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RESPONSES OF TYPE I CELLS DISSOCIATED FROM THE RABBIT CAROTID BODY TO HYPOXIA

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SUMMARY

1. The carotid body chemoreceptors are stimulated *in situ* by hypoxia. We have studied type I cells freshly dissociated from the carotid body of the rabbit. We have used microfluorimetric and patch clamp techniques to examine the responses to hypoxia, to anoxia, and to metabolic inhibition.

2. NADH autofluorescence measured at both 400 and 500 nm increased rapidly and reversibly in response to anoxia or to cyanide (CN^-), reflecting a change in mitochondrial metabolism.

3. Indo-1 was used to measure changes in intracellular calcium, $[Ca^{2+}]_i$. Anoxia reversibly increased $[Ca^{2+}]_i$ from $\approx$ 50–100 to $\approx$ 200–450 nM in all cells tested. The response showed a striking temperature sensitivity. Responses to hypoxic stimuli were barely detectable at 17–20 °C, and were dramatically increased on warming to 36 °C. In contrast, responses to K^+-induced depolarization were only slightly increased in rate of onset and recovery by warming.

4. The rise in $[Ca^{2+}]_i$ originated largely from an intracellular store which was slowly depleted by exposure to nominally Ca^{2+}-free solutions. Responses were unaffected by blockade of Ca^{2+} channels with organic (D600, verapamil) or inorganic (Co^{2+}) blockers, by blockade of Na^+ channels with tetrodotoxin (TTX), or by increasing action potential duration with tetraethylammonium (TEA). Responses to anoxia were increased by the increased $[Ca^{2+}]_i$ loading that follows prior exposure to Ca^{2+}-free solutions.

5. Responses to anoxia, to blockade of electron transport by CN^-, and to the mitochondrial uncoupler, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), were equivalent in amplitude. The response to anoxia was occluded by concurrent application of FCCP, suggesting that the Ca^{2+} originates from the same pool in each case.

6. At 35–36 °C, responses to graded levels of P_{O_2} were also graded. Thresholds varied between cells, but were typically 30–50 mmHg. Stimulus–responses curves were essentially hyperbolic, increasing dramatically as the P_{O_2} approached 0 mmHg.

7. The sensitivity of cells to hypoxic solutions was increased by acidification of the superfusate over the pH range from 7·3 to 6·85.

8. Cell-attached patch clamp recordings showed depression of spontaneous action potentials associated with a rise in $[Ca^{2+}]_i$ during exposure to anoxic solutions.

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Whole-cell recordings showed that anoxia increased a voltage-gated g_K as described previously for CN^-, while producing no change in resting conductance.

9. These data suggest that the rise in $[Ca^{2+}]_i$ originates largely from Ca^{2+} efflux from a mitochondrial pool. This rise in $[Ca^{2+}]_i$ is envisaged to promote transmitter release and activation of sensory nerve terminals. The mitochondrial respiratory chain thus appears to play a central role in chemotransduction.

INTRODUCTION

Chemoreceptors located at the arch of the aorta and at the bifurcation of the carotid arteries monitor the partial pressure of oxygen (P_{O_2}) in the blood of vertebrates. The carotid chemoreceptors are concentrated in the carotid bodies, where they monitor the P_{O_2} of arterial blood *en route* to the brain. A fall in P_{O_2} increases the afferent traffic in the sinus nerve, initiating appropriate compensatory respiratory reflexes (Heymans, Bouckaert & Dautrebande, 1931; Heymans & Bouckaert, 1939; Biscoe, 1971).

Since the work of De Castro (1926, 1928), the type I cell has generally been regarded as the site of transduction although there has been some discussion about this attribution in recent years (Biscoe, 1971; Paintal, 1988). Hypoxia increases the afferent nerve output of the receptor complex *in vivo* (von Euler & Liljestrand, 1937) and *in vitro* (Eyzaguirre & Koyano, 1965), and also increases Ca^{2+}-dependent catecholamine release from the whole carotid body (Fidone, Gonzalez & Yoshizaki, 1982; Obeso, Fidone & Gonzalez, 1987) and from type I cells grown in culture (Fishman, Greene & Platika, 1985). The consistently close correlation between stimulation and catecholamine release suggests that the basic processes underlying the regulation of transmitter release from type I cells must be central in understanding the transduction process, although there remains some debate about the specific role and identity of the transmitter(s) involved.

We have shown that type I cells freshly dissociated from the carotid body of the rabbit are excitable (Duchen, Caddy, Kirby, Patterson, Ponte & Biscoe, 1988), that these cells respond to CN^-, a powerful chemoreceptor stimulant, with an increased voltage- and Ca^{2+}-dependent potassium conductance, $g_{K(Ca)}$ (Biscoe & Duchen, 1989*a*), and that CN^- raises $[Ca^{2+}]_i$, largely from an intracellular pool (Biscoe, Duchen, Eisner, O'Neill & Valdeolmillos, 1989). These data led us to suggest that the electrophysiological response was secondary to an alteration in cellular Ca^{2+} regulation. We have now investigated the effects of hypoxia on these same variables in rather greater detail.

A preliminary report of some of these experiments has been published (Biscoe & Duchen, 1989*b*).

METHODS

Cells were isolated from the adult rabbit carotid body as previously described (Duchen *et al.* 1988). In brief, rabbits were anaesthetized with sodium pentobarbitone (30 mg/kg intravenously). The carotid bifurcation was exposed and removed into ice-cold saline containing (mM): NaCl, 130; $NaHCO_3$, 26; KCl, 3; KH_2PO_4, 1·25; $MgSO_4$, 2; D-glucose, 10, and equilibrated with 95% O_2, 5% CO_2 (solution A). The carotid body was dissected free and incubated in 0·2% protease XIV (Sigma) dissolved in the same saline, incubated at 35 °C, and continuously bubbled with 95% O_2,

5% CO_2. After 30 min the tissue was transferred to a solution containing 0·2% collagenase type II, 0·2% hyaluronidase, 0·07% DNAase and 0·5% bovine serum albumin, BSA (all from Sigma) also dissolved in the same saline and incubated under the same conditions. The tissue was then transferred to an enzyme-free, HEPES-buffered saline containing (mM): NaCl, 156; KCl, 3; KH_2PO_4, 1·25; $CaCl_2$, 2; $MgSO_2$, 2; D-glucose, 10; HEPES, 5; pH 7·3 (solution B) kept on ice. Cells were dispersed by repetitive trituration with fire-polished Pasteur pipettes and suspended on a glass cover-slip used as the base of a Perspex recording chamber. Most of the data shown were obtained from small tight clusters of four to twenty cells, as these are most readily identifiable as type I (Duchen *et al.* 1988). Some of the data come from single cells. The results presented are based on recordings from over 150 groups of cells.

Electrophysiological recordings were made using conventional patch clamp recording techniques (Hamill, Marty, Neher, Sackmann & Sigworth, 1981) using a List EPC-7 amplifier. Voltage commands and data acquisition were controlled using pCLAMP (Axon Instruments, Burlingame, CA, USA) and a microcomputer as previously described (Duchen *et al.* 1988).

Patch pipettes had tip diameters of 1–2 μm and were normally filled with a solution containing (mM): KCl, 130; EGTA, 0·5 and HEPES, 7·5; pH 7·2. The cells were continuously superfused with solution B unless indicated. When high K^+ solutions were used, NaCl was replaced isotonically with KCl. Drugs were added to the superfusate with appropriate correction of pH. Alternatively, drug solutions could be ejected under pressure from micropipettes placed close to the cells, limiting exposure to those cells studied, and sparing the rest of the preparation. The superfusate was made hypoxic by bubbling with nitrogen and the oxygen partial pressure was reduced to zero with addition of sodium dithionite ($Na_2S_2O_4$), 500–700 μM. The solutions were delivered to the recording chamber through stainless-steel tubes as oxygen diffuses through plastic tubing from the air. Superfusates were switched between four reservoirs using solenoid-operated valves and were warmed whilst passing to the chamber. The exchange time for the bath varied between 5 and 15 s. The temperature in the bath was continuously monitored with a thermocouple, and measurements of the bath oxygen partial pressure were made with a home-made oxygen electrode. This was placed at the edge of the bath. Eddying and unevenness of the flow often introduced discrepancies in the timing of the responses of the oxygen electrode and the cell studied, sometimes the cells even responded before the electrode. This means that the relative timing of the responses should not be overinterpreted. The equilibrated P_{O_2} and fluorescence measurements over the time scale used for these experiments should be valid.

Drugs used were carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP, 5 μM; Sigma), sodium cyanide (CN^-, 2 mM; Sigma) and sodium dithionite (500–700 μM; Sigma). Responses to FCCP were slowly reversible under these conditions, but we had the impression that the behaviour of cells altered subtly following exposure, suggesting that recovery was incomplete. For this reason, FCCP application was usually limited to the cells under study by brief pressure ejection from a patch pipette, and this was usually the last manoeuvre before abandoning that group of cells.

Cells were loaded with Indo-1 (Molecular Probes) by incubation at room temperature (15–20 °C) for 15–30 min with the acetoxymethyl ester (1 μM). After loading, cells were kept on ice and aliquots removed for use in the experimental chamber.

For $[Ca^{2+}]_i$ measurements the excitation wavelength used was 365 nm, and fluorescence emission was measured continuously at 400 and 500 nm. A rise in $[Ca^{2+}]_i$ increases fluorescence at 400 nm and decreases it at 500 nm (Grynkiewicz, Poenie & Tsien, 1985), and see Fig. 2. Thus the ratio of fluorescence excited at 400/500 nm compensates for changes in the fluorescence signal due to loss or bleaching of the fluorophore. A change of the individual photomultiplier signals in the same direction usually indicated changes in autofluorescence of the cell due to variations in redox potential and NAD(P)H fluorescence (see below and Fig. 1). Using the 365 nm excitation filter, the change in fluorescence with a rise in $[Ca^{2+}]_i$ was small at 400 nm, but clear at 500 nm. This reflects the relatively small emission of Indo-1 at 400 nm with this exciting wavelength. The 400 nm signal was thus often relatively flat following, for example, a rise in $[Ca^{2+}]_i$ with potassium-induced depolarization. Changes at 400 nm were therefore a good guide to contamination of the signal by autofluorescence, which is otherwise similar at 400 and 500 nm (see Fig. 1).

The fluorescence ratio records obtained from cells have usually been calibrated in terms of $[Ca^{2+}]_i$ using maximum and minimum ratios obtained from *in vitro* fluorescence measurements as described in Biscoe *et al.* (1989). 5 μM-Indo-free acid was added to solutions containing 150 mM-KCl, 5 mM-HEPES (pH 7·3) with either 10 mM-EGTA and no added calcium or with 1 mM-

calcium. After subtraction of the background signal, the fluorescence ratios for minimum and saturating calcium concentrations were obtained, and used in the equation $[Ca^{2+}]_i = K_D (R-R_{min})/(R_{max}-R)$. The value we used for the K_D was 250 nM, quoted by Grynkiewicz *et al.* (1985) for conditions most closely resembling those used in most of our experiments. The K_D is temperature- and pH-dependent, complicating the interpretation of experiments involving changes in these variables, so that alternative mechanisms for raising $[Ca^{2+}]_i$ were used (i.e. high K^+-induced depolarization) as an internal control, to confirm that effects of these variables were due to changes in the response to anoxia etc. and not to changes in the properties of the dye. These difficulties will be further discussed where appropriate. The background signal used for calibration did not include the autofluorescence of the cell. However, under conditions used to measure Indo-1 fluorescence from the cells represented less than 5–8% of the signal, and therefore introduced only small errors into the calculations of $[Ca^{2+}]_i$. Despite the various problems, we have chosen to represent responses in terms of $[Ca^{2+}]_i$ because the calibration curve of ratio to $[Ca^{2+}]_i$ is non-linear. Representing the responses simply in terms of the fluorescence ratio thus alters the apparent rates of change and shape of the response significantly (see, for example, Fig. 2). Clearly, there are numerous difficulties involving calibration, and we have misgivings regarding the absolute values of $[Ca^{2+}]_i$ indicated. Nevertheless, the interpretation of the data and qualitative effects of the manoeuvres shown remain valid.

RESULTS

Most of the data presented here were obtained from small tight clusters of four to ten cells, as these are most readily identifiable as type I (Duchen *et al.* 1988). Some of the data come from single cells. Larger clusters were generally avoided, as loading with Indo-1 was less efficient and the relative contribution of autofluorescence was therefore greater. The results presented are based on recordings from over 150 cells or groups of cells. The values given for n are the numbers of clusters of cells studied in each case, not the number of trials on a cluster nor the number of cells. We have examined and compared the responses of such clusters to a range of stimuli:

(i) Sodium cyanide (CN^-, 2 mM), which blocks the mitochondrial electron transport chain.

(ii) Potassium chloride, applied either by pressure from a pipette (150 mM for 1–2 s) or superfused at 30 mM, which depolarizes the cells and increases Ca^{2+} influx through voltage-gated channels (Biscoe *et al.* 1989).

(iii) A change in the partial pressure of oxygen (P_{O_2}) in the superfusate. We will refer to normoxia as superfusate equilibrated with room air (P_{O_2} of about 150 mmHg); this is in fact hyperoxic compared with the normal P_{O_2} in the tissues (see, for example, Whalen, Savoca & Nair, 1973). Anoxia refers to superfusates with a P_{O_2} of zero obtained by equilibrating solutions with nitrogen and with addition of sodium dithionite. We will refer to solutions as hypoxic when the P_{O_2} was less than 150 mmHg and greater than zero.

(iv) FCCP (5 μM), a proton ionophore and uncoupler of mitochondrial oxidative phosphorylation, which was usually applied by pressure ejection from a pipette placed close to the cells, in order to avoid exposure of other cells in the bath. FCCP was also occasionally bath-applied at the same concentration.

Autofluorescence

When cells were superfused with anoxic solutions without loading with Indo-1, significant changes in their intrinsic autofluorescence were seen under conditions defined for the recording of Indo-1 fluorescence (i.e. excitation at 365 nm, emission measured at 400 and 500 nm), despite the fact that these emission wavelengths are

well removed from the fluorescence peak of around 450 nm (see Chance, Schoener, Oshino, Itshak & Nakase, 1979). For the most part these represent changes in mitochondrial NADH fluorescence and are expected, since anoxia will block the electron transport chain increasing the NADH/NAD ratio and thence the

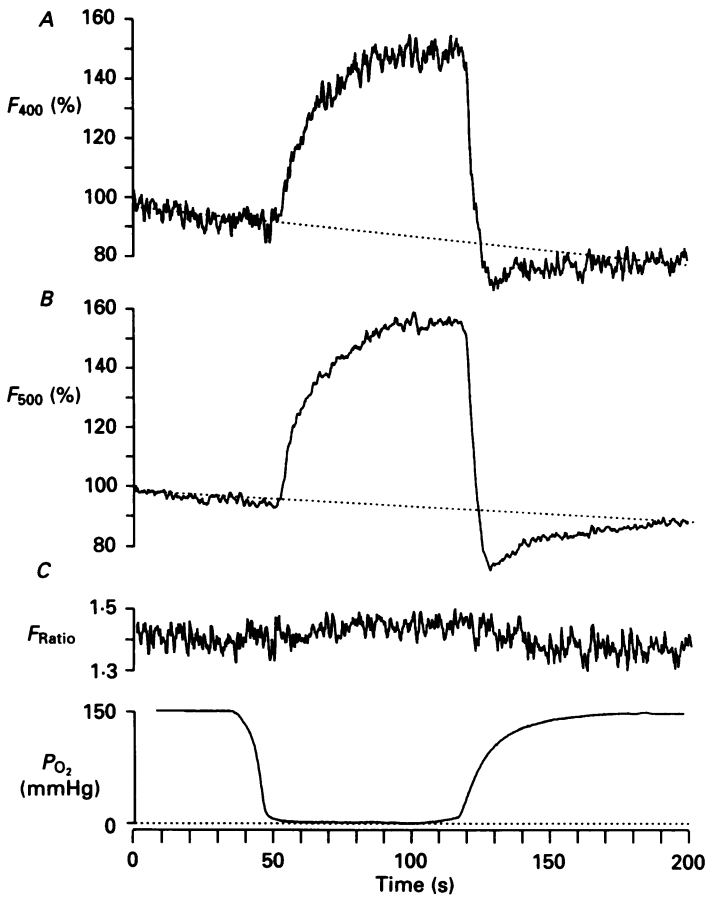

Fig. 1. Changes in autofluorescence of a cluster of type I cells in response to anoxia. The excitation wavelength was 365 nm, and emission was measured at 400 (A, F_{400}) and 500 nm (B, F_{500}). Both signals are superimposed on a slowly decreasing baseline. The lowest trace shows the recording from a P_{O_2} electrode in the bath. The ratio (C, F_{Ratio}) barely changed during the period of anoxia.

autofluorescence. Similar changes in autofluorescence followed superfusion with 2 mM-CN^-. Typically, after the P_{O_2} reached 0 mmHg the fluorescence increased rapidly over 20–30 s and was then maintained at a plateau ($n = 23$; see Fig. 1).

On returning to a normoxic superfusate, the fluorescence decreased within seconds, usually overshooting to a level slightly lower than the initial control, and recovering slowly back to the control level during a 10–20 s period. The increases seen at 400 and 500 nm were similar in amplitude, the change at 500 nm occasionally being slightly larger than that at 400 nm, so that the ratio signal (400/500) remained largely unchanged or decreased slightly. This is the cause of some small decreases in the ratio

for $[Ca^{2+}]_i$ seen on some of our records of Indo-1 emission shortly after the onset of a fall in P_{O_2}. The autofluorescence signal was very much less intense than the fluorescence emission from Indo-1 (see Methods), so that records of Indo-1 fluorescence were generally not distorted by these changes. Brief exposure to FCCP

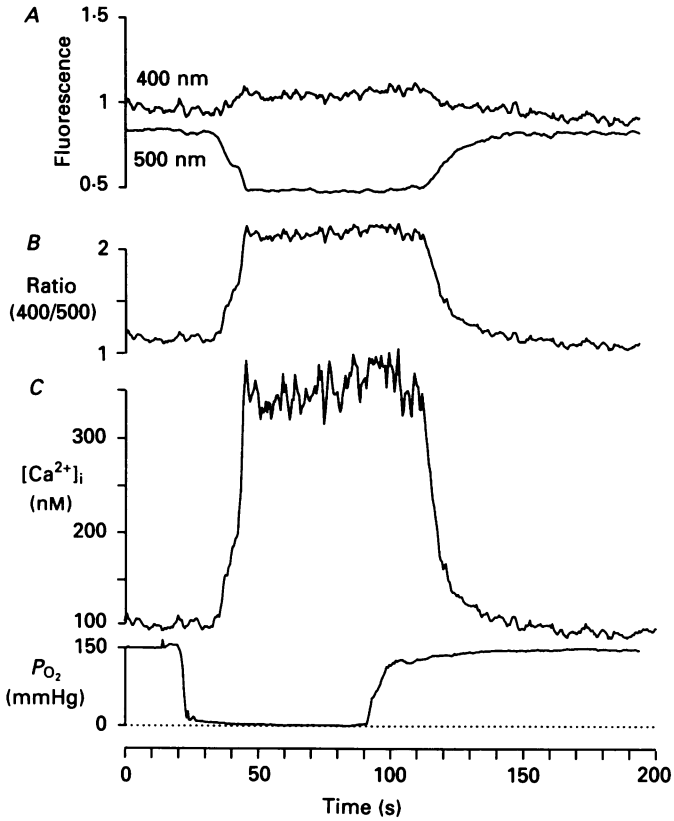

Fig. 2. Changes in Indo-1 fluorescence and in $[Ca^{2+}]_i$ in response to anoxia. The lowest trace shows the response of the P_{O_2} electrode. Signals recorded at 400 and 500 nm are shown in *A*, the ratio in *B*, and the change in $[Ca^{2+}]_i$ computed from the ratio in *C*. The conditions under which these records were obtained were chosen to produce a maximum change in $[Ca^{2+}]_i$ (see text below), i.e. a temperature of 36 °C and a pH of 7·1. The P_{O_2} electrode was placed at the edge of the bath, while the cells were central, accounting to some degree for the time delay between the fall in P_{O_2} and the rise in $[Ca^{2+}]_i$.

rapidly decreased the autofluorescence to a minimum level near background. More detailed examination of these changes will be discussed elsewhere.

Changes in $[Ca^{2+}]_i$ with changes in P_{O_2}

Superfusion with an anoxic solution increased $[Ca^{2+}]_i$ in all cells tested. The signal at 400 nm increased and at 500 nm decreased giving a ratio that increased as shown in Fig. 2, calibrated in terms of $[Ca^{2+}]_i$ as shown in the lowest trace. The rate and extent of the rise of $[Ca^{2+}]_i$ varied considerably between cells, as will become apparent. The time course of the rise in $[Ca^{2+}]_i$ closely paralleled the rise in

autofluorescence, usually increasing over 10–30 s to a plateau. Recovery, usually over 10–20 s on returning to a normoxic superfusate, was rather slower than the recovery of the autofluorescence.

Changes in $[Ca^{2+}]_i$ in response to hypoxia and cyanide are temperature sensitive

The increases in $[Ca^{2+}]_i$ in response to CN^- or anoxia were always of similar magnitude (Fig. 3). At room temperature these changes were relatively small and

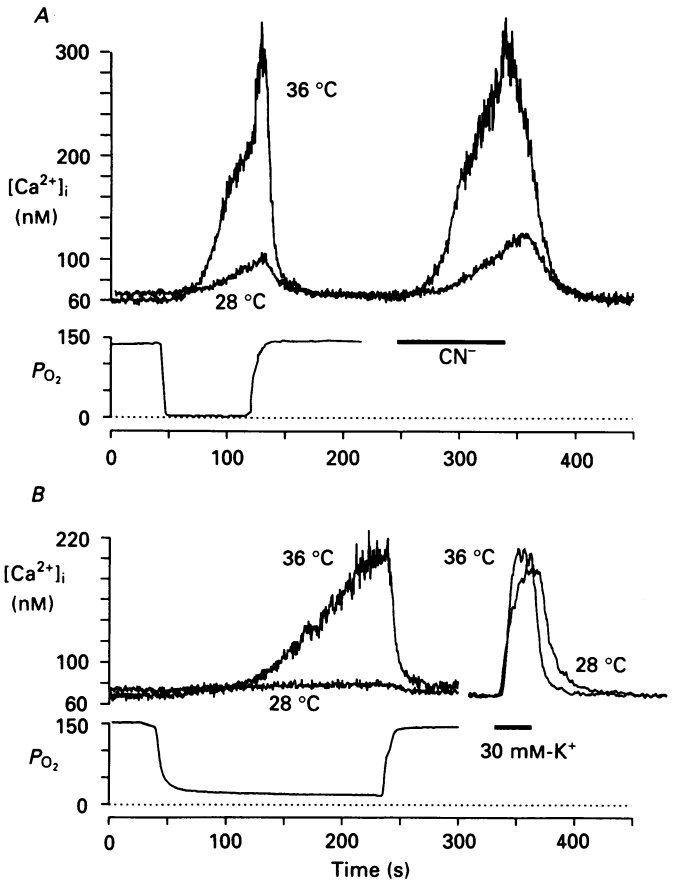

Fig. 3. The rise in $[Ca^{2+}]_i$ in response to anoxia, hypoxia (P_{O_2} about 20 mmHg), CN^-, and 30 mM-K^+ at 28 and 36 °C. Responses to anoxia and CN^- were equivalent in size at either temperature. The slower rate of recovery from CN^- probably reflects the slower diffusion of CN^- from the cell. Warming from 28 to 36 °C greatly increased the responses to anoxia and hypoxia. The rates of rise and of recovery of the response to K^+-induced depolarization were increased slightly on warming.

responses to hypoxic solutions particularly were sometimes barely detectable (Fig. 3*B* at 28 °C). Small increases in the temperature of the superfusate produced similar increases in the responses to both CN^- and to anoxia ($n = 15$; Fig. 3*A*). Furthermore, responses to intermediate levels of hypoxia were very greatly enhanced by warming

(Fig. 3*B*; compare 28 with 36 °C). Most of the data to be shown subsequently were therefore obtained at 35–37 °C.

Depolarization of the cells by exposure to 30 mM-potassium (Fig. 3*B*) invariably raised $[Ca^{2+}]_i$ in healthy cells. The rates of rise and recovery were only slightly affected by a change in temperature. This shows that the predominant action of hypoxia, or of CN^-, must involve a mechanism other than facilitation of Ca^{2+} entry through voltage-gated channels, and that the temperature sensitivity of the response to hypoxia does not simply reflect the temperature sensitivity of $[Ca^{2+}]_i$ buffering mechanisms, or the temperature sensitivity of the fluorophore. In fact, the expected effect of warming on Indo-1 fluorescence would be to decrease the sensitivity of the indicator, as the K_D for Ca^{2+} is raised at higher temperatures (Grynkiewicz *et al.* 1985; and see Bers, Bridge & Spitzer, 1989) and the fluorescence yield is also expected to decrease.

The most striking effect of warming was to increase the rate of rise of the response, as shown in Fig. 4, although the level ultimately achieved was not always increased if the hypoxic or anoxic stimulus was sustained. Clearly at 17 °C the system was very unresponsive. Above this temperature the rate of rise increased about sixfold per 10 °C. With warming above 37 °C, the cells began to deteriorate, with a rise in resting $[Ca^{2+}]_i$.

Hypoxia increases $[Ca^{2+}]_i$ by release from an intracellular pool

We have shown previously that increases in $[Ca^{2+}]_i$ in response to CN^- persist, at least to a degree, both in carotid body type I cells (Biscoe *et al.* 1989) and in isolated dorsal root ganglion neurones (Duchen, Valdeolmillos, O'Neill & Eisner, 1990) following removal of extracellular Ca^{2+}. This is also the case for the response to anoxia ($n = 10$; Fig. 5).

Following replacement of 2 mM-Ca^{2+} with a nominally Ca^{2+}-free superfusate, containing 4 mM-Mg^{2+} to maintain the concentrations of divalent cations, resting $[Ca^{2+}]_i$ fell slightly. Responses to an anoxic superfusate were still clear, although reduced in amplitude. There was variation between cell clusters in the size of the reduction as shown in Fig. 5 (compare *A* with *B*). If exposure to the nominally Ca^{2+}-free superfusate was sustained then repeated exposure to hypoxic solutions tended to produce successively smaller responses. On returning to a normal superfusate (2 mM-Ca^{2+}, 2 mM-Mg^{2+}) $[Ca^{2+}]_i$ rose rapidly (see Biscoe *et al.* 1989). The response to hypoxia seen at this time was invariably exaggerated (Fig. 5*A* and *B*). If 1 mM-EGTA was used to lower extracellular $[Ca^{2+}]_i$ further, the response attenuated more rapidly, but was still detectable. The experiment illustrated in Fig. 5*B* shows that Ca^{2+} loading of the cell, by briefly returning to a Ca^{2+}-containing superfusate after exposure to a Ca^{2+}-free solution, was sufficient to increase the subsequent response to anoxia in a Ca^{2+}-free solution. The response was gradually reduced in the continued presence of the anoxic, Ca^{2+}-free superfusate. Prior K^+-induced depolarization would be expected to load intracellular stores as the raised $[Ca^{2+}]_i$ is sequestered. However K^+-induced depolarization did not increase the subsequent response to anoxia suggesting that an increased Ca^{2+} influx, or an increased resting $[Ca^{2+}]_i$, is required to enhance the response rather than some longer term loading of an intracellular store.

The rise in $[Ca^{2+}]_i$ in response to anoxia was largely unaffected by blockade of voltage-gated Ca^{2+} channels by any one of cobalt (2 mM; $n = 6$), verapamil (100 μM; $n = 2$) or D600 (10 μM; $n = 3$), and was also unaffected by prolonging action potential duration with tetraethylammonium (25 mM; $n = 3$) (not shown).

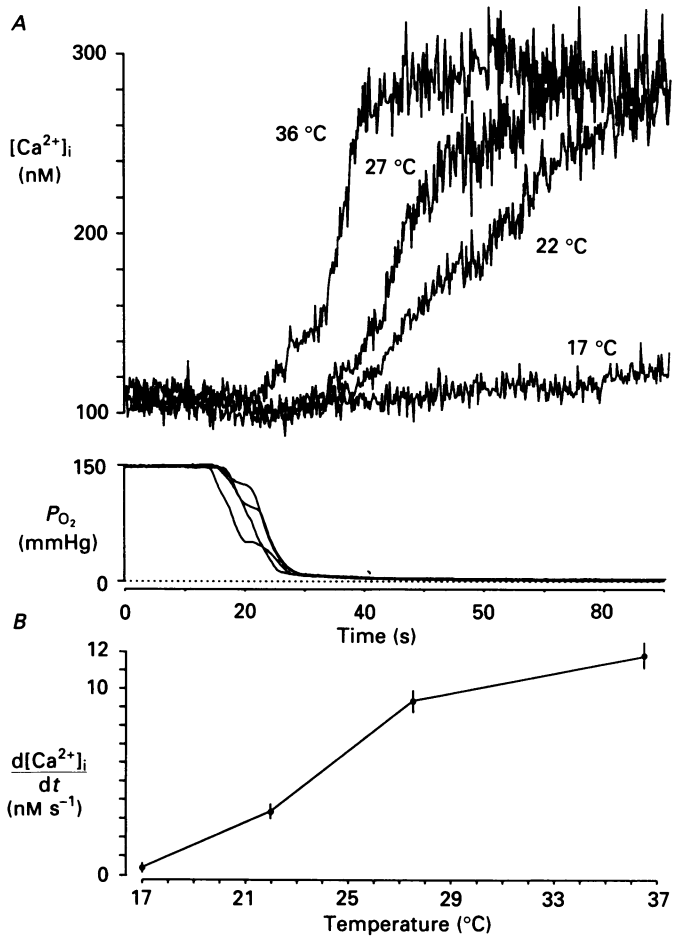

Fig. 4. *A*, the rise in $[Ca^{2+}]_i$ in response to anoxia as the temperature was varied. The rate of rise of $[Ca^{2+}]_i$ is plotted against temperature in *B*. A linear regression line was fitted to the steepest part of each response (in each case, with a correlation of fit $R^2 > 0{\cdot}9$) to obtain the points shown, and the standard error of the fit is indicated on the graph.

It has been suggested that hypoxia might lead to an increase in the frequency of action potentials generated by type I cells (López-Barneo, López-López, Ureña & González, 1988; López-López, González, Ureña & López-Barneo, 1989) raising $[Ca^{2+}]_i$ by an increased influx. While the results illustrated here show that the rise in $[Ca^{2+}]_i$ in our preparation cannot originate predominantly from an increased Ca^{2+} influx, a sufficiently high frequency of action potentials might increase $[Na]_i$, conceivably stimulating Na^+–Ca^{2+} exchange, so raising $[Ca^{2+}]_i$. However, the

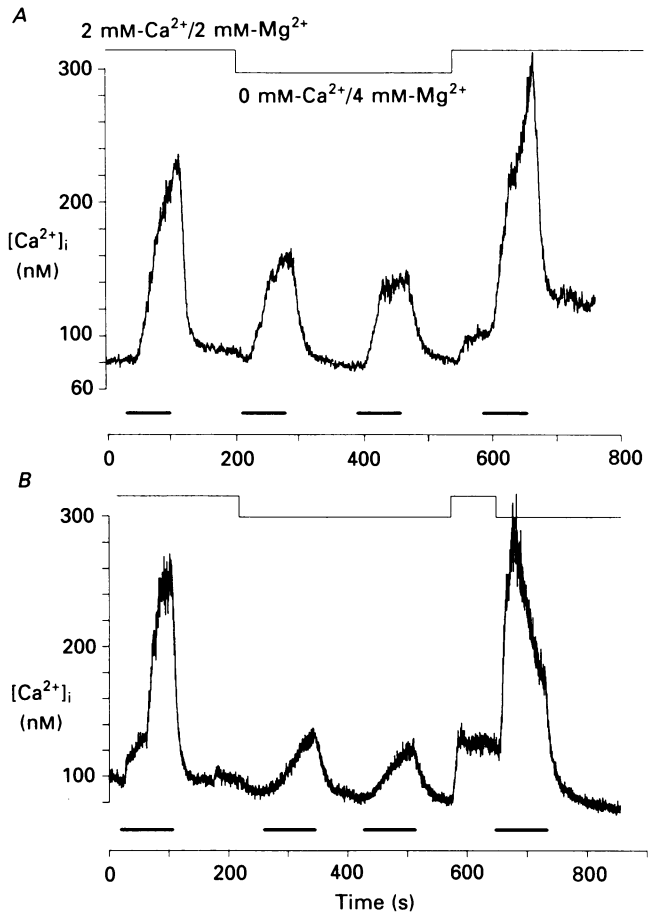

Fig. 5. Responses to anoxia (indicated by horizontal bars) persisted on superfusion with nominally Ca^{2+}-free solutions. *A*, after switching to the Ca^{2+}-free superfusate, the response to anoxia was still seen, although reduced in amplitude. The response to a second exposure was further reduced. $[Ca^{2+}]_i$ rose above the resting level on switching back to the control superfusate, and the response to anoxia was exaggerated. *B*, this experiment illustrates two further points. The initial control response of these cells to anoxia showed a step-like increase (see Fig. 6, in which the same cells were used) which was no longer seen on removal of $[Ca^{2+}]_o$. The reduction in the response was greater than seen in *A*. Switching back to a normal superfusate raised $[Ca^{2+}]_i$, as in *A*, but rather more so. The superfusate was then switched directly to an anoxic Ca^{2+}-free solution, and the response to anoxia was still exaggerated.

response to hypoxia was not impaired by concurrent exposure to 1 μM-tetrodotoxin (TTX, Fig. 6; $n = 4$) which suppresses action potentials by blockade of the fast inward sodium current in these cells (Duchen *et al.* 1988).

The form of the response to anoxia varied between populations of cells. In some cell clusters, there were rapid almost step-like changes, as seen in Figs 3*A* and 6, while in other preparations, the response consisted only of a smooth graded increase in $[Ca^{2+}]_i$, as seen in Figs 4 or 5. The result shown in Fig. 6 shows that the step-like

increases were not due to the cells firing Na^+-dependent action potentials since these had been abolished by TTX. Furthermore, simultaneous electrophysiological and microfluorimetric recordings showed that action potentials in a single type I cell were not sufficient to generate detectable $[Ca^{2+}]_i$ transients in the signal averaged

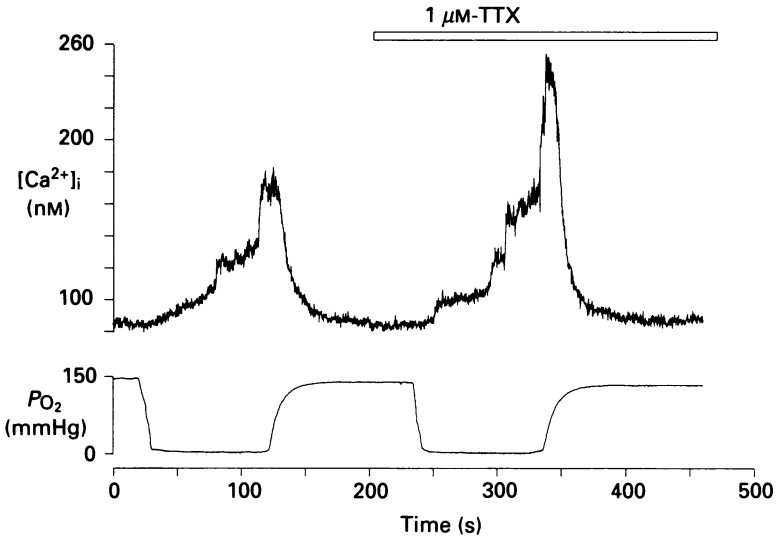

Fig. 6. Records from a cluster of type I cells showing abrupt step-like increases in the control response to anoxia. Inclusion of TTX (1 μM) in the superfusate did not abolish the steps, and seemed to increase the response, not decrease it.

from a cell cluster (see below and Fig. 11). They could therefore only originate from Ca^{2+}-dependent action potentials if the cells were electrically coupled so allowing all of the cells in a cluster to discharge at once. It is conceivable that such coupling may be a variable feature of the preparations. The abrupt, step-like changes in $[Ca^{2+}]_i$ were not seen in Ca^{2+}-free solutions, suggesting that they may originate through Ca^{2+} influx. Unfortunately they have been too inconsistent a feature of the response for systematic study.

Mitochondrial uncouplers and anoxia produce similar increases in $[Ca^{2+}]_i$ and are mutually exclusive

One obvious source for the increase in $[Ca^{2+}]_i$ in response to CN^- or to anoxia is release from a mitochondrial pool as a consequence of a falling mitochondrial membrane potential ($\Delta\psi_m$). Brief exposure of cells to supramaximal concentrations (1–5 μM) of the proton ionophore and mitochondrial uncoupler, FCCP, caused a rapid rise of $[Ca^{2+}]_i$ again to a plateau, from which recovery was slow. The amplitude of the rise in $[Ca^{2+}]_i$ was invariably similar to the rise due to anoxia ($n = 12$). As the responses to FCCP were very slowly, and probably only partially reversible, FCCP was only applied by pressure ejection from a pipette close to the cell under study and was usually the last operation before abandoning a cell.

If the cells were exposed to an anoxic superfusate in the continued presence of FCCP, the response to anoxia was invariably completely occluded ($n = 12$). Only the

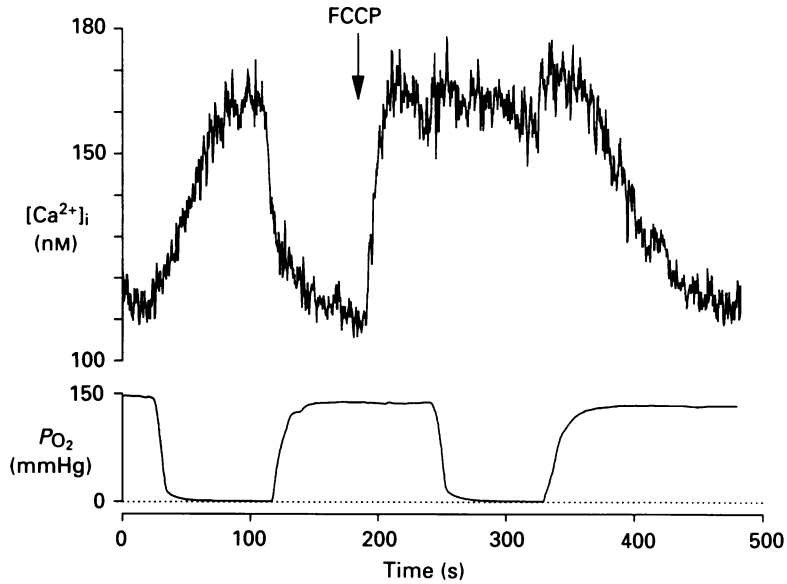

Fig. 7. Effect of FCCP on the response to anoxia. $[Ca^{2+}]_i$ rose in response to anoxia as expected. FCCP (5 μM) raised $[Ca^{2+}]_i$ by the same amount, occluding the response to anoxia.

small increase in autofluorescence remained (seen on the raw traces, but not on the ratio), showing that the activity of the electron transport chain was still altered by the removal of O_2. These findings strongly suggest that both FCCP and anoxia release the same pool of Ca^{2+}, which most probably originates from mitochondria. Attempts to identify a non-mitochondrial Ca^{2+} pool in these cells have failed, as caffeine (10 mM), the agent most likely perhaps to release Ca^{2+} from endoplasmic reticulum (Weber & Herz, 1968), had no effect on $[Ca^{2+}]_i$.

Changes in $[Ca^{2+}]_i$ are graded with graded changes in P_{O_2}

If the rise in $[Ca^{2+}]_i$ during anoxia is relevant for the transduction process, the change in $[Ca^{2+}]_i$ should be related to P_{O_2} in a manner consistent with the activity of the whole structure *in vivo*. The responses shown above have mostly been to a maximum stimulus, i.e. 0 mmHg, but it was also shown in Fig. 3 that, on warming, clear responses could be seen on lowering the P_{O_2} to about 20 mmHg. By varying the intensity with which the superfusates were bubbled with N_2, it proved possible to vary the effective P_{O_2} achieved in the bath. The responses to four out of a larger set of stimuli to one cell group are shown in Fig. 8, where the graded and progressive increase of $[Ca^{2+}]_i$ with lowering of P_{O_2} is shown.

From similar experiments with a range of hypoxic stimuli the maximum $[Ca^{2+}]_i$ was plotted against the minimum P_{O_2} achieved during the set period (usually 60–90 s) for several groups of cells (Fig. 9).

The steepness of the relationship varied between cell groups, but these roughly hyperbolic stimulus–response curves resemble those seen for single afferent fibres from the whole structure, where the response is defined in terms of action potential

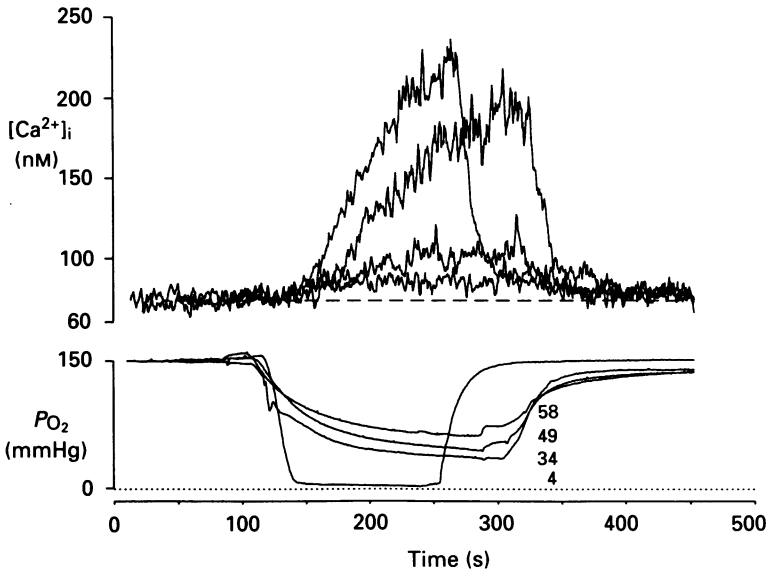

Fig. 8. Four superimposed responses to solutions equilibrated over a range of P_{O_2} values, as shown in the lower traces from the P_{O_2} electrode. The values reached are indicated by each P_{O_2} trace. The temperature was 35 °C.

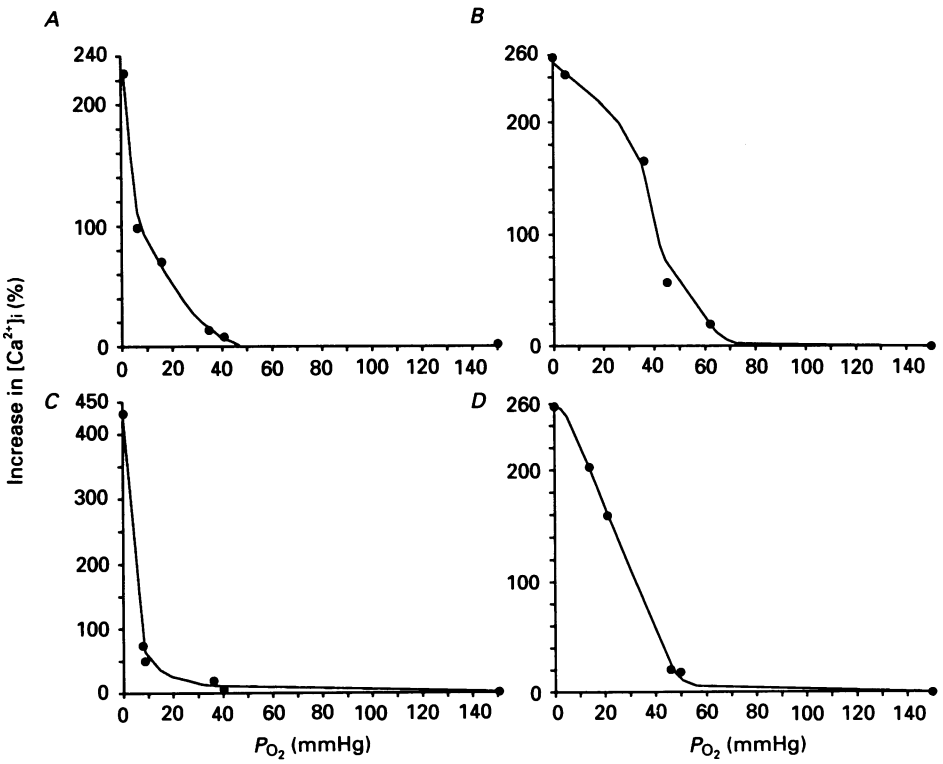

Fig. 9. Peak values of $[Ca^{2+}]_i$ are plotted as a percentage increase against the trough values for P_{O_2} achieved in four separate clusters of cells as shown in Fig. 8. The shape of these stimulus–response curves, and the sensitivity of different cell groups clearly varied. The responses seen in *D* were obtained in a bicarbonate buffer (solution A), variably equilibrated with 95% N_2 and 5% CO_2. All responses were obtained at 35–37 °C.

frequency (Biscoe, Purves & Sampson, 1970), and the variability in the sensitivity of different cell groups also resembles the variability of the responses of single afferent fibres. Such graded changes in $[Ca^{2+}]_i$ with graded changes in P_{O_2} have been documented for more than ten cell clusters.

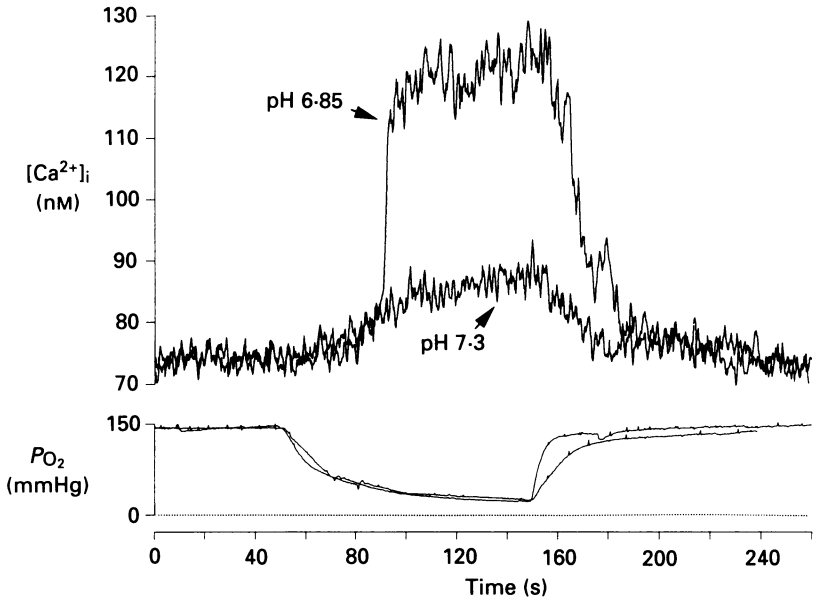

Fig. 10. Two superimposed responses to superfusion with a solution at P_{O_2} of about 30 mmHg, first at pH 7·3, and then at pH 6·85. The sensitivity of the cells to this moderately hypoxic solution was clearly greatly increased in an acid pH.

The sensitivity of the cells to hypoxia is increased by acid pH

The sensitivity of the carotid body *in situ* is increased by either a metabolic or respiratory acidosis. In the present experiments acidification of the HEPES-buffered superfusate by $< 0{\cdot}5$ of a pH unit alone had no significant effect on $[Ca^{2+}]_i$ at the resting P_{O_2} (i.e. about 150 mmHg). However, the rise in $[Ca^{2+}]_i$ when the P_{O_2} fell below about 60 mmHg was much increased (Fig. 10; $n = 12$), shifting the stimulus–response curve to the right. The increased sensitivity followed within 20–30 s of acidification of the superfusate.

When switching from a HEPES-buffered saline (solution B) to a bicarbonate-buffered saline (solution A) at the same pH, a small increase in $[Ca^{2+}]_i$ was seen. This was transient in some cells, sustained in others. The nature of the buffer had only slight and inconsistent effects on the response to hypoxia at any given pH ($n = 6$). Alteration of the pH by varying the bicarbonate concentration had very similar effects to altering the extracellular pH with HEPES; a more acid solution enhanced the response and increased the rate of rise in $[Ca^{2+}]_i$. These effects will be discussed separately in more detail.

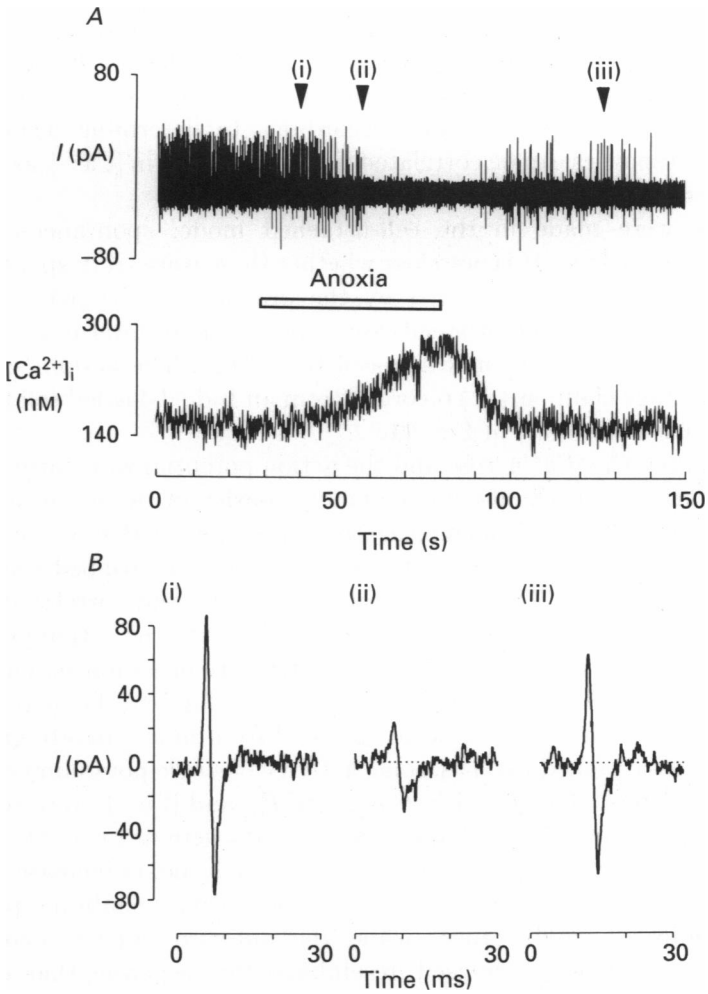

Fig. 11. *A*, cell-attached patch clamp recordings from one cell in a small cluster, showing spontaneous action currents. Exposure to an anoxic superfusate raised $[Ca^{2+}]_i$ (lower trace). The rise in $[Ca^{2+}]_i$ was accompanied by a decrease in the frequency of the action currents, which recovered, although to a slower rate, when the $[Ca^{2+}]_i$ recovered. Examples of the action current waveforms at times indicated (i–iii) are shown in *B*. Note the broadening of the waveform as the frequency began to fall, and $[Ca^{2+}]_i$ began to rise. Note also in *A* that the action potentials in this cell did not generate significant transients on the $[Ca^{2+}]_i$ signal.

Electrophysiological consequences of hypoxia

We have shown that CN^- increases a Ca^{2+}-dependent potassium conductance in type I cells (Biscoe & Duchen, 1989*a*) and have suggested that the electrophysiological response is a consequence of the change in $[Ca^{2+}]_i$, and not its cause. The data presented above argue strongly that the responses to hypoxia and to CN^- are equivalent, sharing a common mechanism. However, it has been suggested by others that the mechanism of the response to hypoxia is distinct, involving the

suppression of a potassium conductance, initiating an increased excitability of the cells with a consequent influx of Ca^{2+} via voltage-gated channels (López-Barneo *et al.* 1988; López-López *et al.* 1989; Delpiano & Hescheler, 1989). This mechanism cannot account for the data that we have presented above for our preparation of adult cells. Nevertheless we have made patch clamp recordings to determine whether the electrophysiological responses can be correlated with the change in $[Ca^{2+}]_i$ as we have shown for CN^- (Biscoe & Duchen, 1989*a*).

When recordings were made in the cell-attached mode, spontaneous action potentials were sometimes seen. It is not clear whether these were truly spontaneous, or whether they were somehow triggered by the presence of the patch pipette. However, they could be used to monitor cell excitability without interfering with the cell cytoplasm, a possible problem in whole-cell recordings. The action 'currents' (the amplifier is in voltage clamp mode) recorded from an Indo-1-loaded cell that was one of a cluster of cells are shown in Fig. 11.

In response to anoxia, the $[Ca^{2+}]_i$ rose and the action potential waveform initially broadened (Fig. 11*B*). This change in shape is consistent with an increased membrane conductance in the cell membrane as a whole, so that less current was generated under the patch. Subsequently, the action potentials stopped completely as the $[Ca^{2+}]_i$ reached its peak. The response was reversible, and correlated clearly with the changes in $[Ca^{2+}]_i$. Note that $[Ca^{2+}]_i$ transients due to the action potentials were not detectable, suggesting that even quite a high rate of action potentials, at least in this cell, was not sufficient to raise $[Ca^{2+}]_i$ to anything like the increase seen in response to hypoxia. Such responses were obtained from four separate groups of cells. In some other cell-attached patches, some activity of single potassium channels was seen, but no consistent changes with changes in P_{O_2} and $[Ca^{2+}]_i$ were seen.

In whole-cell recordings, at 35–36 °C, outward currents were not very stable, and were therefore difficult to examine. However, in five cells, hypoxia increased $[Ca^{2+}]_i$ without changing the holding current at -70 mV, the resting membrane potential or the resting membrane conductance, while the outward current evoked by depolarizations to $+20$ mV was increased, resembling the responses that we have described to CN^- (see Biscoe & Duchen, 1989*a*: Fig. 2) and suggesting that the response to both anoxia and CN^- share a common mechanism.

DISCUSSION

We have shown here that hypoxia raises $[Ca^{2+}]_i$ in type I cells of the rabbit carotid body and that the Ca^{2+} could originate predominantly from a mitochondrial pool. Our data suggest that the response to hypoxia is largely equivalent to the response to CN^-, which we have described previously both in terms of electrophysiology and in terms of altered $[Ca^{2+}]_i$ (Biscoe & Duchen, 1989*a*; Biscoe *et al.* 1989). The sensitivity of this system to graded changes of P_{O_2} and the modulation of the stimulus–response relation by temperature and pH closely parallel the sensitivity of the structure as a whole, suggesting that the changes we describe may well underpin the transduction process in the carotid body.

It is known that the chemoreceptor discharge will follow within-breath changes in

arterial blood gases (Biscoe & Purves, 1967) and so is responding in life within much less than 1 s. This *in vivo* response is from around a tissue P_{O_2} of 40 mmHg to perhaps 5–10 mmHg according to the values given by, for example, Whalen *et al.* (1973). On the other hand the *in vitro* response that we here describe is to changes from a P_{O_2} around 150 mmHg down to between 50 and 0 mmHg. Further, our P_{O_2} electrode is not placed close to the cells and we know that there is turbulent flow in the bath (see Methods). Accordingly the timing of the changes we show should not be taken as an accurate guide to what actually goes on at the surface of, and within, the cells. We are carrying out more experiments to clarify this very issue.

The observations that significant responses to hypoxia are still seen in nominally Ca^{2+}-free solutions, and that the response is occluded by FCCP, strongly suggest that the rise in Ca^{2+} originates from an intracellular pool, and that the pool is mitochondrial, as we suggested previously for the response to CN^-. Ca^{2+} is taken up into mitochondria in response to the large electrochemical potential gradient maintained through the activity of the electron transport chain (Rottenberg & Scarpa, 1974). In turn, Ca^{2+} is exported from mitochondria by a Na^+–Ca^{2+} antiport and possibly by Ca^{2+}–H^+ ion exchange (for reviews, see Crompton, 1985; Carafoli, 1987). This results in a continuous cycle of Ca^{2+} moving between the cytoplasm and mitochondrial matrix, establishing a complex equilibrium between the cytoplasm and mitochondrial matrix on the one hand, and cytoplasm and extracellular space on the other. It is not yet clear by how much mitochondria from different tissues vary in terms of the mechanisms available to express a membrane potential predominantly as a pH gradient or as a potential gradient ($\Delta\psi_m$), or in terms of Ca^{2+} handling. It seems feasible that different populations of mitochondria may effectively cycle Ca^{2+} at different rates so that $[Ca^{2+}]_i$ will rise at different rates as the influx is decreased by a falling $\Delta\psi_m$ or, alternatively, that the rate of change and sensitivity to hypoxia of $\Delta\psi_m$ varies between tissues. The activity of the electron transport chain is not expected to change until the P_{O_2} reaches levels close to the K_D for the oxygen acceptor, cytochrome aa_3, which is < 1 mmHg in most tissues. Mills & Jöbsis (1970, 1972), however, have provided evidence for the expression of an apparently low affinity cytochrome aa_3 in carotid body tissue, which could account for the apparently high sensitivity of electron transport (and therefore of mitochondrial Ca^{2+} efflux) in these cells to falling P_{O_2} (see also Nair, Buerk & Whalen, 1986).

This interpretation of the data is further supported by our observation that when Ca^{2+} influx into the cell is stimulated, for example on returning $[Ca^{2+}]_o$ after exposure to a Ca^{2+}-free superfusate, the response to hypoxia is increased, while it is reduced in the absence of extracellular Ca^{2+}. When extracellular Ca^{2+} is removed, Na^+ moves into the cell through Ca^{2+} channels, loading the cell with Na^+. When extracellular Ca^{2+} is replaced, the Na^+–Ca^{2+} exchange mechanism (which we have demonstrated in these cells previously) extrudes Na^+, and imports Ca^{2+}. Under such conditions, an extra load will be placed on all Ca^{2+} handling processes in the cell, including mitochondrial uptake processes, stimulating mitochondrial Ca^{2+} uptake, diverting energy from the F_1 ATP synthetase, and therefore increasing the effect of hypoxia.

The rise in $[Ca^{2+}]_i$ in response to hypoxia may not require a fall in the ATP/ADP ratio. We have shown previously that inclusion of ATP in the patch pipettes has no

effect on the response to CN^-. A substantial fall in ATP would eventually impair the ability of the cell to maintain all electrochemical potential gradients, with a subsequent influx of Ca^{2+} or loss of Ca^{2+} from an intracellular store that requires ATP to maintain its integrity. In either case the $[Ca^{2+}]_i$ would not be expected to stay at a new plateau level, but would rather rise with increasing rapidity as the ATP levels fall progressively. Furthermore, the increased metabolic insult provided by FCCP would be expected to decrease the rate of ATP production still further and to increase ATP consumption, and therefore to increase the effect of hypoxia, rather than to prevent it, as described here. Clearly, ATP production must be reduced if $\Delta\psi_m$ falls. The actual rates of change of the ATP/ADP ratio will however depend on the rates of consumption, and on the glycolytic capacity of the cells, both of which are unknown.

The above scheme supports a simple model for transduction proposed previously, which requires that graded changes in $[Ca^{2+}]_i$ in response to graded changes in P_{O_2} lead to similarly graded changes in the release of a transmitter from the type I cells onto the terminations of petrosal ganglion neurones, initiating appropriate frequency-coded signals that are then transmitted to the CNS. The nature of the interaction between the type I cell transmitter and the nerve terminals, the identity of that transmitter, and the nature of the coupling between changes in $[Ca^{2+}]_i$ and transmitter release remain somewhat controversial (Jacobs & Comroe, 1968; Pallot, 1987) and must now represent major questions in understanding the details of transduction.

We have documented here a phenomenon for which we can still not account. In some of our preparations, the $[Ca^{2+}]_i$ has risen in a step-like fashion, suggesting abrupt events. These appear to be dependent on Ca^{2+} influx, and are not due to Na^+-dependent action potentials. We have not been able to test the efficacy of Ca^{2+} channel blockers on these responses, as they have not occurred with sufficient regularity. This further suggests that they do not represent a necessary component of the response, but their presence raises interesting possibilities. For example, they may represent a Ca^{2+}-dependent Ca^{2+} influx, as the responses invariably start with a slow graded response which is largely unaffected by Ca^{2+} removal. These phenomena require further study.

Several authors have suggested alternative models for the transduction process in this structure that posit an electrophysiological mechanism as the primary generator of a response to hypoxia. López-López *et al.* (1989) suggested that hypoxia suppresses a voltage-gated potassium conductance, increasing the excitability of the cells, while Delpiano & Hescheler (1989) have suggested that hypoxia suppresses a resting potassium conductance, leading to depolarization of the cells. In both cases, the electrophysiological response is assumed to initiate Ca^{2+} influx through voltage-gated Ca^{2+} channels. Each model, including our own, accepts that a rise in $[Ca^{2+}]_i$ initiating increased transmitter release is a necessary prerequisite for transmission of the signal, but differs in the mechanism suggested to account for the rise in $[Ca^{2+}]_i$.

The models of these other authors clearly cannot account for our findings. The relative independence of the response of $[Ca^{2+}]_o$ immediately serves to exclude both. This is further reinforced by the following: (i) TTX does not block the response to hypoxia; (ii) Ca^{2+} channel blockers fail to block the response; (iii) the responses to

both hypoxia and CN^- are strongly temperature sensitive, suggesting an equivalent metabolic mechanism, while the response to K^+-induced depolarization (and therefore to voltage-gated Ca^{2+} influx) is only minimally affected by the same temperature changes; (iv) if the response to hypoxia were due to Ca^{2+} influx, FCCP should increase the Ca^{2+} transient produced by hypoxia, as Ca^{2+} buffering would be impaired (see, for example, Biscoe *et al.* 1989); instead, the response to hypoxia is completely occluded by FCCP; (v) the equivalence of the rise in $[Ca^{2+}]_i$ seen in response to hypoxia, CN^- and FCCP strongly suggests that all three mobilize Ca^{2+} from the same pool; if the mechanisms differed, there is no reason why they should cause increases in $[Ca^{2+}]_i$ of similar magnitude; (vi) we have documented a decrease in action potential frequency associated with a rise in $[Ca^{2+}]_i$; (vii) we have seen no change in resting current or conductance while documenting a rise in $[Ca^{2+}]_i$, which can thus occur without depolarization of the cells; (viii) 25 mM-tetraethylammonium chloride, which blocks most of the voltage-gated outward current seen in these cells (Duchen *et al.* 1988), only produces a small rise in $[Ca^{2+}]_i$ compared to the response to hypoxia and (ix) while the response to hypoxia is increased by an acid pH, the response to depolarization is decreased. While at present we cannot account for the differences between our results and those quoted above, it seems clear that the properties of the fundamental processes involved in mitochondrial Ca^{2+} regulation and their oxygen dependence may be quite sufficient to serve as a basis for transduction in these cells whatever their electrophysiological response might be.

Our findings lead to the conclusion that, in the type I cells, mitochondrial regulation of $[Ca^{2+}]_i$ serves a physiological role in the regulation of transmitter release. The rise in $[Ca^{2+}]_i$ in response to anoxia or metabolic poisoning is thus not unique to this system, and has been documented also in isolated mammalian neurones (Duchen *et al.* 1990) with similar physiological consequences (Duchen, 1990) and in the squid giant axon (Blaustein & Hodgkin, 1969). In some other secretory systems, increased spontaneous transmitter release has been described as a consequence of hypoxia, anoxia or metabolic poisoning (Alnaes & Rahamimoff, 1975; Nishimura, 1986), although stimulus-coupled release mechanisms may be impaired under these conditions. There are thus precedents for such a mechanism, although the sensitivity of the type I cells suggests that they are specialized for their physiological role, and, perhaps uniquely, that these cells use mitochondrial Ca^{2+} to regulate transmitter release.

Note added in proof. We have recently used the fluorescent dye Rhodamine 123 to monitor $\Delta\psi_m$ directly, and have found that graded hypoxia produces graded depolarizations of $\Delta\psi_m$ as speculated here. These data were presented at the University College meeting of the Physiological Society (Duchen & Biscoe, 1990).

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