnitude to initiate neurosecretion from intact carotid body or to modulate Ca^{2+} transients evoked by cell depolarization in isolated type I cells (Vicario *et al.* 2000). Consequently although both hypoxia and mitochondrial inhibitors may evoke some degree of Ca^{2+} release from internal stores this is likely to be of minor significance to Ca^{2+} signalling overall or consequent neurotransmitter release.

In conclusion, from the perspective of calcium signalling events, the mechanisms of action of a wide range of mitochondrial inhibitors are indistinguishable from that of hypoxia, i.e. membrane depolarization followed by voltage-gated calcium entry. Calcium release from internal, unidentified, stores although detectable plays only a minor and probably insignificant role.

Effects of mitochondrial inhibitors on membrane currents

Previous work in this laboratory has shown that neonatal rat type I cells possess O_2-sensitive TASK-1-like K^+ currents; these currents are inhibited by both hypoxia and mitochondrial uncouplers (Buckler, 1997; Buckler & Vaughan-Jones, 1998) resulting in membrane depolarization and Ca^{2+} entry. In order to ascertain whether the mitochondrial inhibitors used here could also be inhibiting the TASK-1-like K^+ current, membrane currents were observed under voltage clamp conditions. Rotenone, myxothiazol, cyanide and oligomycin all significantly inhibited a background K^+ current (see Figs 7 and 8) an effect reminiscent of that seen with hypoxia. The observation that hypoxia had no additional effect in the presence of cyanide, rotenone and the uncoupler FCCP, further argues that inhibitors of mitochondrial

metabolism are inhibiting the same background K^+ current as that modulated by hypoxia.

In addition to their effects upon background K^+ current, inhibitors of mitochondrial metabolism may well modulate other ionic currents as well. As we have previously reported for mitochondrial uncouplers, the reversal potentials for the inhibitor-sensitive currents of some of the compounds tested here showed some deviation from the predicted reversal potentials for a pure K^+ current (see results). Indeed it is known that in adult guinea-pig chromaffin cells both anoxia and cyanide can activate non-selective cation channels (Inoue *et al.* 1998, 1999). A similar action in the type I cells would inevitably shift the reversal potential of total inhibitor-sensitive current to more negative potentials. The identification of additional currents that may be activated by mitochondrial inhibitors is currently under investigation.

Mechanism of action of mitochondrial inhibitors

A key question posed by the data presented in this paper is how does inhibition of mitochondrial function lead to modulation of ion channel activity. In principle there are a number of facets of mitochondrial activity that could be altered by metabolic inhibitors. Here we consider some of the main ones and whether they could account for our observed results.

ATP synthesis. All inhibitors studied here would be expected to inhibit the synthesis of ATP and most (i.e. all bar oligomycin) may be expected to accelerate its consumption via reversal of the ATP synthase secondary to mitochondrial depolarization. It has recently been reported that the activity of background K^+ channels in inside out patches is increased by the application of solutions containing ATP to the intracellular aspect of the membrane patch (Williams & Buckler, 2004). These observations suggest that, in principle, changes in cytosolic ATP could provide a link between background K^+ channel activity and mitochondrial energy metabolism. Whether inhibition of mitochondrial energy metabolism leads directly to significant changes in cytosolic ATP (or ADP, AMP or P_i) in type I cells remains to be determined, but such studies in chromaffin cells suggest that mitochondrial inhibition does lead to rapid changes cytosolic ATP levels (Inoue *et al.* 2002). Changes in *mitochondrial* ATP levels/synthesis could also alter the production of an unknown signalling molecule that is involved in the regulation of ion channel function.

Mitochondrial depolarization. In their original papers Duchen & Biscoe (1992*a,b*) postulated that mitochondrial depolarization could be the key event leading to the generation of a calcium signal (originally postulated to be via Ca^{2+} release from internal stores). The principle argument against this is that whilst electron transport inhibitors and uncouplers depolarize mitochondrial membrane potential, oligomycin causes a hyperpolarization (Duchen & Biscoe, 1992*b*).

Change in mitochondrial redox NAD^+/NADH. Electron transport inhibitors will decrease NAD^+/NADH but uncouplers have the opposite effect. This was demonstrated in rabbit type I cells by Duchen & Biscoe (1992*a*) and we have observed the same effect in neonatal rat type I cells in which anoxia increases and FCCP decreases NADH autofluoresence (data not shown). Consequently we would reject changes in NAD^+/NADH levels as a likely signalling mechanism.

Changes in reactive oxygen species (ROS) generation. A number of studies have suggested that modulation of mitochondrial superoxide (O_2^-) production plays a role in hypoxic pulmonary vasoconstriction in the lung and in the inhibition of potassium channels in pulmonary vascular myocytes. There are, however, two diametrically opposed views, (1) that hypoxia increases ROS (Waypa *et al.* 2001) and (2) that hypoxia decreases ROS (Archer *et al.* 1993). This raises the question as to whether changes in ROS production could be involved in mediating the response of type I cells to metabolic inhibitors.

O_2^- is generated at two points within the mitochondrial respiratory chain, complex I (Turrens & Boveris, 1980) and complex III (Cadenas *et al.* 1977; Turrens *et al.* 1985; Turrens, 1997). Various inhibitors of electron transport including cyanide, rotenone and myxothiazol promote O_2^- production by NADH dehydrogenase (Turrens & Boveris, 1980). Antimycin A also increases O_2^- production at complex III (see Turrens, 1997 and refs therein). In contrast both rotenone and myxothiazol inhibit O_2^- production by complex III (Turrens *et al.* 1985), as does cyanide ($IC_{50} = 30 \mu M$; Cadenas & Boveris, 1980). Mitochondrial uncouplers also decrease mitochondrial O_2^- production (Skulachev, 1996; Boveris & Chance, 1973); O_2^- production at complex III is believed to be highly sensitive to mitochondrial membrane potential (Demin *et al.* 1998). Whereas oligomycin is reported to have no effect upon O_2^- production (Boveris & Chance, 1973). Thus the range of mitochondrial inhibitors used in this study, all of which mediate an increase in type

I cell intracellular calcium, are expected to have diverse effects upon mitochondrial O_2^- production. Furthermore any increase in O_2^- production by complex I in response to electron transport inhibition should be antagonized by a reduction in oxygen levels (O_2^- production at both complex I and III is a non-enzymatic process that should be governed by the laws of mass action, Turrens, 1997). We saw no such antagonism of the effects of either rotenone or cyanide when P_{O_2} was lowered from 150 Torr to around 5 Torr (Fig. 9). Thus the effects of all the inhibitors of mitochondrial metabolism used in this study cannot be accounted for by either an increase or decrease in O_2^- production.

In summary the most likely candidate for mediating the effects of mitochondrial inhibitors seems to be a reduction in mitochondrial ATP synthesis. The above discussion, and our conclusion, is however, based upon two assumptions (a) that there is only one unifying mechanism responsible for mediating the effects of electron transport inhibition, uncoupling, and inhibition of ATP synthase and (b) that none of the compounds used are having 'non-specific' effects.

Role of mitochondria in O₂ sensing

The data presented in this, and previous studies (Buckler, 1997; Buckler & Vaughan-Jones, 1998) show that hypoxia and many different inhibitors of oxidative phosphorylation have very similar effects upon isolated type I cells. If the transduction pathways for hypoxia and mitochondrial inhibitors are independent, one might expect the effects of hypoxia and mitochondrial inhibitors to be additive. It has recently been reported that the secretory response to hypoxia in carotid body slices is additive to that evoked by cyanide, myxothiazol or antimycin A; but that rotenone selectively occludes sensitivity to hypoxia (Ortega-Sáenz *et al.* 2003). On the basis of these observations it has been suggested that mitochondrial electron transport flow is not directly involved in acute oxygen sensing but that a rotenone-sensitive molecule is critical to oxygen sensing (Ortega-Sáenz *et al.* 2003). It is important to note, however, that additivity between the effects of hypoxia and of electron transport inhibitors can only be considered definitive evidence that oxygen sensing is independent from electron transport if the inhibition of electron transport is complete. We have found that the volatility of cyanide in buffered solutions requires the use of very high initial concentrations of CN^- and regular replacement of such solutions if full inhibition of electron transport is to be achieved (Fig. 1). We have also observed that the effects

of some electron transport inhibitors, e.g. myxothiazol, are slow in onset such that several minutes' exposure may be required before electron transport is fully inhibited (data not shown). In our study, we have found no evidence for additivity between hypoxia and the effects of cyanide, rotenone or the uncoupler FCCP on background K^+ currents (see Fig. 9*A*, *B* and *C*). Similar observations have also been made in the intact carotid body in which the increase in chemosensory nerve discharge in response to hypoxia is abolished in the presence of antimycin A, oligomycin or FCCP (Mulligan *et al.* 1981; Mulligan & Lahiri, 1982; Mosqueira & Iturriaga, 2002). This mutual exclusivity between the actions of mitochondrial inhibitors and hypoxia suggests that they share some common mode of action.

In order to explore a possible role for mitochondrial H_2O_2 production in mediating the response to hypoxia we investigated the effects of applying exogenous H_2O_2 upon oxygen sensing. Brief application of 30 μM H_2O_2, a concentration several orders of magnitude higher than that expected to be normally present within cells (Cadenas & Davies, 2000; Chance *et al.* 1979), had no effect upon intracellular calcium alone ($[Ca^{2+}]_i = 31 \pm 2$ nM, control and 30 ± 3 nM in the presence of H_2O_2; $n = 6$, n.s.) and neither did it antagonize the $[Ca^{2+}]_i$ response to a hypoxic stimulus (hypoxia evoked rise in $[Ca^{2+}]_i = 270 \pm 68$ nM, control, and 267 ± 56 nM in the presence of H_2O_2; $n = 6$, n.s.); see Fig. 10. Thus neither increase nor decrease in H_2O_2 generation would appear to play a major role in mediating the acute effects of hypoxia upon type I cells. With respect to the suggestion that a rotenone-sensitive molecule critically participates in oxygen sensing (Ortega-Sáenz *et al.* 2003), we have found that whilst rotenone does indeed occlude oxygen sensitivity it is not unique in this respect. Moreover, oxygen sensitivity can be restored in the continued presence of rotenone by the addition of the electron donor TMPD. These data suggest that if a rotenone-sensitive molecule does have a specific role in oxygen sensing, the site of action of rotenone must be distinct from the site of interaction of oxygen. These data are, however, consistent with the effect of rotenone being mediated through inhibition of mitochondrial electron transport as TMPD can restore electron transport by donating electrons to cytochrome *c*. At present therefore we can only suggest two general mechanisms which could account for the apparent role of mitochondria in oxygen sensing.

(1) The oxygen-sensing pathway may be dependent upon mitochondrial energy metabolism. For example ATP may be required to generate the signal that indicates the presence of oxygen. Thus either a lack of ATP (due

to metabolic inhibition) or a lack of oxygen will result in the failure to generate this signalling molecule and the activation of a common response. In this context it is of interest to note that oxygen sensing in some prokaryotes is mediated via oxygen-sensitive kinases and phosphorylation cascades (see Bunn & Poyton, 1996, for a review).

(2) Oxygen sensing is a direct function of mitochondrial energy metabolism and ATP synthesis.

ATP synthesis and oxygen sensing. The hypothesis that oxygen sensing itself could be mediated via changes in ATP synthesis is not new but is controversial. The principle theoretical argument against a direct role for ATP in oxygen sensing is that the level of hypoxia required to significantly impair electron transport and ATP synthesis (approx <3 Torr) should be well below the physiological range for oxygen sensing. Optical measurements of mitochondrial membrane potential and NADH levels in isolated type I cells, however, have shown that type I cell mitochondria depolarize and NADH accumulates as P_{O_2} is reduced below about 60 Torr (Duchen & Biscoe, 1992*a*,*b*). This surprising sensitivity of type I cell mitochondrial function to hypoxia is comparable to that of key events in the oxygen signalling cascade (i.e. elevation of intracellular calcium, neurosecretion and inhibition of background K^+ currents; Buckler & Vaughan-Jones, 1994; Montoro *et al.* 1996; Buckler, 1997) and to estimates of oxygen sensitivity in the intact organ when expressed as a function of microvascular P_{O_2}, as opposed to arterial P_{O_2} (Lahiri *et al.* 1993). Thus the issue of whether mitochondria sense hypoxia through a reduced oxygen affinity cytochrome
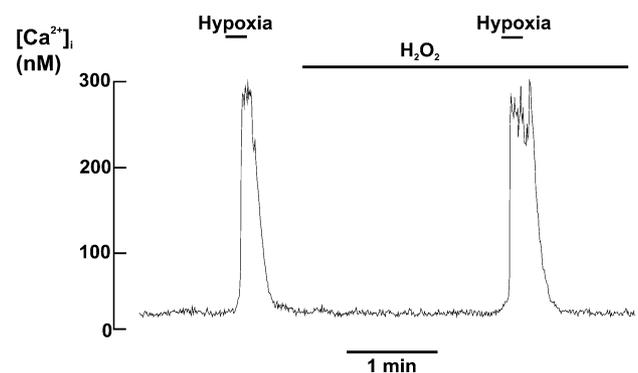
Figure 10. Effect of H_2O_2 on $[Ca^{2+}]_i$ in type I cells
Effect of hypoxia on intracellular calcium (indo-1) in the presence and absence of 30 μM H_2O_2. Note that H_2O_2 alone has no effect upon $[Ca^{2+}]_i$ nor does it antagonize the abrupt increase in $[Ca^{2+}]_i$ seen in response to a hypoxic stimulus.

oxidase and subsequent variation in mitochondrial ATP synthesis remains open.

In summary, the data presented in this paper suggests that the mitochondria play an essential role in the oxygen-dependent regulation of background K^+ channels in type I cells. Whether mitochondria act in an accessory role (e.g. by supplying ATP to the real oxygen sensing pathway) or form an integral part of the oxygen sensing pathway itself remains to be determined.

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Acknowledgements