Hypoxia-induced catecholamine secretion in isolated newborn rat adrenal chromaffin cells is mimicked by inhibition of mitochondrial respiration

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1. In newborn mammals, systemic hypoxia provokes catecholamine secretion from the adrenal medulla. In contrast to adults, this release is independent of sympathetic innervation. We have studied the cellular processes involved in hypoxia-induced catecholamine secretion, employing fluorimetric techniques to measure changes in $[Ca^{2+}]_i$, NADH and mitochondrial potential, and voltammetric techniques to record changes in P_{O_2} and catecholamine secretion.

2. In adrenal chromaffin cells freshly dissociated from newborn rats, severe hypoxia increased $[Ca^{2+}]_i$ and secretion of catecholamines, indicating that the response of the newborn adrenal medulla to hypoxia is an intrinsic property of these cells. Discrete quantal secretory events were identifiable, suggesting an exocytotic mechanism of secretion.

3. Hypoxia-induced secretion was only seen when P_{O_2} fell below 5 mmHg, similar to the threshold arterial P_{O_2} reported to stimulate release *in vivo*. Such oxygen tensions also inhibited mitochondrial metabolism, shown by an increase in NADH autofluorescence. We therefore explored the involvement of mitochondria in oxygen sensing. Inhibition of mitochondrial respiration either by CN^- at complex IV or by rotenone at complex I mimicked severe hypoxia, reversibly increasing both $[Ca^{2+}]_i$ and catecholamine secretion. The CN^--induced depolarization of the mitochondrial inner membrane potential preceded the increase in $[Ca^{2+}]_i$ by ~6 s.

4. The effects of severe hypoxia and CN^- on $[Ca^{2+}]_i$ and catecholamine secretion were not additive, suggesting a common mechanism.

5. Chemical anoxia failed to increase $[Ca^{2+}]_i$ in a significant proportion of cells dissociated from 2- to 4-week-old rats. Thus, the sensitivity to hypoxia is specific to adrenal chromaffin cells dissociated from newborn rats.

6. These data indicate that hypoxia-induced catecholamine secretion in the newborn adrenal medulla is mediated by reversible inhibition of mitochondrial respiration, leading to an increase in $[Ca^{2+}]_i$ and catecholamine secretion.

From fetal to adult life, the release of catecholamines from the adrenal medulla plays an important role in surviving hypoxic stress (for review see Lagercrantz, 1996). In addition, during vaginal delivery, a surge of catecholamine secretion in the fetus plays an important role in the initiation of respiration, by inducing the lung endothelium to switch from a state of net fluid secretion to one of net fluid absorption (Olver, Ramsden, Strang & Walters, 1986). The catecholamines are presumably released from the adrenal medulla: it is the major site of catecholamine synthesis and secretion, it is relatively enlarged in the perinatal period, and hypoxia represents a major stimulus of adrenal catecholamine secretion in the neonate (Girard & Zeghal, 1975).

The mechanisms mediating hypoxia-induced catecholamine secretion from the adrenal medulla have been of long standing interest (Houssay & Molinelli, 1926; Bülbring, Burn & De Elio, 1948; Comline, Silver & Silver, 1965; Comline & Silver, 1966; Seidler & Slotkin, 1985, 1986*a*, *b*). It is now clear that two distinct mechanisms must operate at different stages of development. During the fetal period, the adrenal medulla is not functionally innervated; hence hypoxia can only stimulate the adrenal medulla either directly or via an endocrine mechanism. In contrast, after innervation is established, catecholamine secretion in response to hypoxia is entirely dependent on sympathetic outflow, and is abolished by sectioning the nerves (Comline & Silver, 1966; Seidler & Slotkin, 1985). In the calf, the transition from the direct to the neurogenic response occurs within 24 h of birth (Comline & Silver, 1966). Both the newborn and the mature adrenal response to hypoxia can be

studied more conveniently in the rat, as in this species the transition occurs 4–7 days after birth (Seidler & Slotkin, 1985). Studies in this species suggest that impulse activity in the adrenal nerves inactivates the ability of the medulla to mount a direct response to hypoxia. It is as yet not clear whether the direct response is an intrinsic property of the adrenal medulla, or whether it reflects a response to changes in the endocrine environment. Even assuming some direct cellular response, the cellular mechanism remains unknown. One possibility is that compromised metabolic activity leads to the reversal of a carrier, as suggested in sympathetic neurons in the heart (Schömig, Kurz, Richardt & Schömig, 1988); alternatively, rather indirect evidence suggests an exocytotic mechanism (Seidler & Slotkin, 1986*b*).

In the present study, we have isolated chromaffin cells from the rat adrenal medulla. We have used fluorimetric and voltammetric techniques, and show that hypoxia-induced catecholamine secretion is an intrinsic property of newborn adrenal chromaffin cells. We then studied the cellular mechanism underlying the response, addressing the following questions: (i) is the release based on exocytosis or on metabolic inhibition of a catecholamine uptake carrier? (ii) is catecholamine secretion accompanied by an increase in $[Ca^{2+}]_i$? and (iii) what is the specific oxygen sensitivity of the response? When it became apparent that only severe hypoxia leads to catecholamine secretion, we considered the possible involvement of mitochondrial respiration in the process of oxygen sensing. Finally, we examined the developmental differences by studying the effect of chemical anoxia in chromaffin cells isolated from 2- to 4-week-old rats.

Preliminary results have been presented to the Physiological Society (Mojet, Mills & Duchen, 1995, 1996).

METHODS

Preparation

Adrenal glands from one Sprague–Dawley rat (either less than 24 h old (6–7 g) or 2–4 week old) were quickly removed after decapitation (2- to 4-week-old animals were first stunned by cervical dislocation). While in ice-cold isolation saline (IS, see below), any remaining fatty tissue was removed, and the adrenal glands were cut into small pieces. To allow comparison between newborn and 2- to 4-week-old rats, special care was taken to obtain tissue pieces of the same size. Likewise, the rest of the isolation procedure was identical for newborn and 2- to 4-week-old rats.

The tissue was treated first for 30 min with 4 mg ml^{-1} collagenase I (EC 3.4.24.3; Sigma) in IS, nominally free of Ca^{2+}, and then for another 30 min with 10 μl ml^{-1} papain (Worthington) in IS activated with 11 mM L-cysteine hydrochloride (Sigma); both solutions were maintained at 30–32 °C and continuously bubbled with 100% oxygen. After gentle trituration in IS at room temperature with flamed Pasteur pipettes, cells were plated out on untreated glass coverslips, allowed to settle for at least 30 min, and kept for the day on the benchtop in recording saline (RS, see below). Under phase contrast microscopy chromaffin cell clusters were easily distinguishable from cortical cells, and experiments were performed on clusters varying in number from four to approximately thirty cells.

Microfluorimetry

To record changes in $[Ca^{2+}]_i$, cells were loaded with the Ca^{2+} indicators fura-2 or indo-1 by incubation with their AM esters (Molecular Probes, 5 μg ml^{-1} in RS, supplemented with 50 μg ml^{-1} pluronic acid; final dimethyl sulphoxide (DMSO) concentration, 0·75%) at room temperature for 30 min. In order to examine changes in the mitochondrial inner membrane potential ($\Delta\Psi_m$), cells were loaded with rhodamine 123 (Rh 123, Molecular Probes, 10 μg ml^{-1} RS) for 10 min at room temperature. As a cationic dye, Rh 123 accumulates in mitochondria, where the concentration of the dye causes quenching of the fluorescence. A decrease in mitochondrial inner membrane potential leads to dye efflux from the mitochondria, dequenching and hence an increase in fluorescence (Emaus, Grunwald & Lemasters, 1986; Bunting, 1992). Changes in the Rh 123 signal routinely give reliable and reproducible qualitative changes in signal with a wide range of manipulations consistent with changes in $\Delta\Psi_m$ predicted from chemiosmotic theory (e.g. Duchen & Biscoe, 1992*b*).

Experiments were performed using an inverted epifluorescence microscope (Nikon TMD300), under continuous visual control using phase contrast with red light (> 610 nm) transmitted through the preparation and monitored with a video camera as previously described (e.g. Duchen, 1992). After dye loading, the cells were washed with RS. In all experiments cells were continuously perfused with RS, at a rate of approximately 10 ml min^{-1}, and maintained at 30–32 °C. To achieve severe hypoxia (oxygen partial pressure (P_{O_2}) below 4 mmHg or 0·5 kPa), coverslips were mounted in an open, essentially rectangular chamber designed to minimize local turbulence, and cells were superfused with saline equilibrated with argon, which has a higher specific density than air and therefore effectively forms a blanket above the open bath. To avoid the formation of bubbles in the perfusion lines, media were preheated in a water bath and recirculated if not led to the cells. The perfusion medium could be rapidly switched using solenoid valves, and was passed through a custom built feedback controlled heating element for accurate temperature control. In order to reach low P_{O_2} values, 10 mg ml^{-1} glucose oxidase was included in some early experiments. This enzyme catalyses the oxidation of glucose, depleting the medium of the last traces of molecular oxygen while producing hydrogen peroxide. However, usually no additional suppression of P_{O_2} was measured, and it was therefore often omitted.

Fluorescence was excited using a 75 W xenon arc lamp. Both indo-1 and NADH fluorescence were excited at 350 nm. Fluorescence emission from indo-1 was measured using two photomultipliers screened by 10 nm bandpass filters, one centred at 410 nm, the other at 490 nm. The ratio of the signals obtained at 410 nm to that at 490 nm was presented to reflect changes in $[Ca^{2+}]_i$. NADH autofluorescence was recorded at 450 ± 40 nm.

Simultaneous recording of the changes in $[Ca^{2+}]_i$ and $\Delta\Psi_m$ is possible by dual loading the cells with fura-2 AM and Rh 123, as their excitation spectra do not overlap and both dyes emit at 530 nm. The excitation light was passed through a filter wheel rotating at 12 Hz (Cairn Research Ltd, Faversham, UK) and equipped with filters centred at 340, 360, 380 (for fura-2) and 490 nm (for Rh 123). Using a 510 dichroic block, the fluorescence emission was measured at 530 nm, using a 10 nm bandpass filter. As the photomultiplier measures the result of rapid sequential excitation at different wavelengths, the fluorescence signal at each excitation

wavelength was extracted using a synchronized, integrating sample and hold circuit (Cairn Research). The ratio of the fluorescence excited at 340 nm to that at 380 nm is presented to reflect changes in $[Ca^{2+}]_i$.

The fluorescence signals, the P_{O_2} signal and the bath temperature were recorded on-line using a Tecmar DMA Labmaster-TL1 interface (Scientific Solutions Inc., Solon, OH, USA) controlled through Labtech Notebook data acquisition software (Laboratory Technologies Corp., Wilmington, MA, USA). Data are presented as means ± S.D., and levels of statistical significance (P values) are the result of a one-population Student's t test, unless stated otherwise.

The Rh 123 signal is difficult to calibrate in terms of specific changes in $\Delta\Psi_m$, as it is a single wavelength indicator. Therefore the signal was presented as a percentage change from the resting level, in which an increase in signal reflects mitochondrial depolarization. The signals from the Ca^{2+} indicator dyes were also not calibrated, and have been presented either as fura-2 or indo-1 ratios, or as percentage change from resting values. Despite the use of ratiometric dyes, many problems remain with calibrations, especially in the context of hypoxic exposures. Thus, approximately 10–20% of the fluorescence signal in indo-1-loaded cells was attributable to NADH autofluorescence. The emission spectrum of NADH peaks at 450 nm and extends from 400 to 500 nm (Chance & Schoener, 1966). Indeed, inhibition of mitochondrial respiration led in some clusters to a clear increase in the emission measured at both 410 and 490 nm, affecting the indo-1 ratio and underestimating the change in $[Ca^{2+}]_i$; such data were discarded. Moreover, indo-1 and fura-2 calibrations also vary with the ambient P_{O_2} (see Discussion; Stevens, Fouty, Cornfield & Rodman, 1994). And finally, we routinely found that greater changes in signal were obtained with depolarization of the plasma membrane potential than during attempts to obtain R_{max} using calcium ionophores.

Electrophysiology

Catecholamine secretion and P_{O_2} were recorded electrochemically, using carbon fibre electrodes.

Carbon fibre electrodes were manufactured essentially according to Millar (1992) and Stamford, Crespi & Marsden (1992). Briefly, borosilicate glass tubing was filled with a single carbon fibre (outer diameter, 8 μm) and pulled on a vertical electrode puller. Electrical connection between the carbon fibre and insulated wire was achieved with silver conductive paint and the carbon fibre was spark-etched to a length of approximately 20 μm. Carbon fibre electrodes were tested with fast cyclic voltammetry as described by Stamford *et al.* (1992), using a Millar voltammeter (P. D. Systems International Ltd, West Molesey, UK). Useful carbon fibre electrodes showed a high resistance and large capacitative charging currents in response to rapid changes of the electrode voltage. Often the response of the electrodes improved substantially by offsetting the ramps above the redox potential for water (about 1 V) for a few minutes.

Changes in P_{O_2} were recorded with a carbon fibre electrode positioned within 100 μm of the cell cluster under study, and −600 mV (relative to a Ag–AgCl pellet) was applied using an elementary home-made amplifier. The electrode was calibrated by equating the recorded current in saline equilibrated with air to 150 mmHg, and by assuming that the electrode passes no current during true anoxia. The response properties of the electrode were tested by recording simultaneously the fall in P_{O_2} with a carbon fibre electrode and a home-made platinum wire electrode (Duchen & Biscoe, 1992*a*, *b*). Figure 1 shows the current passed by the carbon fibre electrode plotted as a function of the current passed by the platinum wire electrode. The responses were largely equivalent at high P_{O_2} values but deviated significantly as the P_{O_2} levels fell, where the platinum wire electrode appeared to record lower values of P_{O_2} than the carbon fibre electrode. Presumably this discrepancy was due to diffusional limitations in combination with a difference in O_2 consumption at the surface of the electrodes. These data suggest that the carbon fibre electrode was more accurate than the platinum wire. In addition, the small dimensions of carbon fibre electrodes allow the measurement of changes in P_{O_2} in the immediate vicinity of the cell, avoiding problems related to O_2 gradients or to turbulence in the bath.

For the detection of catecholamine secretion, a (second) carbon fibre electrode was positioned adjacent to or touching a chromaffin cell cluster. Using either a List EPC-7, a Biologic 300 or a home-built patch-clamp amplifier, a constant voltage of 500 mV (relative to a Ag–AgCl pellet) was applied. This voltage is well above the redox potential of catecholamines, which are therefore oxidized on the carbon fibre surface. In fact, the redox potential for catecholamines is between 150 and 250 mV, measured against a Ag–AgCl reference electrode (Stamford *et al.* 1992). Here 500 mV was used to enhance the amplitude of currents attributable to individual secretory events. The redox current was measured with the patch-clamp amplifier, and recorded on tape for off-line analysis. Secretory events were identified by inspection using Fetchan from the pCLAMP suite (Axon Instruments).

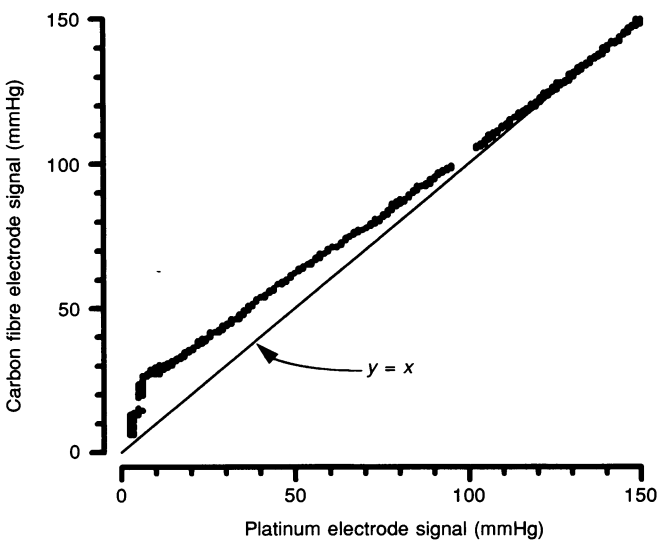

Figure 1. Comparison of carbon fibre and platinum O_2 electrodes

The carbon fibre electrode signal is plotted as a function of the signal measured at the platinum electrode. The oxygen tension was allowed to ramp slowly down towards zero by bubbling a superfusing saline with argon. The progressive deviation from linearity is presumably due to differences in local oxygen gradients and oxygen consumption.

In order to characterize the nature of the secreted species, fast differential ramp voltammetry was applied, using the Millar voltammeter in differential, double diphasic mode (Stamford *et al.* 1992; or Williams & Millar, 1990). In this mode a set of four biphasic triangular asymmetric voltage ramps were applied in succession (duration, 37 ms; repetition frequency, 8 Hz). The first two ramps run each from 0 mV via 1000 and −400 mV to 0 mV (i.e. 560 mV ms^{-1}). The second pair of ramps was offset by −200 mV from the first pair. The last ramp (waveform d) is displayed in Fig. 2*C*. As the redox or faradaic current is very small relative to the charging current, the latter is removed by subtracting electronically the redox current resulting from the second pair of ramps from the first pair. Any catecholamine present is oxidized during the positive phase of the ramps, and reduced again during the negative phase. A chemical species that is oxidized during the positive phase but not reduced during the negative phase (e.g. ascorbate) will not contribute to the redox current during the last waveform. The characteristic waveform for catecholamines is remarkably specific; as shown in Fig. 2*A*, noradrenaline and adrenaline are quite clearly distinguishable. However, in our hands fast differential ramp voltammetry at cell surfaces is not practical for measuring relatively slow long lasting changes as occur during hypoxia.

Chemicals and solution

All chemicals were obtained from BDH, unless otherwise specified. Isolation saline (IS) contained (mM): 160 NaCl, 4·5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 Hepes (Sigma), 10 D-glucose, 10 ascorbate and 0·1 mg ml^{-1} bovine serum albumin (Sigma, Cohn fraction V, 96–99% pure). Recording saline (RS) contained (mM): 156 NaCl, 3 KCl, 2 $MgSO_4$, 1·25 KH_2PO_4, 2 $CaCl_2$, 10 Hepes and 10 D-glucose. Both IS and RS were adjusted to pH = 7·35 with NaOH. To depolarize the plasma membrane potential a medium similar to RS was used, containing 50 mM K^+ obtained by replacing the appropriate amount of NaCl by KCl. L-Noradrenaline, L-adrenaline and glucose oxidase (EC 1.1.3.4) were obtained from Sigma. NaCN was simply added to the solution, and the pH was corrected with HCl. Anoxia was achieved by adding sodium dithionite ($Na_2S_2O_4$, 132 μg ml^{-1} RS) after equilibration with N_2. Rotenone (Sigma) was dissolved in DMSO to 10 mM, and used at 1 μM.

RESULTS

Newborn adrenal chromaffin cells are capable of catecholamine exocytosis

It is well established that the adrenal medulla in newborn rats releases catecholamines during periods of hypoxia *in vivo* (Girard & Zeghal, 1975; Seidler & Slotkin, 1985, 1986*a*, *b*). In order to study the cellular mechanism *in vitro*, we first examined whether adrenal chromaffin cell clusters isolated from newborn rats are capable of releasing catecholamines upon depolarization of the plasma membrane.

Using fast differential ramp voltammetry (see Methods; Williams & Millar, 1990; Stamford *et al.* 1992) voltage ramps were applied to obtain redox current waveforms that are specific for the electroactive species present. As shown in Fig. 2*A*, the method is sensitive enough to discriminate between exogenously applied noradrenaline, adrenaline, or a mixture of the two at a ratio of 0·3, similar to that found in the newborn adrenal (Coulter, McMillen & Browne, 1988). Figure 2*B* shows the waveform obtained at the surface of a chromaffin cell cluster that was depolarized by pressure ejecting a medium containing 50 mM K^+. For comparison, the 'signature' recorded in the presence of the noradrenaline/adrenaline mixture is superimposed; the similarity is striking, and shows that adrenal chromaffin cells isolated from the newborn rat are capable of non-selective release of noradrenaline and adrenaline.

To establish whether the secretion is due to exocytosis, we used DC voltammetry (Millar, 1992; Stamford *et al.* 1992)

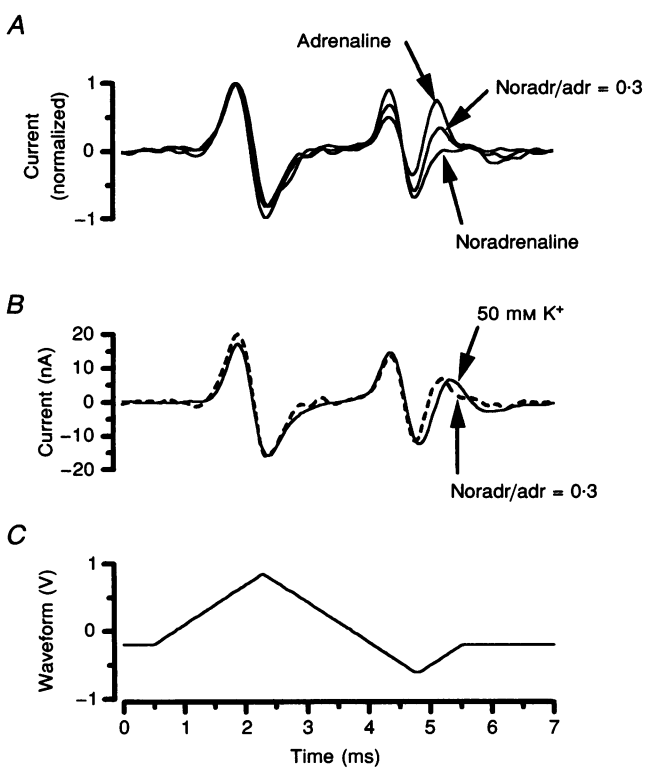

Figure 2. Adrenal chromaffin cells from newborn rats secrete adrenaline and noradrenaline

Fast differential ramp voltammetry was used to identify the electroactive species secreted from the newborn adrenal chromaffin cells in response to potassium-induced depolarization. *A*, normalized responses to exogenous application of adrenaline, noradrenaline and a mixture of noradrenaline and adrenaline at a ratio of 0·3 (as indicated). *B*, waveform recorded at the surface of a cluster of chromaffin cells, while pressure ejecting a medium containing 50 mM K^+. The trace obtained with the exogenous mixture is appropriately scaled and superimposed for comparison. *C*, part of the voltage waveform used (see Methods). Each trace is the average of 5 sweeps.

in combination with fluorimetry. As shown in Fig. 3, depolarization of the plasma membrane with a pulse of 50 mM K^+ evoked a brisk transient increase in $[Ca^{2+}]_i$ and a burst of discrete transient events on the voltammetry signal, each of which reflects a single exocytotic event (Wightman *et al.* 1991; Chow, von Ruden & Neher, 1992; Terakawa, Kumakura & Duchen, 1995). The $[Ca^{2+}]_i$ transient and the burst of catecholamine secretion were quite reproducible, both in the same cell cluster and between clusters ($n \gg 100$). Qualitatively similar responses were observed in adrenal chromaffin cells isolated from 2- to 4-week-old animals.

The rise in $[Ca^{2+}]_i$ followed the K^+ stimulus with a delay of a few hundred milliseconds. The recovery was typically biphasic, first falling to a plateau slightly above the resting value (time constant, in the order of 2–4 s), and then reaching resting levels in 2–3 min. When recordings were maintained from cell clusters for 60 min, or when recordings were made from clusters more than 6 h after isolation, the initial decrease in $[Ca^{2+}]_i$ was much slower (time constant, several tens of seconds), suggesting impaired $[Ca^{2+}]_i$ clearance. The response kinetics to depolarization with 50 mM K^+ were therefore used to assess the cell viability, and clusters with slow recovery were discarded.

Whereas the vigorous barrage of secretory events in response to depolarization was highly reproducible, the waveform of the individual events within such a barrage was variable. It seems likely that this variability reflects spatial variation in the origin of the exocytotic events. Presumably due to diffusional processes, events occurring away from the electrode result in small slow redox currents at the tip of the electrode, while larger events with a faster rise time probably stem from release sites closer to the electrode (Wightman *et al.* 1991; Terakawa *et al.* 1995). The inset in Fig. 3 shows three of these larger and faster events, with amplitudes of approximately 100–200 pA, duration at half-maximum of 10–20 ms and a maximal duration of less than 50 ms. A more detailed analysis of the amplitude distribution and time course of the individual secretory events will be the subject of a separate publication.

The voltammetric record in Fig. 3 also shows a slow transient increase of the baseline, underlying the barrage of quantal events and mirroring the transient increase of $[Ca^{2+}]_i$. This envelope is partly due to the summation of many slow events, but could also in part represent changes in the ionic environment at the tip of the electrode during the application of the medium containing 50 mM K^+, because applying the same stimulus directly onto the electrode without any cells present resulted in some cases in a similar slow transient increase.

Hypoxia-induced catecholamine secretion

Having established that the exocytotic machinery of chromaffin cells isolated from the newborn animal was fully functional, we then studied the effect of hypoxia, by measuring simultaneously catecholamine release, changes in $[Ca^{2+}]_i$, and P_{O_2} within 100 μm of the cell cluster. A typical

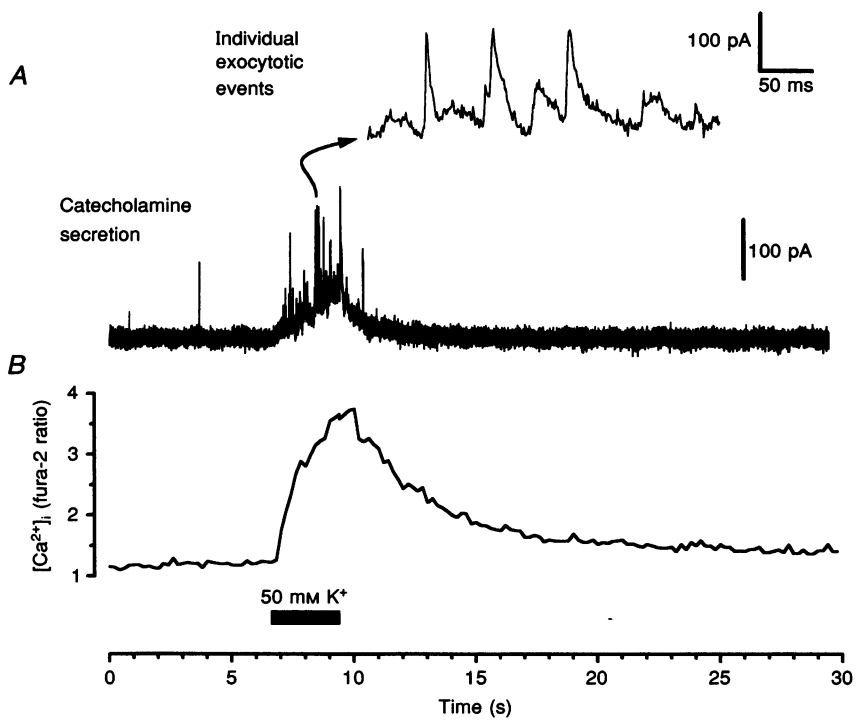

Figure 3. Adrenal chromaffin cells from newborn rats have fully functional exocytotic machinery
Illustration of a typical response of a cluster of cells to potassium-induced depolarization. *A*, DC voltammetric signal; *B*, change in $[Ca^{2+}]_i$ measured with fura-2. Individual exocytotic events are shown in the inset on an expanded time scale.

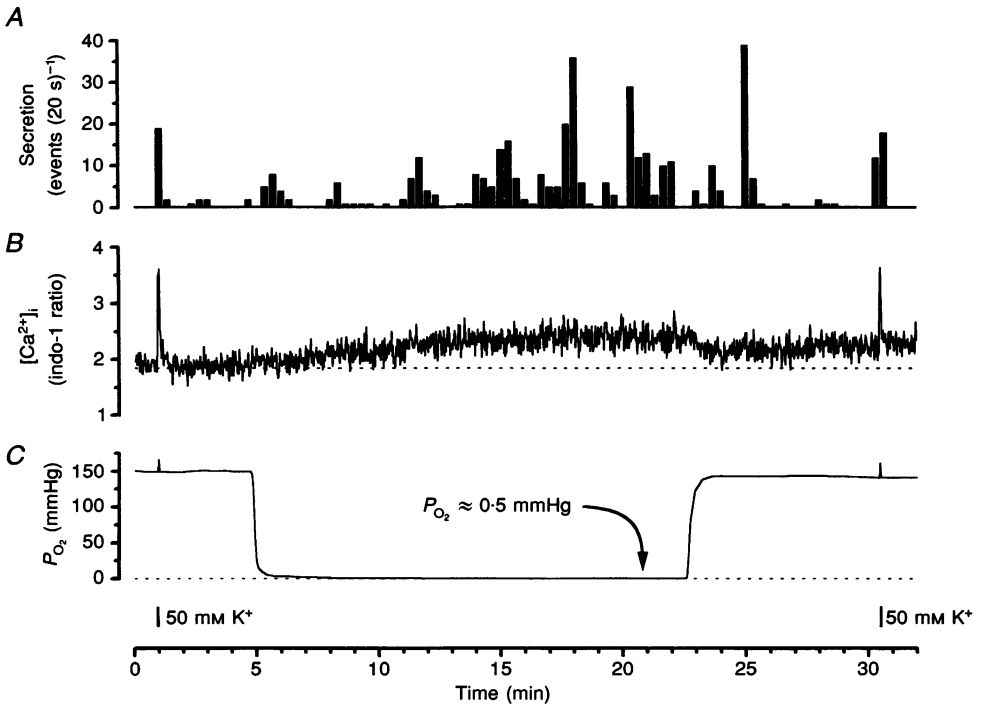

Figure 4. Severe hypoxia increases $[Ca^{2+}]_i$ and catecholamine secretion

A, changes in catecholamine secretion, presented as number of secretory events in 20 s bins; *B*, changes in $[Ca^{2+}]_i$, presented as indo-1 ratio; *C*, changes in P_{O_2}, measured with a carbon fibre electrode within 100 μm of the cell cluster. To establish their viability, cells were first depolarized by pressure ejecting 50 mM K^+, which resulted in a brisk $[Ca^{2+}]_i$ transient and secretory response. Switching to a hypoxic superfusate led to a slow increase in $[Ca^{2+}]_i$ and bursts of secretory events. On restoration of the P_{O_2}, $[Ca^{2+}]_i$ recovered only partially, but a further challenge with 50 mM K^+ established that the cells were still viable.

experiment is presented in Fig. 4, and similar results were obtained in seven other cell clusters.

Cells were stimulated first with 50 mM K^+ to establish their viability. The superfusate was then switched to a saline equilibrated with argon. Only when the P_{O_2} fell sufficiently low did we observe a small increase in $[Ca^{2+}]_i$ and increased catecholamine secretion. The catecholamine release was clearly quantal, with a variation in amplitude and time course similar to events seen in response to depolarization; as events occurred much less frequently and were clearly individually distinguishable, secretion could be presented with frequency histograms. Interestingly, the secretory events seemed to be clustered in bursts. These bursts were not reflected in oscillatory changes in $[Ca^{2+}]_i$. However, as the fluorescence from the whole cluster was measured using photomultipliers, changes of $[Ca^{2+}]_i$ in individual cells could not be resolved. Upon re-oxygenation, the secretion of catecholamines stopped and the increase in $[Ca^{2+}]_i$ partially recovered. Although in the experiment illustrated, the $[Ca^{2+}]_i$ did not return to baseline upon re-oxygenation, the

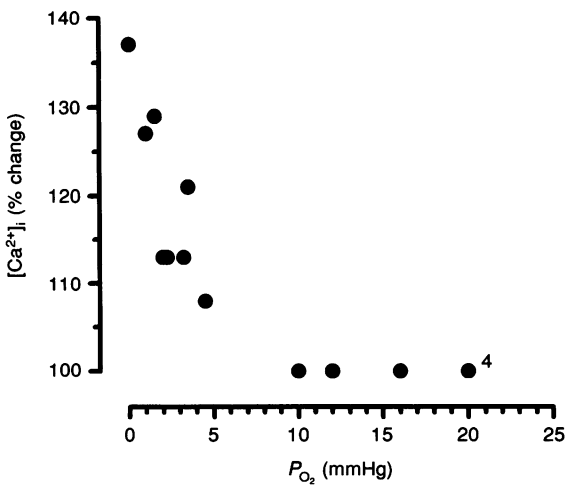

Figure 5. Only severe hypoxia (P_{O_2} < 5 mmHg) leads to an increase in $[Ca^{2+}]_i$

Results of hypoxic challenges to 15 adrenal chromaffin cell clusters isolated from newborn rats. Maximal hypoxia-induced increases in $[Ca^{2+}]_i$ (measured with indo-1 and presented as the percentage change from resting value) are plotted against prevailing local P_{O_2}. The data point marked '4' represents 4 different cell clusters.

response to 50 mM K^+ applied after the hypoxic challenge is indistinguishable from the initial control response.

Figure 5 shows the results of a hypoxic challenge in fifteen adrenal chromaffin cell clusters, by plotting the increase in $[Ca^{2+}]_i$ against the local P_{O_2}. An increase in $[Ca^{2+}]_i$ was only observed if the P_{O_2} fell below *ca* 5 mmHg.

Hypoxia-induced catecholamine secretion coincides with inhibition of mitochondrial metabolism

The range of oxygen pressures that cause an increase in $[Ca^{2+}]_i$ and catecholamine secretion is of the same order of magnitude as that expected to inhibit mitochondrial respiration. To assess the specific oxygen dependence of mitochondrial function in these cells, we studied hypoxia-induced changes in the NADH/NAD^+ ratio and changes in the mitochondrial inner membrane potential ($\Delta\Psi_m$) using rhodamine 123 (Rh 123). Hypoxic challenges as severe as 1–2 mmHg did not change the Rh 123 fluorescence (possibly because Rh 123 is not sensitive enough; Ubl, Chatton, Chen & Stucki, 1996), but NADH autofluorescence did increase during moderate hypoxia ($n = 10$). An increase in autofluorescence indicates an increase in the ratio of NADH over NAD^+, because NADH fluoresces and NAD^+ does not (Chance & Schoener, 1966). An increase in the NADH/NAD^+ ratio can be due either to an increase in substrate oxidation, or to a decrease in the flux through the electron transfer chain and/or availability of molecular oxygen. The NADH autofluorescence is predominantly derived from mitochondrial NAD(P)H (for review see Balaban & Mandel, 1990).

Figure 6 illustrates the response of a chromaffin cell cluster first to hypoxia and then to anoxia. The NADH autofluorescence started to increase as the P_{O_2} fell below *ca* 20 mmHg, as shown in the top left inset. The abrupt transition to anoxia was associated with a second large increase in the NADH/NAD^+ ratio. In this particular experiment a local perturbation of the P_{O_2} occurred, and the P_{O_2} transiently increased to approximately 12 mmHg. This

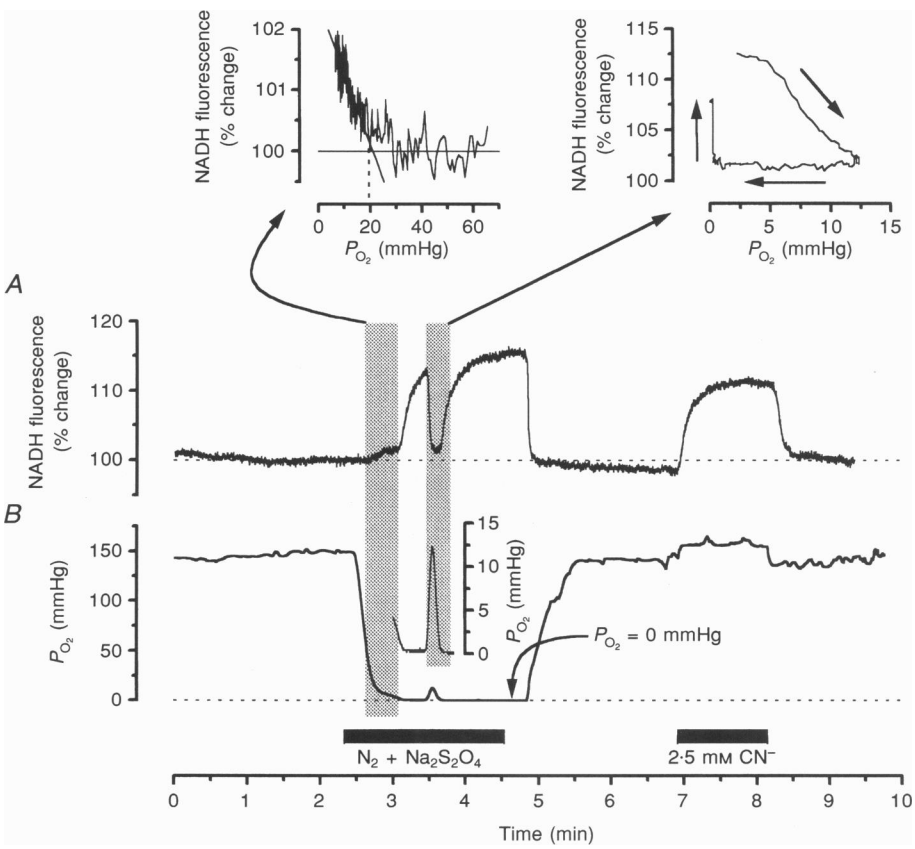

Figure 6. Mitochondrial respiration is impaired during hypoxia-induced catecholamine secretion
A, changes in NADH autofluorescence; *B*, changes in P_{O_2}, induced by perfusion with a medium equilibrated with N_2 and supplemented with sodium dithionite. Note that as the P_{O_2} fell below about 20 mmHg, this was associated with a small increase in the fluorescence signal. Arrival at effectively zero P_{O_2} caused a much larger, rapid increase in signal. For clarity, the data included by the shaded bars are replotted in the panels inset above; here the NADH fluorescence signal is shown as a function of local P_{O_2}. The hypoxic challenge was followed by application of cyanide (2·5 mM); this caused an increase in the fluorescence signal very similar to that seen with anoxia. The slightly slower rate of decay may reflect the slower diffusion of cyanide from the cell compared with the rapid movement of oxygen.

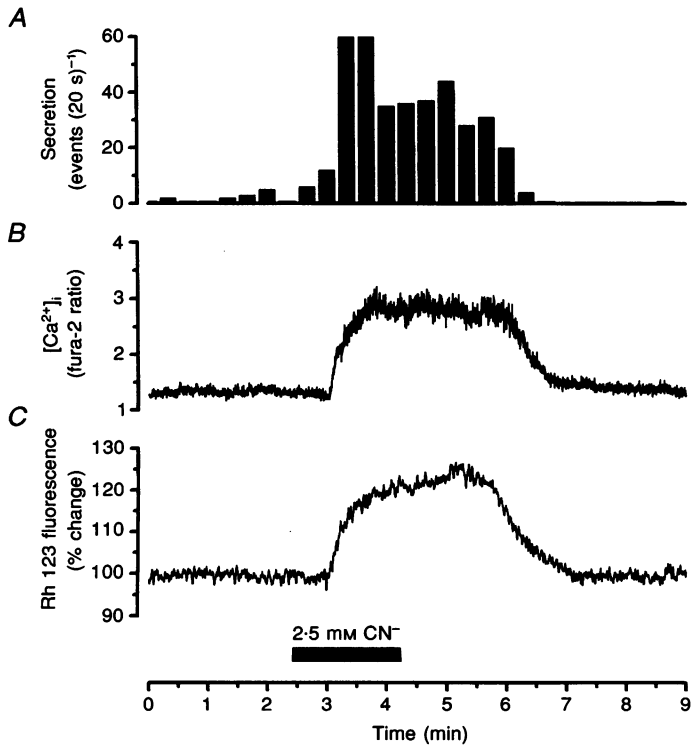

Figure 7. Application of CN^- mimics hypoxia-induced catecholamine secretion

A, catecholamine secretion as number of events in 20 s bins; *B*, changes in $[Ca^{2+}]_i$, presented as fura-2 ratio; *C*, changes in Rh 123, an increase indicating mitochondrial depolarization. On switching to a perfusate containing 2·5 mM CN^-, the mitochondrial potential depolarized, $[Ca^{2+}]_i$ rose and a brisk secretory response was evoked. Recovery in this case was delayed because of slow flow through the bath. The mitochondrial potential was clearly the first signal to recover. Once the Rh 123 signal was close to baseline, then both the $[Ca^{2+}]_i$ and secretory response returned to basal levels.

was associated with a brisk recovery of the NADH autofluorescence, but not to resting levels. Only when the P_{O_2} reached values below about 2 mmHg did the NADH autofluorescence increase again (see top right inset, Fig. 6). Although this correlation clearly showed hysteresis, it exemplifies how levels of hypoxia sufficient to induce catecholamine secretion will also inhibit mitochondrial respiration.

The threshold P_{O_2} at which NADH autofluorescence started to increase varied between clusters, ranging from 4 to 30 mmHg ($n = 6$). Possible reasons for this variation are: (i) variations in the size of the cell cluster, as larger clusters might have a steeper oxygen gradient between the core and the surface than smaller clusters at an equivalent bath P_{O_2}; (ii) a delay between the fall in P_{O_2} and the increase in NADH autofluorescence (reflecting the time taken for the

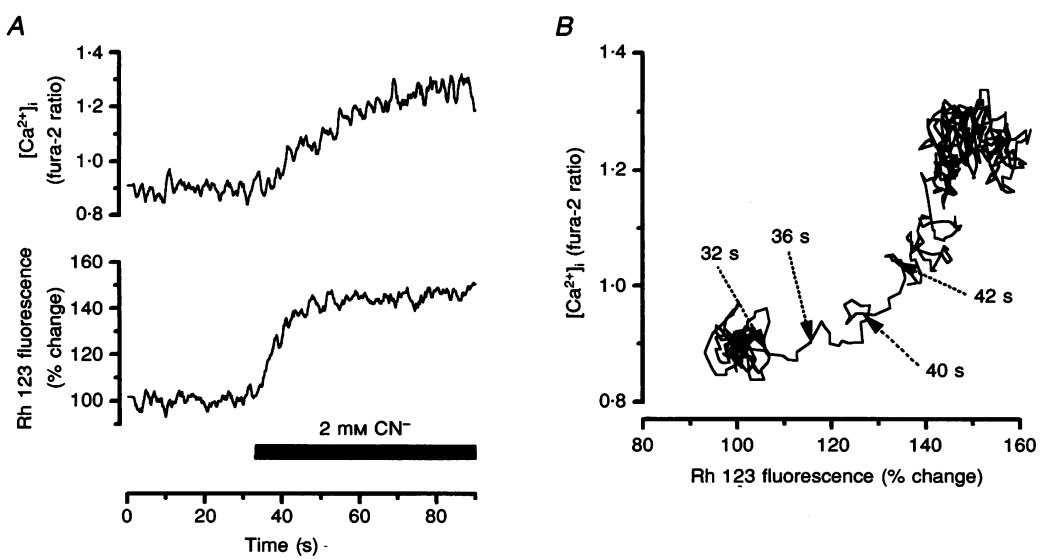

Figure 8. The CN^--induced increase in Rh 123 precedes the CN^--induced increase in $[Ca^{2+}]_i$

A, CN^--induced increase in Rh 123 and $[Ca^{2+}]_i$ on an expanded time scale; *B*, change in $[Ca^{2+}]_i$ plotted against the change in Rh 123. Clearly, after pressure ejection of 2 mM CN^- the Rh 123 signal was the first variable to change, followed closely by a rise in $[Ca^{2+}]_i$. In 9 cell clusters, the CN^--induced depolarization of $\Delta\Psi_m$ precedes the CN^--induced increase in $[Ca^{2+}]_i$ by 6·1 ± 5·5 s.

accumulation of the reduced form); this delay is likely to be more important in experiments with faster changes in local P_{O_2}; and (iii) a variation in the basal ATP turnover rate, higher rates depending more heavily on the O_2 supply.

CN^- induces catecholamine secretion and raises $[Ca^{2+}]_i$

The parallel between the levels of hypoxia that induce catecholamine secretion and those that result in inhibition of mitochondrial function raised the question whether the hypoxia-induced secretory response might be directly related to the inhibition of mitochondrial respiration. If so, alternative mechanisms that attenuate mitochondrial function should increase $[Ca^{2+}]_i$ and evoke catecholamine secretion. In the first instance, we used CN^- to inhibit mitochondrial respiration. The right-hand part of Fig. 6 shows that application of 2·5 mM CN^- by pressure ejection resulted in an increase in the NADH autofluorescence that is comparable to that seen with anoxia ($n = 3$).

Figure 7 shows the results of an experiment in which catecholamine release was measured simultaneously with changes in $\Delta\Psi_m$ and changes in $[Ca^{2+}]_i$, using Rh 123 and fura-2 fluorescence, respectively. Changing the superfusate to a saline containing 2·5 mM CN^- induced a depolarization of $\Delta\Psi_m$, an increase in $[Ca^{2+}]_i$ and a rapid and profound increase in catecholamine secretion. Similar results were obtained in nine cell clusters (Rh 123 fluorescence increased to $127 \pm 17\%$, $P = 0{\cdot}002$; fura-2 ratio increased to $154 \pm 30\%$, $P < 0{\cdot}001$).

The analysis in Fig. 8 illustrates the temporal relationship between the changes in $[Ca^{2+}]_i$ and $\Delta\Psi_m$ seen in response to CN^-. Figure 8*A* shows the CN^--induced increase in Rh 123 and $[Ca^{2+}]_i$ on an expanded time scale. In Fig. 8*B* the fura-2 ratio was plotted as a function of the Rh 123 signal, showing clearly that $[Ca^{2+}]_i$ began to rise only after the Rh 123 signal was beginning to reach a plateau. In general, mitochondrial depolarization preceded a rise in $[Ca^{2+}]_i$ by $6{\cdot}1 \pm 5{\cdot}5$ s ($n = 9$, $P = 0{\cdot}01$).

CN^-- and hypoxia-induced effects are not additive

While these results are compatible with the hypothesis that hypoxia-induced catecholamine secretion in the newborn rat adrenal chromaffin cells is due to inhibition of

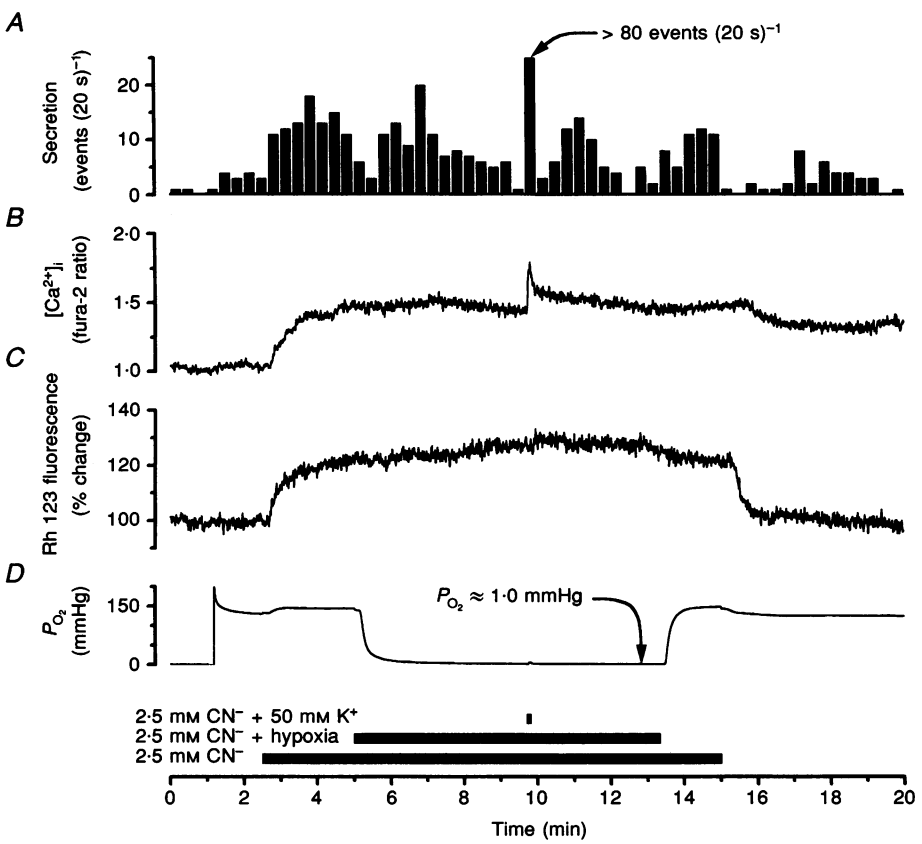

Figure 9. CN^-- and hypoxia-induced effects are not additive

A, changes in catecholamine secretion in number of events per 20 s; *B*, changes in $[Ca^{2+}]_i$, presented as changes in fura-2 ratio; *C*, changes in Rh 123 fluorescence, an increase indicating mitochondrial depolarization; *D*, changes in P_{O_2}, within 100 μm of the cell cluster. When the perfusate was changed to a medium containing 2·5 mM CN^-, $\Delta\Psi_m$ depolarized, $[Ca^{2+}]_i$ rose and catecholamine secretion increased. On switching to a superfusate containing 2·5 mM CN^-, and in addition equilibrated with argon, all cellular responses remained effectively unchanged, even though the local P_{O_2} fell to *ca* 1 mmHg. The viability of the preparation was tested and any possible saturation of signal excluded by pressure ejecting 50 mM K^+. On removal of the CN^-, all signals recovered to baseline.

mitochondrial respiration, it remains possible that an independent mechanism sensitive to very low oxygen tensions is expressed in these cells and that this mediates the secretory response. If two independent sensors were involved, one might expect that they would be additive. To test this, cells were first exposed to 2·5 mM CN^- and then, in addition, made hypoxic (P_{O_2}, 1–2 mmHg; Fig. 9). In five cell clusters, the additional stimulus of hypoxia failed to induce any significant change in any of the variables measured – mitochondrial potential, $[Ca^{2+}]_i$ or secretion. It is clear that this was not due to saturation of the cellular secretory machinery, as pressure ejection of 50 mM K^+ during combined CN^-–hypoxia perfusion evoked an additional transient increase in $[Ca^{2+}]_i$ and catecholamine secretion. Similarly, pressure ejection of CN^- during the combined CN^-–hypoxia perfusion had no effect (not presented), showing that the argon had not washed out the CN^-.

Rotenone also raises $[Ca^{2+}]_i$ and promotes catecholamine secretion

Cyanide is a widely used inhibitor of mitochondrial respiration; its advantages are reversibility, diffusibility and completeness of inhibition because it acts on complex IV of the electron transport chain. However CN^- can also inhibit some of the superoxide dismutases, enzymes which act as scavengers of reactive oxygen species. More specifically, CN^- inhibits the extramitochondrial copper- and zinc-containing, but not the mitochondrial magnesium-containing, isozymes (Beauchamp & Fridovich, 1973). Reactive oxygen species have been implicated in physiological responses to hypoxia in other cell types, for example pulmonary vascular smooth muscle cells (Archer, Huang, Henry, Peterson & Weir, 1993) or carotid body type I cells (Acker, 1994). Therefore we examined the effects of rotenone, another inhibitor of mitochondrial respiration with a completely different structure and pharmacology. Rotenone inhibits complex I of the respiratory chain, blocking the electron transport from NADH to O_2; it still allows electron flux from complex II to complex IV.

Figure 10 shows the effect of 1 μM rotenone, applied by pressure ejection. As expected, $\Delta\Psi_m$ depolarized, $[Ca^{2+}]_i$ increased and catecholamine secretion was enhanced. The Rh 123 signal increased to 112 ± 12% ($n = 17$, $P < 0{\cdot}001$) and the fura-2 ratio to 132 ± 21% ($n = 16$, $P < 0{\cdot}001$). Interestingly, application of both mitochondrial inhibitors on the same cell cluster showed that CN^- increased the Rh 123 fluorescence 109 ± 8% more than rotenone did

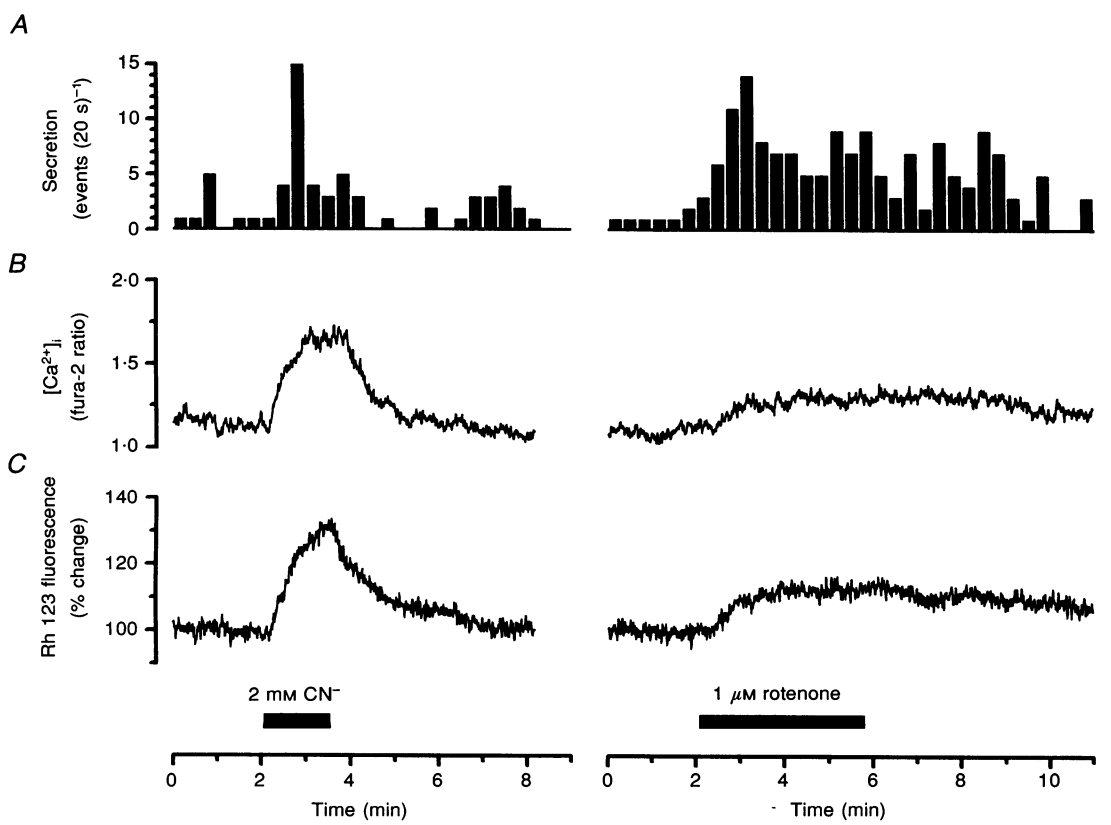

Figure 10. Rotenone also mimics hypoxia-induced catecholamine secretion

A, changes in catecholamine secretion in number of events per 20 s; *B*, changes in $[Ca^{2+}]_i$, presented as changes in fura-2 ratio; *C*, changes in Rh 123 fluorescence, an increase indicating mitochondrial depolarization. The mitochondrial, $[Ca^{2+}]_i$ and secretory responses to CN^- (2 mM, applied by pressure ejection) were tested first. After a period of recovery, the cells were exposed to 1 μM rotenone (also by pressure ejection), following which the mitochondria depolarized, accompanied by an increase in $[Ca^{2+}]_i$ and catecholamine secretion.

($n = 6$; $P = 0{\cdot}043$), indicating that some electron flux from complex II to complex IV was maintained. However, this electron flux was not sufficient to lessen the increase in $[Ca^{2+}]_i$, as the rotenone-induced increase in $[Ca^{2+}]_i$ was not significantly different from that induced by CN^- (CN^- increased the fura-2 ratio $105 \pm 14\%$ more than did rotenone, but this difference is not significant; $P = 0{\cdot}34$, $n = 7$). The effect of rotenone was reversible, but much slower than the effect of CN^-.

The fact that both CN^- and rotenone, two pharmacologically and structurally distinct inhibitors of mitochondrial respiration, mimic hypoxia-induced catecholamine secretion strongly suggests that the hypoxia-induced response is mediated by mitochondrial inhibition. In addition, it is rather unlikely that reactive oxygen species are implicated in the rise in $[Ca^{2+}]_i$ and catecholamine secretion that occur as a consequence of inhibition of cellular respiration.

2- to 4-week-old rats do not respond to hypoxia or inhibition of mitochondrial respiration

Seidler & Slotkin (1985) have shown in rats *in vivo* that the adrenal medulla secretes catecholamines during periods of hypoxia (1–2 h breathing 5–8% oxygen in nitrogen, i.e. 40–60 mmHg). In newborn rats the secretion is not affected by denervating the adrenal glands, indicating a non-neurogenic mechanism. This property is lost in rats more than 8–10 days old, where the adrenal medulla is only capable of catecholamine secretion during hypoxia through neurogenic, cholinergic stimulation. Consistent with this observation, we found that the CN^--induced increase in $[Ca^{2+}]_i$ in isolated adrenal chromaffin cell clusters was significantly different between newborn and 2- to 4-week-old rats (see Fig. 11; newborn, $148 \pm 25\%$ ($n = 18$); 2- to 4-week-old, $128 \pm 26\%$ ($n = 19$); $P = 0{\cdot}023$, two population independent Student's t test). In contrast, the CN^--induced depolarization of the mitochondrial inner membrane potential was not significantly different (newborn $126 \pm 17\%$, $n = 21$; 2- to 4-week-old, $123 \pm 13\%$, $n = 21$; $P = 0{\cdot}58$). In fact, while CN^- had no effect on $[Ca^{2+}]_i$ in only one out of eighteen cell clusters from the newborn, in 2- to 4-week-old rats eight out of nineteen cells (42%) showed no change in $[Ca^{2+}]_i$. The frequency distribution is presented in Fig. 11*C* and *D*.

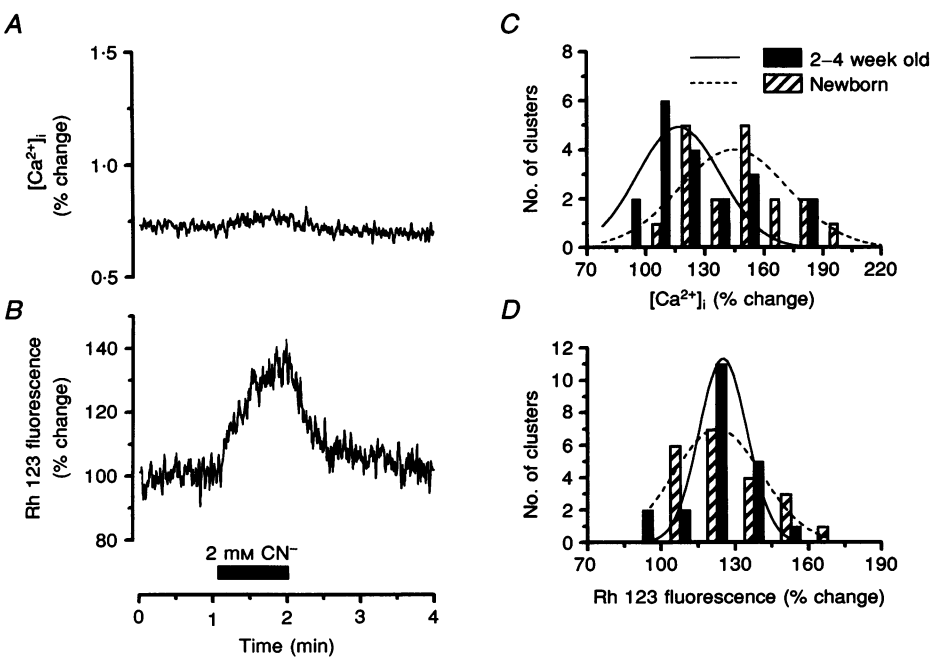

Figure 11. CN^--induced changes in $\Delta\Psi_m$ and $[Ca^{2+}]_i$ in adrenal chromaffin cells isolated from 2- to 4-week-old rats

A and *B*, characteristic response to CN^- (2 mM, applied by pressure ejection) of a cluster of chromaffin cells isolated from a 28-day-old animal. The Rh 123 signal (*B*) changed as expected, but there was no significant change in $[Ca^{2+}]_i$ (*A*). *C* and *D*, comparison between newborn and 2- to 4-week-old rats ($n = 18$ and 21, respectively). The frequency distribution of Rh 123 signals (*D*) from 2- to 4-week-old (filled bars) and newborn cells (hatched bars) overlap. Both data sets are adequately fitted by a Gaussian distribution with an equivalent mean. In contrast, the distribution of $[Ca^{2+}]_i$ responses (*D*) for 2- to 4-week-old (filled bars) and for newborn cells (hatched bars) are significantly different. The Gaussian distributions (continuous line for 2- to 4-week-old, dashed line for newborn chromaffin cells) are clearly distinct; the mean increase in $[Ca^{2+}]_i$ for cells from 2- to 4-week-old rats lies at 128%, with a substantial number of cells not responding, whereas the mean for newborn cells lies at 148%, with only 1 non-responding cell. For significance levels see text.

DISCUSSION

Hypoxia-induced catecholamine secretion in newborn adrenal chromaffin cells

We have shown that: (i) adrenal chromaffin cells isolated from the newborn rat possess a fully functional exocytotic machinery, even though these cells are not functionally innervated *in vivo*; (ii) hypoxia-induced catecholamine secretion is an intrinsic property of adrenal chromaffin cells of newborn rats – this property seems much diminished in cells from the 2- to 4-week-old rats, in which only about half the cells showed any response to chemical anoxia; (iii) hypoxia-induced catecholamine secretion was quantal, and therefore presumably involves exocytotic fusion; (iv) newborn adrenal chromaffin cells respond only to severe hypoxia, of the same order of magnitude as that required to inhibit mitochondrial respiration and raise the $NADH/NAD^+$ ratio; (v) responses to either severe hypoxia or chemical inhibition of mitochondrial respiration appear to be equivalent; (vi) the effects of severe hypoxia and chemical anoxia are not additive; and finally, (vii) the responses to either severe hypoxia or chemical anoxia appear to involve first inhibition of mitochondrial respiration, then a rise in $[Ca^{2+}]_i$, and finally exocytotic secretion of catecholamine.

The threshold P_{O_2} required to increase $[Ca^{2+}]_i$ and catecholamine secretion was low, about 5 mmHg. However these values were not simply cytotoxic, as (i) the hypoxic challenge is completely reversible (Fig. 4), and (ii) cells isolated from 2- to 4-week-old rats with exactly the same procedures were not similarly affected. Indeed, these values are equivalent to those observed *in vivo*: the threshold arterial P_{O_2} for stimulating the direct response in the sheep was also 3–4 mmHg, and the arterial P_{O_2} often fell to 0 mmHg during the period in which the catecholamine output was measured in the adrenal venous blood (Comline *et al.* 1965; Comline & Silver, 1966). Parallel to the present study, Thompson, Jackson & Nurse (1997) have investigated hypoxia-induced catecholamine secretion in newborn adrenal chromaffin cells maintained in short term culture. These authors found effectively no P_{O_2} threshold: during 1 h of moderate hypoxia ($P_{O_2} \approx 75$ mmHg), catecholamine secretion was ~3-fold higher than during 'normoxia' (atmospheric P_{O_2}). These data appear to contrast with results obtained *in vivo*: newborn adrenal glands only secrete catecholamine when the inspired oxygen pressure is below *ca* 50 mmHg. This paradox possibly stems from the lack of accurate measurements of the P_{O_2} near the cells in the culture dish. Indeed, Dickman & Mandel (1989) presented results indicating a steep oxygen gradient in the culture medium: kidney proximal tubule cells in primary culture showed pronounced glycolytic activity, but reverted to oxidative metabolism when cultured in shaking Erlenmeyer flasks.

In addition, Thompson *et al.* (1997) found that acute moderate hypoxia ($P_{O_2} \approx 40$ mmHg) depolarized the membrane potential *ca* 15 mV, and reversibly suppressed voltage-dependent K^+ currents to ~75%. At present we cannot account for these differences; however, they are potentially very interesting, as the only difference between the two studies appears to be the isolation procedure and the fact that Thompson *et al.* (1997) maintained cells in short-term culture (2–4 days), whereas in the present study cells were used on the same day, to avoid possible de-differentiation in culture.

Hypoxia-induced catecholamine secretion is an intrinsic property of newborn adrenal chromaffin cells and involves exocytosis

Hypoxia-induced catecholamine secretion was observed in isolated cell clusters. This suggests that at least part of the hypoxia-induced catecholamine secretion observed *in vivo* (Seidler & Slotkin, 1985) reflects an intrinsic property of chromaffin cells of the newborn rat. It still seems possible that other, perhaps humoral, factors may modulate this response to generate the full response seen *in vivo*. However, our data clearly indicate that isolated adrenal chromaffin cells from newborn rats are sufficiently equipped to secrete catecholamines during a period of hypoxic stress, and are in all probability responsible for the bulk of the catecholamine secretion observed *in vivo*, especially since the responsiveness to hypoxia is substantially reduced in adrenal chromaffin cells from 2- to 4-week-old rats.

The secretion was shown to be exocytotic in nature. Catecholamine release could also be due to reversal of an uptake carrier, as has been shown to be the case for catecholamine release by sympathetic nerve endings in the heart during an ischaemic period (Schömig *et al.* 1988). We cannot exclude the possibility that the catecholamine release is partially due to reversal of an uptake carrier. However, the contribution of such a mechanism (if present) is likely to be small. CN^--induced catecholamine release from bovine adrenal chromaffin cells kept in short term culture was not affected by inhibition of catecholamine uptake carriers (Dry, Phillips & Dart, 1991). Furthermore, judging from the K_m values reported for cultured bovine adrenal chromaffin cells (Kenigsberg & Trifaro, 1980), a maximum secretion of about 20 amol min^{-1} for a cluster of ten cells seems possible. Thus we confirm indirect evidence obtained by Seidler & Slotkin (1986*b*), who have shown *in vivo* that during hypoxia the catecholamine content in adrenal chromaffin cells is reduced. In addition they showed that during hypoxia [^{3}H]adrenaline uptake into granules is reduced, a result that was interpreted as being consistent with exocytotic secretion.

We have shown that depolarization of the plasma membrane leads to an increase in $[Ca^{2+}]_i$ and exocytosis of adrenaline and noradrenaline in a ratio of 3 : 1. This is exactly the same proportion as present in the newborn rat adrenal medulla at birth (Coulter *et al.* 1988). We have not been able to measure the ratio of adrenaline to noradrenaline secreted during hypoxia due to limitations of the method of fast differential

ramp voltammetry (see Methods); however, Shaul, Cha & Oh (1989) have shown using biochemical techniques that the proportions of noradrenaline and adrenaline released during hypoxia mimic the ratio of the amines in the adrenal medulla.

Hypoxia-induced increase in $[Ca^{2+}]_i$

The hypoxia-induced increase in $[Ca^{2+}]_i$ was small compared with the increase due to depolarization of the plasma membrane with 50 mM K^+ or during application of 2·5 mM CN^-. The following points are relevant. First, the hypoxia-induced increase in $[Ca^{2+}]_i$ may be expected to be smaller than that induced by CN^-, because application of CN^- is equivalent to anoxia. Second, changes in fluorescence over the cluster were sampled using photomultipliers, and so changes in signal were effectively averaged over the whole cluster. It is likely that application of 50 mM K^+ depolarized the plasma membrane of all cells, while hypoxia may have increased $[Ca^{2+}]_i$ and catecholamine secretion in only a subset of cells in the cluster, if individual cells have different sensitivities to hypoxia. Third, we cannot exclude the possibility of an underestimate due to contamination of the indo-1 signal with NADH autofluorescence (see Methods). However, also when using fura-2, we observed that the hypoxia-induced increase in $[Ca^{2+}]_i$ was substantially smaller than that induced by CN^- or 50 mM K^+.

A recent publication showed that changes in the behaviour of indo-1 and fura-2 in the presence of hypoxia may lead to overestimates of hypoxia-induced changes in $[Ca^{2+}]_i$, due to an ill-understood process that is independent of changes in the oxygen pressure itself, pH_i, viscosity, redox state or photobleaching (Stevens *et al.* 1994). However, the increases in $[Ca^{2+}]_i$ upon inhibition of mitochondrial respiration that we have described here can be abolished by preventing influx of extracellular Ca^{2+} (Mojet *et al.* 1996). In addition, the fura-2 ratio did not change when (in the presence of 2·5 mM CN^-) the P_{O_2} was reduced to 1–2 mmHg (see Fig. 8).

Hypoxia-induced effects are due to inhibition of mitochondrial respiration

The nature of the O_2 sensor in newborn adrenal chromaffin cells is not known. Several different cellular oxygen sensing mechanisms have been described: hypoxia-induced gene expression in a variety of mammalian cells (reviewed by Fandrey, 1995), which possibly involves oxygen sensitive haem proteins; hypoxic pulmonary vasoconstriction, possibly involving mitochondrial production of reactive oxygen species (e.g. Archer *et al.* 1993); and the oxygen sensing chemoreceptors in the carotid body and pulmonary neuroepithelial bodies. The transduction mechanism in the chemoreceptors is a matter of dispute, with different hypotheses proposing as primary sensor (i) a plasmalemmal oxygen sensitive K^+ channel (Delpiano & Hescheler, 1989), which is Ca^{2+} sensitive (Peers, 1990) or Ca^{2+} insensitive (Ganfornina & Lopez Barneo, 1992*a*, *b*), (ii) a plasmalemmal NADPH oxidase (Cross, Henderson, Jones, Delpiano, Hentschel & Acker, 1990; Acker, 1994) or (iii) mitochondria with a reduced oxygen affinity (Mills & Jöbsis, 1970, 1972; Duchen & Biscoe, 1992*a*, *b*).

Adrenal chromaffin cells isolated from newborn rats appear to respond only to severe hypoxia. Not surprisingly, at these low levels of P_{O_2} mitochondrial respiration was also affected. As shown in Fig. 6, the NADH/NAD^+ ratio increased, indicating that the oxidation of NADH was attenuated. The threshold P_{O_2} at which NADH autofluorescence started to increase varied between clusters, ranging from 4 to 30 mmHg ($n = 6$). Paradoxically, the increase in $[Ca^{2+}]_i$ and catecholamine secretion were only seen at P_{O_2} values below 5 mmHg. However, these results are not incompatible: as the increase in $[Ca^{2+}]_i$ is small, the threshold is very difficult to determine; Fig. 4 certainly does not exclude the possibility that $[Ca^{2+}]_i$ increases while the P_{O_2} is falling. In addition, a reduction in O_2 availability will lead to an increased amount of reduced cytochrome a_3 and an increased NADH/NAD^+ ratio; this increased substrate concentration can keep the reaction rate unchanged; consequently, the total electron flux and the ensuing H^+ translocation and ATP production may remain constant. In other words, we may expect the change in $[Ca^{2+}]_i$ to trail behind the change in mitochondrial metabolism.

If the hypoxia-induced catecholamine secretion represents a direct response to inhibition of mitochondrial respiration, we can make several predictions. First, pharmacological blockade of the respiratory electron transport chain should lead to an increase in $[Ca^{2+}]_i$ and catecholamine secretion. Indeed, application of 2·5 mM CN^- or 1 μM rotenone (Figs 7 and 10 respectively) was sufficient to depolarize the mitochondrial inner membrane potential, to increase $[Ca^{2+}]_i$ and to induce substantial secretion of catecholamines. The depolarization of the mitochondrial inner membrane potential is not the result of the increase of $[Ca^{2+}]_i$, as application of 50 mM K^+ had only marginal effects on $\Delta\Psi_m$. Also, interference with Ca^{2+} influx over the plasma membrane reduced the increase in $[Ca^{2+}]_i$ but had no effect on $\Delta\Psi_m$ (Mojet *et al.* 1996).

Second, mitochondrial depolarization should precede the increase in $[Ca^{2+}]_i$. We were able to test this prediction in the case of chemical anoxia, because of the high rate with which the cellular parameters changed. As illustrated in Fig. 8, the onset of the increase in the Rh 123 fluorescence and the increase in fura-2 ratio differ by *ca* 6 s.

A third prediction we have tested is that the effects of CN^- and severe hypoxia should not be additive. As shown in Fig. 9, a hypoxic challenge to a cell cluster that was perfused with 2·5 mM CN^- did not have any effect on $\Delta\Psi_m$, $[Ca^{2+}]_i$ or catecholamine secretion. This test is not without caveats. (i) Leading argon through a reservoir containing CN^- might wash out HCN, reducing the effective concentration of CN^-. Therefore we pressure ejected CN^- onto cells that were exposed both to CN^- and severe hypoxia. The additional

CN^- had no effect on $\Delta\Psi_m$, $[Ca^{2+}]_i$ or catecholamine secretion. (ii) The onset of hypoxia is slow, making it difficult to detect subtle changes in $\Delta\Psi_m$, $[Ca^{2+}]_i$ and secretion. However, during the pressure ejection of CN^-, the P_{O_2} increased abruptly, because the saline in the puffer pipette was not depleted of O_2; indeed, the P_{O_2} electrode clearly registered a local increase in P_{O_2}. Whereas similar changes in P_{O_2} had a profound effect on the NADH autofluorescence (see Fig. 6), we recorded no changes in $\Delta\Psi_m$, $[Ca^{2+}]_i$ or secretion. (iii) Non-additivity does not necessarily imply that the same O_2 binding site is involved; it merely shows that the effect of CN^- and severe hypoxia is brought about via a common final pathway, including changes in $[Ca^{2+}]_i$ and secretion. It remains possible that CN^- completely inhibits mitochondrial respiration at the level of cytochrome a_3, leading to changes in $\Delta\Psi_m$, $[Ca^{2+}]_i$ and secretion, whereas severe hypoxia affects some other receptor, but because of saturation in the common pathway has no additional effect on $[Ca^{2+}]_i$ and secretion. The nature of the intermediate steps between the inhibition of mitochondrial respiration and the increase of $[Ca^{2+}]_i$ and catecholamine secretion is largely unknown and may involve a fall in cellular [ATP] (Mojet *et al.* 1996); however, we can exclude saturation at the stage of $[Ca^{2+}]_i$ and catecholamine secretion, as an additional increase in $[Ca^{2+}]_i$ and secretion could be produced by depolarizing the plasma membrane with 50 mM K^+ (Fig. 9).

In summary, we have shown that adrenal chromaffin cells isolated from newborn rats (< 24 h old) are sensitive to hypoxia, which is likely to be due to inhibition of cellular respiration. In contrast chemical anoxia, which is the ultimate form of mitochondrial inhibition, only leads to an increase in $[Ca^{2+}]_i$ and catecholamine secretion in about half of the adrenal chromaffin cells isolated from 2- to 4-week-old rats. The hypoxia-induced catecholamine secretion is an intrinsic property of the adrenal medulla, is due to inhibition of mitochondrial respiration, and leads via a rise in $[Ca^{2+}]_i$ to exocytosis of catecholamines.

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