

Rotenone selectively occludes sensitivity to hypoxia in rat carotid body glomus cells

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Carotid body glomus cells release transmitters in response to hypoxia due to the increase of excitability resulting from inhibition of O₂-regulated K⁺ channels. However, the mechanisms involved in the detection of changes of O₂ tension are unknown. We have studied the interaction between glomus cell O₂ sensitivity and inhibition of the mitochondrial electron transport chain (ETC) in a carotid body thin slice preparation in which catecholamine release from intact single glomus cells can be monitored by amperometry. Inhibition of the mitochondrial ETC at proximal and distal complexes induces external Ca²⁺-dependent catecholamine secretion. At saturating concentration of the ETC inhibitors, the cellular response to hypoxia is maintained. However, rotenone, a complex I blocker, selectively occludes the responsiveness to hypoxia of glomus cells in a dose-dependent manner. The effect of rotenone is mimicked by 1-methyl-4-phenylpyridinium ion (MPP⁺), an agent that binds to the same site as rotenone, but not by complex I inhibitors acting on different sites. In addition, the effect of rotenone is not prevented by incubation of the cells with succinate, a substrate of complex II. These data strongly suggest that sensitivity to hypoxia of carotid body glomus cells is not linked in a simple way to mitochondrial electron flow and that a rotenone (and MPP⁺)-sensitive molecule critically participates in acute oxygen sensing in the carotid body.

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Glomus cells are the major O₂-responsive elements in the carotid body. These are excitable neurosecretory cells that release transmitters in response to hypoxia to activate afferent sensory nerve fibres conveying the information to the respiratory centre (for recent review, see López-Barneo *et al.* 2001). It has been demonstrated that glomus cells sense O₂ tension (P_{O₂}) through regulation of membrane ion channels and several types of K⁺ channels whose activity is inhibited by low P_{O₂} have been described (López-Barneo *et al.* 1988; Delpiano & Hescheler, 1989; Peers, 1990; Stea & Nurse, 1991; Buckler, 1997). Reduction of K⁺ conductance by hypoxia causes cell depolarization and an increase of action potential firing frequency (López-López *et al.* 1989; Buckler & Vaughan-Jones, 1994; Wyatt & Peers, 1995; Montoro *et al.* 1996; Pérez-García *et al.* 2000), leading to extracellular Ca²⁺ influx through plasmalemmal voltage-gated channels (López-Barneo *et al.* 1993; Buckler & Vaughan-Jones, 1994; Ureña *et al.* 1994). Quantal catecholamine release induced by hypoxia in single glomus cells is absolutely dependent on extracellular Ca²⁺ influx (Ureña *et al.* 1994; Carpenter *et al.* 2000) and is mimicked by some K⁺-channel blockers (Pardal *et al.* 2000).

Despite the progress in the understanding of glomus cell electrophysiology and responsiveness to hypoxia, the molecular nature of the O₂ sensor remains unknown.

Because modulation of some K⁺ channels by P_{O₂} is maintained in excised patches (Ganformina & López-Barneo, 1991; Riesco-Fagundo *et al.* 2001) it was postulated that O₂ sensing in glomus cells depends on membrane-delimited mechanisms (see López-Barneo, 1994; Lewis *et al.* 2002). On the other hand, several investigators have traditionally considered mitochondria as the site for glomus cell O₂ sensing because, similarly to hypoxia, inhibitors of the electron transport chain (ETC) or mitochondrial uncouplers increase the afferent activity of the sinus nerve (see, for instance, Mills & Jöbsis, 1972; Mulligan *et al.* 1981). It has also been reported that cyanide and anoxia release Ca²⁺ from mitochondria in isolated rabbit glomus cells (Biscoe & Duchon, 1990; Duchon & Biscoe, 1992) and that mitochondrial uncouplers raise cytosolic Ca²⁺ and reduce background K⁺ permeability in rat carotid body cells (Buckler & Vaughan-Jones, 1998). Besides in the carotid body, mitochondria have also been postulated to participate in O₂ sensing in other acutely responding systems, such as pulmonary vascular smooth muscle (Archer *et al.* 1993; Leach *et al.* 2001; Waypa *et al.* 2001) or chromaffin cells (Mojet *et al.* 1997; Inoue *et al.* 2002).

A major limitation for the study of the carotid body O₂ sensors is the difficulty in obtaining a preparation with reproducible sensitivity to hypoxia, as this is easily altered

by dispersion of the cells (see López-Barneo *et al.* 1998). We have recently developed the carotid body thin slice preparation in which the response of glomus cells to low P_{O_2} can be studied in almost optimal physiological conditions (Pardal *et al.* 2000; Pardal & López-Barneo, 2002). Taking advantage of this technique we designed the present research to investigate whether sensitivity of intact glomus cells to hypoxia is altered by mitochondrial dysfunction. We report here that, as occurs with hypoxia, mitochondrial ETC inhibitors evoke an extracellular Ca^{2+} -dependent secretory response from glomus cells. Sensitivity to lowering P_{O_2} is not altered by blockade of mitochondrial electron flow, however responsiveness to hypoxia is selectively abolished by rotenone and 1-methyl-4-phenylpyridinium ion (MPP⁺). Thus the data suggest that a rotenone (and MPP⁺)-binding protein is part of the O_2 sensor. A preliminary account of part of this work has previously been published (Pardal & López-Barneo, 2000).

METHODS

Preparation of carotid body thin slices

Wistar rats of ages between postnatal days 10 and 30 were deeply anaesthetized by injection of 100 mg kg⁻¹ pentobarbital. Carotid bodies were dissected and placed on ice-cooled and O_2 -saturated modified Tyrode solution (mM: 148 NaCl, 2 KCl, 3 MgCl₂, 10 Hepes, 10 glucose, pH 7.4). Slices were taken after inclusion of the carotid bodies in agarose as described in Pardal *et al.* (2000). Slices 150 μ m thick were placed on 35 mm Petri dishes with culture medium and maintained at 37 °C in a 5% CO_2 incubator for 24–96 h before use. All experiments were performed according to the institutional animal care committee guidelines.

Experimental setup and measurement of secretion from single cells

For the experiments, a slice was transferred to the chamber (~0.2 ml volume) placed on the stage of an upright microscope (Axioscope, Zeiss, Göttingen, Germany) and continuously perfused by gravity (flow 1–2 ml min⁻¹) with a solution containing (mM): 117 NaCl, 4.5 KCl, 23 NaHCO₃, 1 MgCl₂, 2.5 CaCl₂, 5 glucose and 5 sucrose. The 'normoxic' solution was bubbled with a gas mixture of 5% CO_2 , 20% O_2 and 75% N_2 ($P_{O_2} \approx 150$ mmHg). The 'hypoxic' solution was bubbled with 5% CO_2 and 95% N_2 to reach a P_{O_2} in the chamber of ~20 mmHg. To attain lower P_{O_2} values in the chamber, the use of oxygen scavengers is required. As these agents interfere with the amperometric measurements they were not used in our study. Equilibration of solutions in the chamber, determined with an O_2 electrode, required ~40 s. In the high K^+ solutions, KCl replaced NaCl equimolarly. When external solution with 5 mM sodium succinate or methylsuccinate was used, the required amount of NaCl was removed. The pharmacological agents used were dissolved in distilled water, DMSO or ethanol as required. From these stock solutions they were added to the external solution at the desired concentration. At the concentrations used (lower than 1/1000 dilution), the vehicles (DMSO or ethanol) did not have any effect on glomus cell catecholamine secretion. Osmolality of solutions was ~300 mosmol kg⁻¹ and pH was 7.4. All the experiments were carried out at a temperature in the chamber of ~36 °C. Secretory events were recorded with a 10 μ m carbon-fibre electrode positioned near a cell under visual

control (Ureña *et al.* 1994; Pardal *et al.* 2000). Amperometric currents were recorded with an EPC-8 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfaltz, Germany), filtered at 100 Hz and digitized at 250 Hz before storage on computer. Data acquisition and analysis were done with an ITC-16 interface (Instrutech Corporation, NY, USA) and PULSE/PULSEFIT software (HEKA Electronics). Samples were statistically compared with a Student's *t* test and their differences were considered to be significant if $P < 0.05$. Unless otherwise noted, the data are expressed as means \pm S.E.M.

RESULTS

Electron transport chain inhibitors induce external Ca^{2+} -dependent secretion from glomus cells

The secretory response to hypoxia ($P_{O_2} \approx 20$ mmHg) of glomus cells in carotid body slices is shown in Fig. 1A. As described before (Pardal *et al.* 2000; Pardal & López-Barneo, 2002), low P_{O_2} induced spike-like quantal events corresponding to catecholamine release from individual vesicles. The cumulative secretion signal (lower trace in Fig. 1A) is a value of electric charge obtained by the sum of the time integral of successive amperometric events, which is proportional to the number of catecholamine molecules oxidized. To estimate the magnitude of the cell secretory responses to hypoxia or any other stimulus, we used the secretion rate (femtocoulombs (fC) min⁻¹) calculated as the amount of charge transferred to the recording electrode during the 60 s after the solutions were equilibrated in the recording chamber. In our initial set of experiments we investigated if, as occurs with hypoxia, blockade of mitochondrial ETC also induced secretion from glomus cells. We studied the effect of inhibition at complex I with rotenone, at complex II with thenoyltrifluoroacetone (TTFA), at complex III with myxothiazol or antimycin A (respectively proximal and distal inhibitors of this complex) and at complex IV with cyanide. These agents were used in a broad range of concentrations, however as our experimental protocol required the application of saturating concentrations of the drugs, the lowest concentrations used were at least 5 to 10 times the reported K_{50} values (see Degli Esposti, 1998; Vaux *et al.* 2001). All the ETC inhibitors induced an exocytotic response from glomus cells in 1–3 min (Fig. 1B–F; see also Fig. 3). The difference in the secretagogue potency of applied ETC inhibitors was not very marked as they were used at concentrations above saturation. The fact that mitochondrial inhibition did not induce rapid cellular degranulation and preserved the responses to hypoxia or high potassium, indicates that ETC inhibitors did not produce major non-specific pharmacological alterations of the cells. The mean areas of individual quantal events (in fC, mean \pm s.d.) triggered by the ETC inhibitors (rotenone: 40 \pm 18, $n = 245$ spikes in 5 cells; myxothiazol: 42 \pm 30, $n = 102$ spikes in 7 cells; antimycin A: 40 \pm 22, $n = 132$ spikes in 5 cells; cyanide: 48 \pm 33, $n = 116$ spikes in

7 cells) were not significantly different ($P > 0.1$) from the average values estimated with events evoked by hypoxia (43 ± 26 , $n = 576$ spikes in 14 cells). Therefore, hypoxia and the ETC inhibitors appeared to induce the release of a common type of catecholaminergic vesicle. Secretion evoked by all the ETC inhibitors was completely abolished by blockade of membrane Ca^{2+} channels with Cd^{2+} (Fig. 1B–F). Only the secretory response induced by concentrations of cyanide in the millimolar range was partially maintained in the presence of 0.3 mM extracellular Cd^{2+} , thus suggesting Ca^{2+} release from intracellular stores. These data indicate that, as described in cells exposed to hypoxia (Ureña *et al.* 1994; Pardal *et al.* 2000; Carpenter *et al.* 2000), activation

of carotid body glomus cells by mitochondrial ETC inhibitors largely depends on extracellular Ca^{2+} influx through channels of the plasma membrane.

Responsiveness of glomus cells to hypoxia is occluded by rotenone but unaffected by other electron transport chain inhibitors

The interaction between hypoxia and the mitochondrial electron flow was studied in cells exposed to low P_{O_2} before and during application of ETC inhibitors acting at either proximal or distal mitochondrial complexes. The rationale behind these experiments was that if hypoxia exerts its effect through alteration of the mitochondrial electron flow, preincubation with saturating concentrations of

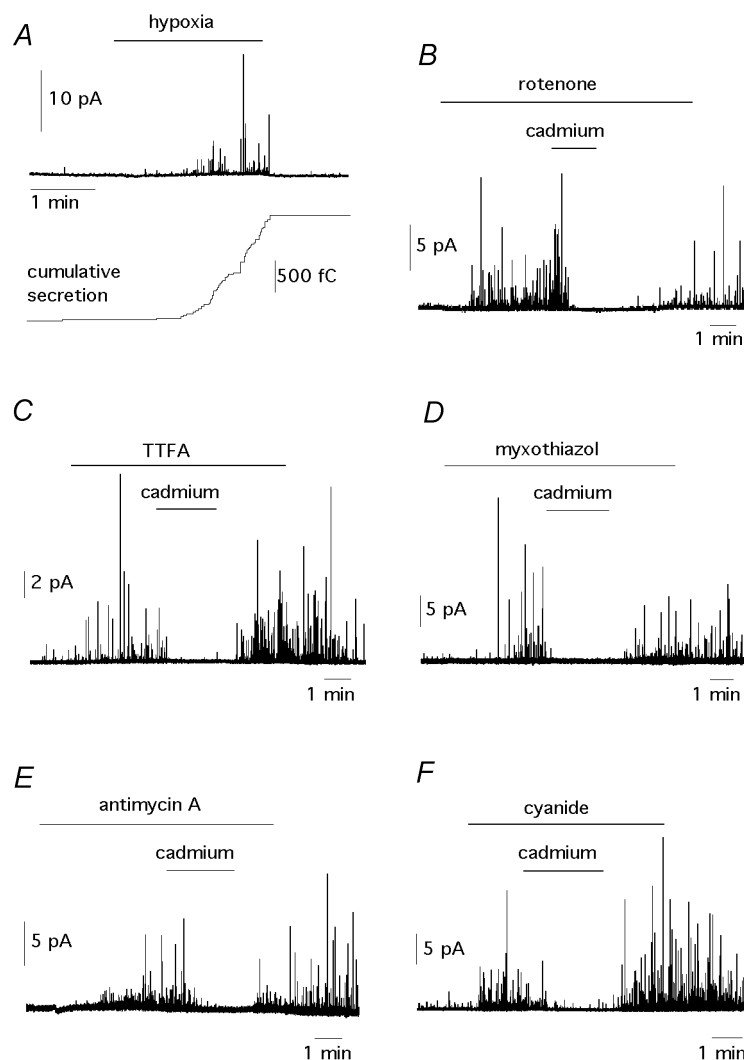


Figure 1. Secretory responses of glomus cells to hypoxia and to the inhibition of the mitochondrial electron transport

A, top, amperometric signal showing catecholamine release from a glomus cell exposed to low P_{O_2} (≈ 20 mmHg). Each spike represents an exocytotic event; bottom, cumulative secretion signal (in femtocoulombs, fC) resulting from the time integral of the amperometric recording. B–F, catecholamine release induced by exposure to several electron transport inhibitors. The concentrations were: rotenone ($5 \mu\text{M}$), TTFA ($0.3 \mu\text{M}$), myxothiazol ($1 \mu\text{g ml}^{-1}$), antimycin A ($1 \mu\text{g ml}^{-1}$) and cyanide ($100 \mu\text{M}$). In all cases secretion was reversibly abolished by external application of 0.3 mM Cd^{2+} .

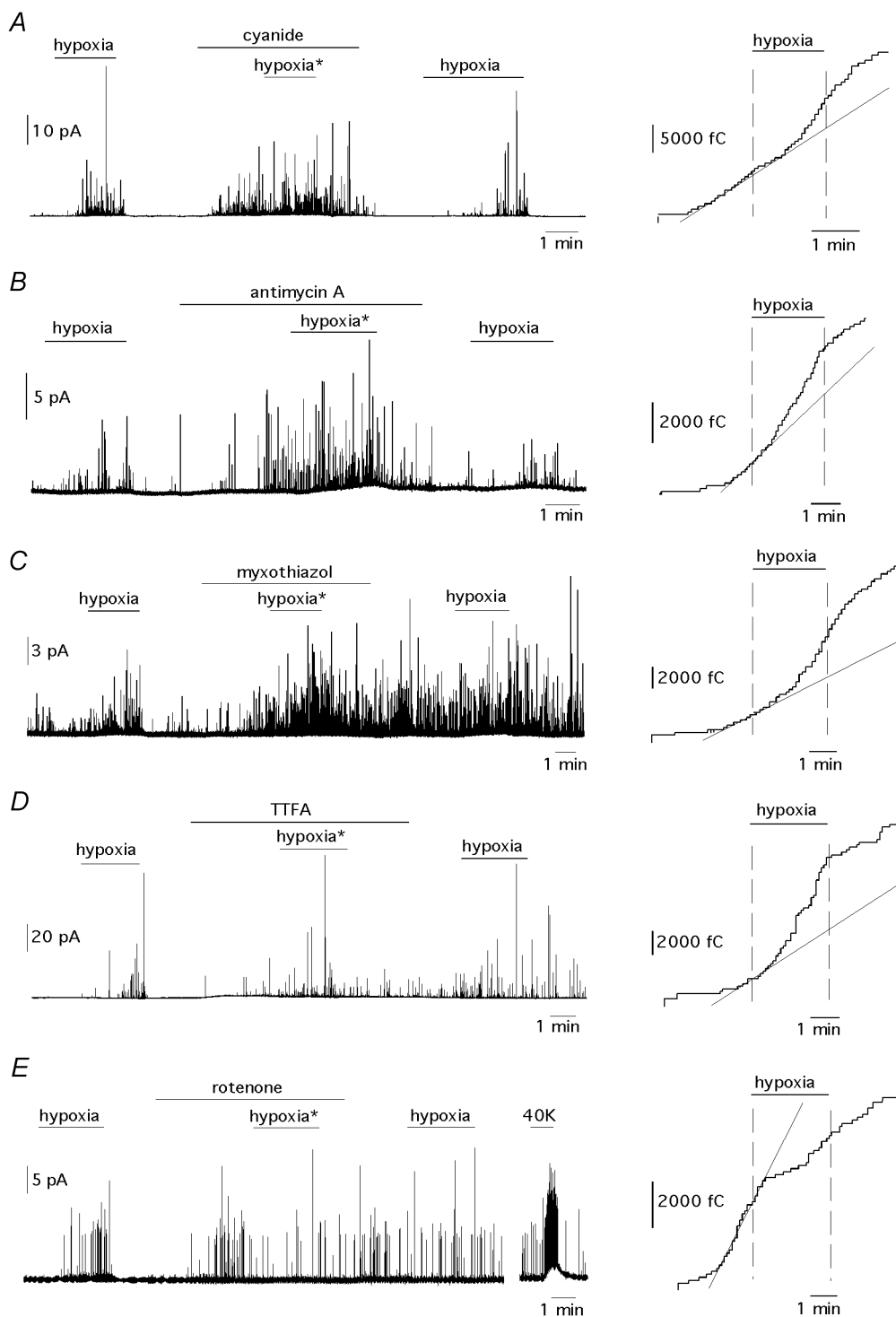


Figure 2. Secretory responses of glomus cells exposed concomitantly to hypoxia ($P_{O_2} \approx 20$ mmHg) and to blockade of mitochondrial electron transport

A–E, left panels, amperometric recordings. The concentration of drugs used in each experiment was: cyanide (A, $100 \mu\text{M}$), antimycin A (B, $1 \mu\text{g ml}^{-1}$), myxothiazol (C, $1 \mu\text{g ml}^{-1}$), TTFA (D, $0.1 \mu\text{M}$) and rotenone (E, $5 \mu\text{M}$). A–E, right panels, cumulative secretion signals before, during and after exposure to hypoxia in the presence of the ETC inhibitors. The exposures to hypoxia are marked with an asterisk in the left panels. The straight lines represent the slopes (secretion rates) of the cumulative secretion signals immediately before exposure to hypoxia.

ETC blockers would prevent any further effect of low P_{O_2} . In contrast, the effects of hypoxia and ETC inhibition would be additive, at least partially, if they were acting through separate pathways. Fig. 2A–D illustrates that when O_2 -responsive cells were treated with cyanide, antimycin A, myxothiazol or TTFA, the concomitant exposure to low P_{O_2} elicited further increase in the secretory activity. In each case the amperometric recordings are shown on the left panel of the figure. The right panels show on an expanded time base, the cumulative secretion signals recorded during exposures to hypoxia (marked with an asterisk in the left panels) in the presence of the different ETC inhibitors. The rates of secretion immediately before hypoxia are illustrated diagrammatically by the slopes of the cumulative secretion signals (straight lines). The amperometric recordings show that responsiveness to hypoxia was preserved in cells treated with ETC inhibitors, even when a marked exocytotic activity was maintained after removal of the drugs. Similarly, the cumulative secretion traces clearly illustrate that in the presence of ETC inhibitors, hypoxia induced a reversible increase in the slope of the signals. An exception among the mitochondrial inhibitors tested in this set of experiments was rotenone, a flavo-protein inhibitor that blocks mitochondrial complex I. Figure 2E shows that, like other ETC inhibitors, rotenone elicited secretion from the cells; however, previous exposure to rotenone abolished any further increase of secretion by hypoxia. Therefore, rotenone occluded the response to hypoxia without mimicking it. In some cases, as in the example of Fig. 2E, the application of hypoxia in

the presence of rotenone even reduced the secretory activity of the cells. In cells treated with rotenone the secretory response to depolarization with high potassium was unaltered (Fig. 2E).

Average secretion rates measured in several cells exposed to hypoxia and the ETC inhibitors are given in the summary plot of Fig. 3. Inhibition at various sites along the ETC with saturating concentrations of mitochondrial inhibitors induced a secretory activity in glomus cells of a magnitude comparable to that evoked by low P_{O_2} (~20 mmHg). With the exception of rotenone, the effects of hypoxia and ETC inhibitors were additive, thus suggesting that they might act through separate signalling pathways. Selective occlusion of hypoxia sensitivity by rotenone was observed in all the cells studied ($n = 28$) with concentrations of the drug between 0.1 and 5 μM . The lowest concentration used in these experiments (0.1 μM) can produce full blockade of complex I (Degli Esposti, 1998; Vaux *et al.* 2001) or saturation of rotenone-binding sites (Higgins & Greenamyre, 1996).

Rotenone-induced secretion and sensitivity of glomus cells to hypoxia

The effects of rotenone on the sensitivity to hypoxia in glomus cells were analysed in greater detail following the same experimental protocol as in Fig. 2E but using lower concentrations of the drug. Figure 4A–C illustrates that the potency of rotenone to evoke secretion decreased progressively at concentrations below 100 nM. Rotenone is a poorly reversible drug and washout from the cells was

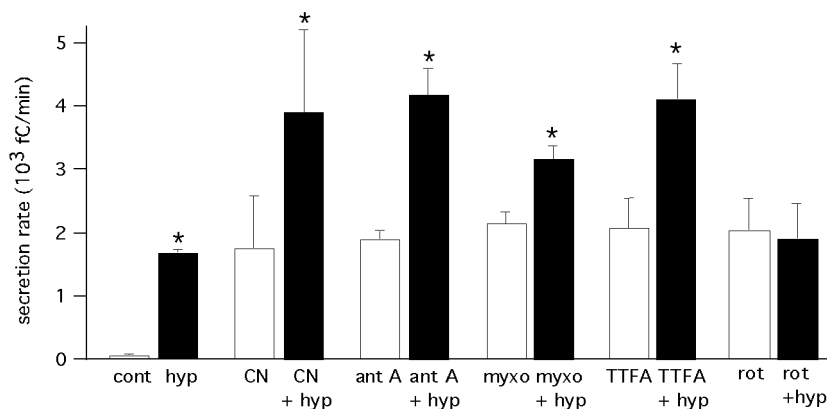


Figure 3. Average secretion rate measured in cells in various experimental conditions

Secretion rate on the ordinate is expressed in $fC \text{ min}^{-1}$ (mean \pm s.e.m.). Experimental conditions from left to right in the figure: control (cont; $P_{O_2} \approx 150$ mmHg; $75 \pm 15 fC \text{ min}^{-1}$; $n = 17$ cells) and hypoxia ($P_{O_2} \approx 20$ mmHg; $1710 \pm 65 fC \text{ min}^{-1}$; $n = 17$ cells); cyanide (CN; 100 μM ; $1771 \pm 842 fC \text{ min}^{-1}$; $n = 4$ cells) and cyanide plus hypoxia (CN + hyp; $3932 \pm 1339 fC \text{ min}^{-1}$; $n = 4$ cells); antimycin A (ant A; 0.1–1 $\mu g \text{ ml}^{-1}$; $1910 \pm 151 fC \text{ min}^{-1}$; $n = 13$ cells) and antimycin A plus hypoxia (ant + hyp; $4201 \pm 421 fC \text{ min}^{-1}$; $n = 7$ cells); myxothiazol (myxo; 0.1–1 $\mu g \text{ ml}^{-1}$; $2167 \pm 199 fC \text{ min}^{-1}$; $n = 6$ cells) and myxothiazol plus hypoxia (myxo + hyp; $3188 \pm 240 fC \text{ min}^{-1}$; $n = 6$ cells); TTFA (0.1–0.3 μM ; $2093 \pm 488 fC \text{ min}^{-1}$; $n = 5$ cells) and TTFA plus hypoxia (TTFA + hyp; $4134 \pm 587 fC \text{ min}^{-1}$; $n = 5$ cells); rotenone (rot; 0.1–5 μM ; $2058 \pm 550 fC \text{ min}^{-1}$; $n = 14$ cells) and rotenone plus hypoxia (rot + hyp; $1915 \pm 552 fC \text{ min}^{-1}$; $n = 12$ cells). Asterisks indicate a statistically significant difference ($P < 0.05$) between each pair of samples.

very slow in most experiments. The decrease of rotenone-evoked secretion was inversely related with its efficacy in occluding the responsiveness to hypoxia. This changed from full blockade of the cellular response to hypoxia at 100 nM (Fig. 4A) to lack of effect when the concentration of rotenone was reduced to 20 nM (Fig. 4C). With intermediate concentrations of rotenone (50 nM), blockade of responsiveness to hypoxia was only partial (Fig. 4B). Another observation suggesting a relationship between rotenone and hypoxia sensitivity was that cells insensitive to hypoxia were either unaffected or showed a minor response to rotenone (at concentrations between 0.1 and 5 μM ; $n = 8$ cells), although they were healthy since they responded with a powerful burst of secretion to high $[\text{K}^+]$ (Fig. 5A). In contrast, O_2^- - and rotenone-insensitive cells appeared to respond normally to myxothiazol, antimycin A or cyanide (Fig. 5B). It has been noticed before that glomus

cells, either dispersed or in slices, can lose hypoxia responsiveness while maintaining normal ionic currents and secretory responses to other agents (López-Barneo *et al.* 1998; Pardal *et al.* 2000; see Discussion).

To further investigate the relationships between hypoxia, occlusion of hypoxia responsiveness by rotenone and mitochondrial electron flow we tested to see if the effect of rotenone was prevented by the presence of succinate (5 mM), a substrate for complex II (see Leach *et al.* 2001). Similar experiments were also performed with methylsuccinate, a derivative with higher membrane permeability than succinate. Figure 6A shows that in O_2^- -responsive cells, succinate did not prevent the abolition of responsiveness to hypoxia by rotenone ($n = 3$ cells). Similarly, in the presence of methylsuccinate both sensitivity to hypoxia ($n = 7$ cells, Fig. 6B) and its occlusion by rotenone ($n = 6$ cells, Fig. 6C) were observed. The amperometric trace in Fig. 6C (left)

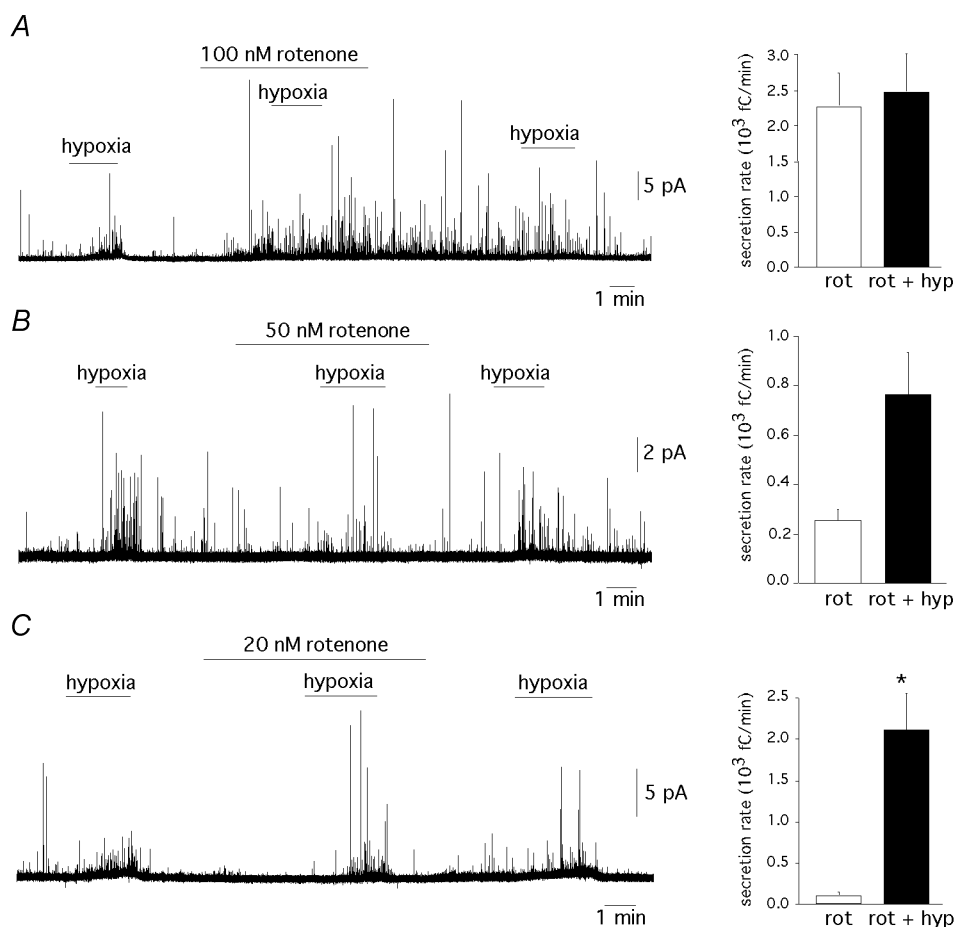


Figure 4. Dose-dependent occlusion of the responsiveness to hypoxia by rotenone

A–C, left panels, amperometric recordings illustrating the decrease in the potency of rotenone in blocking sensitivity to hypoxia as the concentration of the drug decreases from 100 to 20 nM. A–C, right panels, average secretion rates in the various experimental conditions were: A, rotenone 100 nM; 2300 \pm 484 fC min $^{-1}$; rotenone plus hypoxia, 2491 \pm 566 fC min $^{-1}$; $n = 5$ cells. $P = 0.8$. B, rotenone 50 nM; 264 \pm 54 fC min $^{-1}$; rotenone plus hypoxia, 772 \pm 179 fC min $^{-1}$; $n = 3$ cells. $P = 0.05$. C, rotenone 20 nM; 111 \pm 50 fC min $^{-1}$; rotenone plus hypoxia, 2130 \pm 462 fC min $^{-1}$; $n = 3$ cells. $P < 0.05$. Statistically significant difference ($P < 0.05$) indicated by an asterisk.

illustrates a full representative experiment in a cell with a clear response to hypoxia that was maintained in the presence of methylsuccinate but abolished by the addition of rotenone. The cumulative secretion signal is shown in the figure to facilitate its comparison with the results presented in Fig. 2. Therefore, occlusion of glomus cells' responsiveness to hypoxia by rotenone is not prevented by feeding electrons to the mitochondrial ETC through complex II.

Effects of other complex I inhibitors

The action of rotenone appeared to depend on its interaction with specific targets rather than on general inhibition of electron flow in complex I because the effect of this agent was not mimicked by myxothiazol (Figs 2 and 3), which also inhibits complex I (Degli Esposti, 1998). We also tested the effects of three other complex I inhibitors: diphenyleneiodonium (DPI), a flavoprotein inhibitor that acts on a site at complex I separated from the rotenone binding site; rhein, a competitive inhibitor of NADH; and 1-methyl-4-phenylpyridinium ion (MPP⁺), an agent that binds to the same site as rotenone (Higgins & Greenamyre, 1996; Degli Esposti, 1998). DPI used in a broad range of concentrations (0.2–5 μM , $n = 5$ cells) induced glomus cell secretion but, as other ETC inhibitors, did not prevent the stimulatory effect of hypoxia (Fig. 7A). A similar effect was observed for rhein when used at intermediate concentrations (10–25 μM , $n = 7$ cells; Fig. 7B). At higher concentrations, rhein produced inhibition of spontaneous and potassium-evoked secretion, thus suggesting that, as shown before for DPI in patch-clamped glomus cells (see Wyatt *et al.* 1994) this agent blocks extracellular Ca²⁺ entry through membrane channels. Therefore, responsiveness to hypoxia in the presence of fully saturating concentrations of rhein could not be studied in detail. Sensitivity of glomus cells to hypoxia was, however, occluded in the presence of MPP⁺ (50–150 μM , $n = 7$ cells).

DISCUSSION

The major observations in this study are: (1) inhibitors of proximal and distal complexes of the mitochondrial ETC elicit, as does hypoxia, a powerful external Ca²⁺-dependent secretory response in intact rat carotid body glomus cells; (2) cellular sensitivity to hypoxia is maintained after blockade of the mitochondrial ETC; and (3) rotenone and MPP⁺ selectively suppress responsiveness to hypoxia. These findings strongly suggest that mitochondrial electron flow is not directly involved in acute O₂ sensing by glomus cells. However, a rotenone (and MPP⁺)-inhibitable molecule participates critically in this process.

The observations at the cellular level reported in this study explain the well-known stimulatory action of mitochondrial poisons on the carotid body chemoreceptors (Mills & Jöbsis, 1972; Mulligan *et al.* 1981). It had been reported before

that cyanide increases cytosolic [Ca²⁺] in rabbit glomus cells due to release of the cation from mitochondria (Biscoe & Duchon, 1990); our results indicate that at moderate doses, cyanide-induced secretion is totally dependent on extracellular Ca²⁺. Similar dependence of external Ca²⁺ has been described for the effects of mitochondrial inhibitors on cytosolic [Ca²⁺] in rat glomus cells (Buckler & Vaughan-Jones, 1998) or the secretory activity in PC12 cells (Taylor *et al.* 2000). Taken together, these results could indicate that mitochondria can signal the cell membrane to regulate transmembrane Ca²⁺ influx. In support of this idea it has been reported that metabolic uncouplers reduce resting K⁺ permeability in rat glomus cells (Buckler & Vaughan-Jones, 1998) and that large concentrations of rotenone, antimycin A or DPI decrease the amplitude of voltage-dependent K⁺ currents in

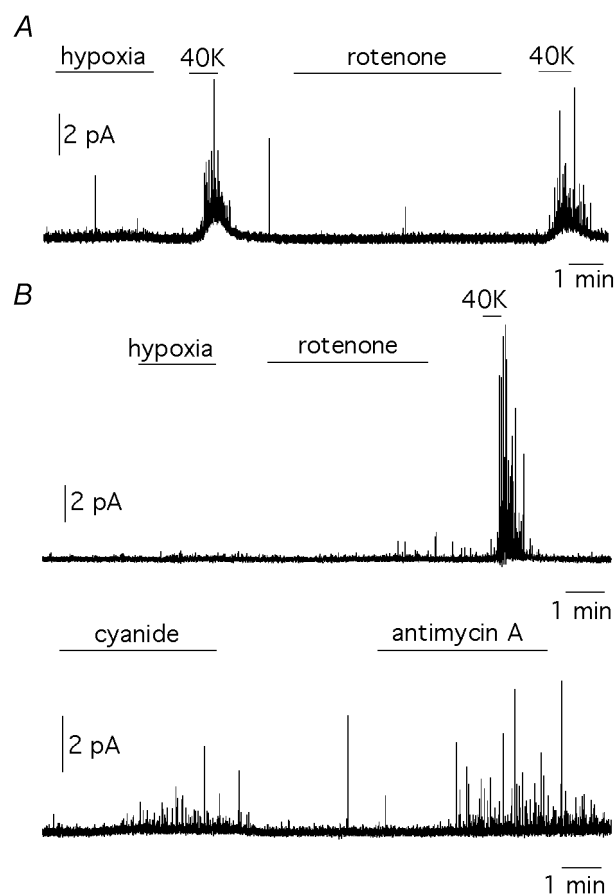


Figure 5. Cells insensitive to hypoxia respond weakly to rotenone

A, representative recording from a cell that did not respond to hypoxia ($P_{\text{O}_2} \approx 20$ mmHg) or rotenone (5 μM) but was stimulated to secrete by membrane depolarization with 40 mM potassium. B, top, representative recordings from another cell that did not respond to either hypoxia ($P_{\text{O}_2} \approx 20$ mmHg) or rotenone (5 μM) but produced a potent burst of secretory activity by membrane depolarization with 40 mM potassium. Bottom, in the same cell, secretion was induced by exposure to cyanide (0.1 mM) and antimycin A (1 $\mu\text{g ml}^{-1}$).

pulmonary arterial smooth muscle (Archer *et al.* 1993) and the carotid body (Wyatt *et al.* 1994; our own unpublished results) or model airway chemoreceptor (Searle *et al.* 2002) cells. Mitochondrial signalling could depend on either the production by these organelles of reactive oxygen species that modify the redox state of membrane channels (Archer *et al.* 1993; Chandel & Schumacker, 2000), or the level of cytosolic ATP concentration (Inoue *et al.* 2002). However, the effects of ETC inhibitors on membrane ionic conductances must be interpreted cautiously since at the concentrations used in most studies (several orders of magnitude higher than that required to inhibit mito-

chondrial ETC) some of them may behave as non-specific inhibitors of ion channels in glomus cells (Wyatt *et al.* 1994; our own unpublished results). An alternative explanation for the secretory response induced by mitochondrial inhibition is the existence in resting glomus cells of a maintained Ca^{2+} influx, which is normally counterbalanced by Ca^{2+} uptake in mitochondria located adjacent to the cell membrane. As described by Montero *et al.* (2000) in chromaffin cells, inhibition of submembranous mitochondria and the loss of their Ca^{2+} -buffering capacity, would then raise cytosolic Ca^{2+} levels and trigger catecholamine release.

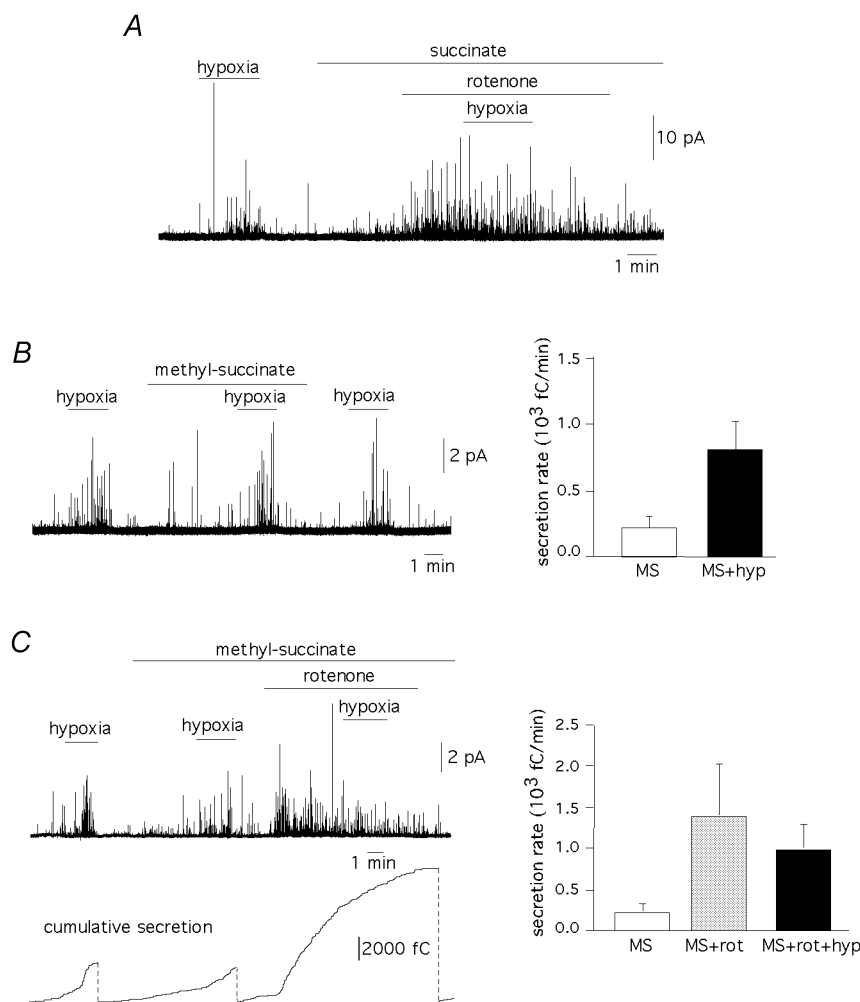


Figure 6. Hypoxia and mitochondrial electron flow

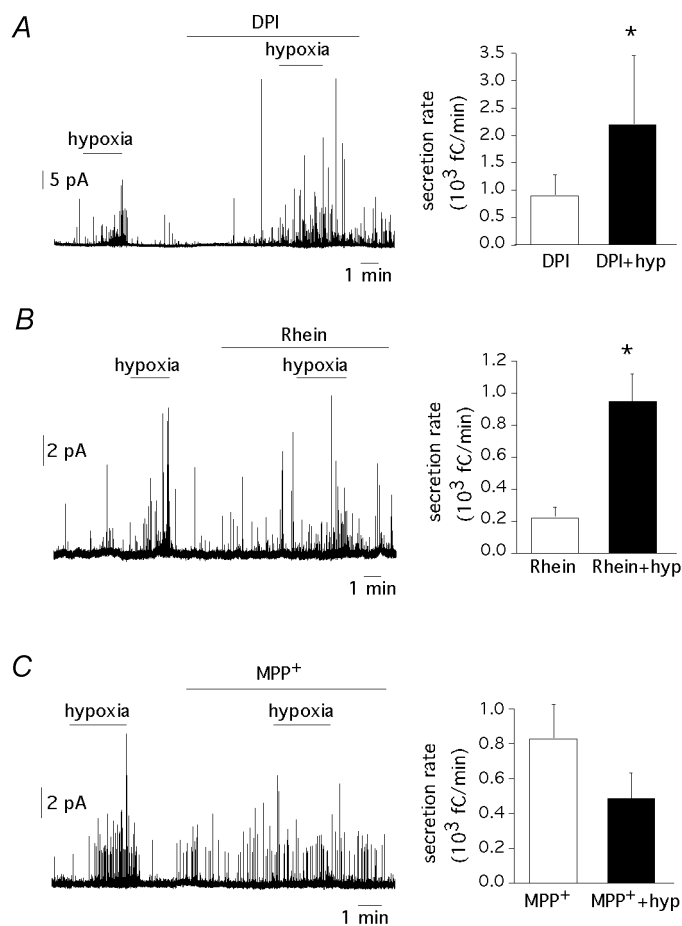
A, occlusion of responsiveness to hypoxia ($P_{\text{O}_2} \approx 20$ mmHg) by rotenone ($0.1 \mu\text{M}$) in the presence of 5 mM sodium succinate in the external solution. Similar qualitative results were obtained in two other cells tested. **B**, left, amperometric recording showing that methylsuccinate does not alter sensitivity to hypoxia; right, average secretion rates (fC min^{-1}) in cells ($n = 7$) exposed to 5 mM methylsuccinate (230 ± 115) and methylsuccinate plus hypoxia (816 ± 229), $P < 0.05$. **C**, left, occlusion of responsiveness to hypoxia ($P_{\text{O}_2} \approx 20$ mmHg) by rotenone ($1 \mu\text{M}$) in the presence of 5 mM methylsuccinate in the external solution. The vertical dotted lines in the lower panel indicate the resetting of the integrator used to calculate the cumulative secretion signal; right, average secretion rates (fC min^{-1}) in cells treated with 5 mM methylsuccinate (230 ± 115 , $n = 7$ cells); methylsuccinate plus rotenone (0.1 to $1 \mu\text{M}$) (1400 ± 648 , $n = 6$ cells) and methylsuccinate plus rotenone plus hypoxia (1000 ± 309 , $n = 6$). Statistical comparison between the two last pair of samples, $P = 0.47$.

Although hypoxia and mitochondrial ETC inhibitors induce similar external Ca^{2+} -dependent secretory responses from glomus cells in carotid body slices, our results indicate that activation of these cells by low P_{O_2} is not directly dependent on mitochondrial electron flow. Mitochondria have been classically postulated to participate in carotid body O_2 sensing due to the similarity of the stimulatory effects of hypoxia and mitochondrial poisoning (Mulligan *et al.* 1981). They have also been ascribed a role in acute O_2 sensing in chromaffin cells (Mojet *et al.* 1997; Inoue *et al.* 2002) and pulmonary arterial smooth muscle (Archer *et al.* 1993). Using this last preparation it has recently been hypothesized that O_2 tension regulates superoxide production at the semiubiquinone site of mitochondrial complex III and that an increase of radical production during exposure to low P_{O_2} is the signal that mediates the acute phase of hypoxic pulmonary vasoconstriction (HPV) (Leach *et al.* 2001; Waypa *et al.* 2001). A major argument in favour of this model is the differential effect of blockers of the ETC acting upstream and downstream of the semiubiquinone site. Antimycin A, a downstream blocker that leaves complex III reduced, does not alter HPV whereas this response is blocked by upstream ETC inhibitors, such as rotenone and myxothiazol, that maintain complex III permanently oxidized. In addition, occlusion of HPV by rotenone is reversed by stimulation of electron transport via complex II with succinate (Leach *et al.*

2001). In contrast with these data, we observe in carotid body cells that several blockers acting upstream of the semiubiquinone site at complexes I (DPI, rhein and myxothiazol), II (TTFA) and III (myxothiazol) are able to trigger a powerful secretory response but they do not prevent further increase of secretion by hypoxia. Moreover, the occlusion of hypoxia responsiveness by rotenone is unaltered after incubation of the cells with succinate, which feeds electrons to the ETC through complex II. The effect of rotenone is mimicked by MPP^+ , a drug that binds to the same site as rotenone (Higgins & Greenamyre, 1996). In parallel with the present study focused on intact glomus cells in carotid body slices, we have observed in preliminary experiments that at sub-micromolar concentrations rotenone, but not antimycin A, reduces the amplitude of the macroscopic K^+ current and blocks the inhibition of this same current by hypoxia. Altogether, these observations strongly suggest that although a rotenone-sensitive molecule appears to be necessary, mitochondrial electron transport *per se* is not directly involved in acute O_2 sensing by carotid body glomus cells. This conclusion, however, is compatible with the data reported for pulmonary smooth muscle (Leach *et al.* 2001; Waypa *et al.* 2001) as it is generally agreed that distinct cell types may use different strategies to sense O_2 . For example, knocking out the NADPH oxidase gene abolishes O_2 sensitivity in neuroepithelial cells of the lung but leaves

Figure 7. Responsiveness to hypoxia and complex I inhibitors

A, left, response to hypoxia in a cell treated with DPI (0.2 μM); right, increase in the secretory activity (fC min^{-1}) induced by DPI (0.2–5 μM ; 918 ± 389 ; $n = 5$ cells; $P < 0.05$) and by DPI plus hypoxia (2195 ± 1259 ; $n = 5$ cells). **B**, left, response to hypoxia in a cell treated with rhein (25 μM); right, increase in secretory activity (fC min^{-1}) induced by rhein (10–25 μM ; 228 ± 64 ; $n = 5$ cells; $P < 0.05$) and by rhein plus hypoxia (957 ± 179 ; $n = 5$ cells; $P < 0.05$). **C**, left, abolition of the responsiveness to hypoxia by 1-methyl-4-phenylpyridinium ion (MPP^+ , 50 μM); right, secretory activity (fC min^{-1}) induced by MPP^+ (50–150 μM ; 831 ± 198 ; $n = 7$ cells) and by hypoxia plus MPP^+ (489 ± 150 ; $n = 7$ cells; $P = 0.3$). Statistically significant difference ($P < 0.05$) indicated by an asterisk.



unaltered the responses to hypoxia in pulmonary smooth muscle or carotid body cells (for review and discussion, see López-Barneo *et al.* 2001). In addition, sensitivity of macroscopic outward currents to hypoxia in model airway chemoreceptor cells is maintained in the presence of high concentrations of rotenone and antimycin A (Searle *et al.* 2002). Cross *et al.* (1990), have reported the abolition of the hypoxia-induced increase in carotid body sinus nerve chemoreceptor discharge by DPI (that inhibits both mitochondrial complex I and NADPH oxidase). These results can, however, be explained by the non-specific blocking effect of this agent on Ca^{2+} channels at the glomus cell-afferent fibre synapse (Wyatt *et al.* 1994).

Besides the effects of rotenone and MPP^+ on the fast response of glomus cells to acute hypoxia described here, there are several reports suggesting that rotenone-binding proteins might be involved in chronic cellular responses to low P_{O_2} . Erythropoietin gene induction by hypoxia, although unaffected by downstream mitochondrial inhibitors like azide or cyanide, is blocked by rotenone (Necas & Thorling, 1972; Goldberg *et al.* 1988; Pugh *et al.* 1991). Similarly, activation and cell accumulation of hypoxia-inducible factor 1 α in low P_{O_2} is prevented by rotenone (Chandel *et al.* 1998; Semenza, 1999) and MPP^+ (Agani *et al.* 2000). These effects of rotenone are, however, unclear because they are only observed at concentrations higher than that required to inhibit oxygen consumption by the cells (Vaux *et al.* 2001).

The nature and location of the rotenone (and MPP^+)-inhibitable molecule involved in glomus cell O_2 sensing is unknown. It could be a membrane K^+ channel subunit with a flavoprotein modified by rotenoids that changes its conformation in response to lowering P_{O_2} . This hypothesis, although speculative, is in accord with the existence of cytosolic aggregates of pre-assembled complex I proteins of unknown function (Schulte, 2001), which in glomus cells could be associated with membrane ion channels. However, we cannot discount a more elaborate arrangement constituted by mitochondria with complex I proteins regulated by changes of O_2 tension in close contact with membrane K^+ channels. As described in other systems (Kennedy *et al.* 1999; Montero *et al.* 2000), peripheral mitochondria may form functional units with membrane proteins to delimit submembraneous cytosolic microdomains. These ideas are compatible with previous observations describing the modulation of membrane K^+ channels by O_2 tension in excised membrane patches (Ganforina & López-Barneo, 1991; Riesco-Fagundo *et al.* 2001; Lewis *et al.* 2002) as these are known to contain mitochondria and other intracellular organelles (Sather *et al.* 1992; Rustenbeck *et al.* 1999). The existence of a separate sensor that confers O_2 sensitivity to the K^+ channels helps to explain why in some conditions, in which the sensor is possibly detached from the channels,

glomus cells lose the responsiveness to hypoxia while maintaining normal electrophysiological properties (López-Barneo *et al.* 1998). This scheme of glomus cell O_2 sensing could also account for the existence of O_2 -insensitive cells with low responsiveness to rotenone. Detachment of mitochondria from the cell membrane would render cells O_2 -insensitive and make mitochondrial signalling to the membrane more difficult. These cells would then require complete blockade of the ETC (with cyanide or antimycin A) to induce significant secretion.

In conclusion, we report that, as with hypoxia, mitochondrial inhibitors activate external Ca^{2+} -dependent catecholamine secretion from carotid body glomus cells. However, our results indicate that mitochondrial electron flow is not linked in a simple way to acute regulation of glomus cell activity by changes of O_2 tension. Hypoxia and mitochondrial inhibitors, acting through separate pathways, converge to raise cytosolic $[\text{Ca}^{2+}]$, which triggers secretion. We have identified rotenone (and MPP^+) as highly selective and specific inhibitors of the responsiveness to hypoxia. Therefore, these drugs and their derivatives could be used as tools to pursue investigation of the molecular characterization and location of the O_2 sensing mechanism in glomus cells.

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