Multifocal animated imaging of changes in cellular oxygen and calcium concentrations and membrane potential within the intact adult mouse carotid body ex vivo

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The carotid body (CB) located at the bifurcation of the common carotid artery transforms changes in arterial oxygen partial pressure into nervous discharges activity. Action potentials travel along sinus and glossopharyngeal nerves to the brain stem for increasing ventilation and blood pressure under hypoxia to improve oxygen supply and to avoid or reduce hypoxic tissue damages. Under hypoxic conditions, various oxygen-sensitive potassium channels have been described for the mouse CB and other species such as Kv3 (19) and the TASK-1/3 K⁺ channels (2, 13, 16) colocalizing with nicotinergic, purinergic, and dopaminergic receptors in mouse type I cells (10). The closing of potassium channels as the first step for hypoxia-induced transmitter release might be mediated, for example, by oxygen radicals (9), CO (12, 29), H₂S (15), or an AMP kinase (22). The inhibition causes membrane depolarization, the opening of calcium channels, and a subsequent increase in cytosolic calcium concentrations, whereupon type I cells enveloped by glia cell-like type II cells release transmitter molecules to induce synaptic depolarizing potentials (SDP) with subsequent action potentials (AP) in the adjacent sinus nerve endings. These basic mechanisms have been detected in studies of isolated type I cells and thin CB tissue slices (3, 30). However, Donnelly and Khohlwadel (8) described a decrease of intracellular calcium upon hypoxia in freshly isolated rat type I cells and about 3 h after isolation of two type I cells, one cell showed increasing and the other cell decreasing intracellular calcium under hypoxia (see Fig. 8 in Ref. 8). Furthermore, Bright et al. (1) found that only 20% of single isolated rat type I cells responded with an increase of intracellular calcium to hypoxia. All type I cells isolated in clusters failed to respond. Pang and Eyzaguirre (17) described that, under hypoxia, >80% of isolated clustered rat type I cells depolarized and their input resistance decreased, whereas ~60% of single isolated cells hyperpolarized and their input resistance increased. The same authors (18) showed that rat type I cells, isolated as a cluster and not as single cells, decreased as well as increased intracellular pH under hypoxia. According to Donnelly (7), AP generation is not due to SDP events; rather, AP generation is likely to depend on a process endogenous to the nerve terminals of the intact rat CB ex vivo. To circumvent the putative methodological pitfalls during isolation of type I cells, we describe here a multifocal microscopy method for studying the oxygen-sensing of type I cells within the intact mouse CB ex vivo. This method does not disturb the interaction between type I cells, type II cells, adjacent nerve endings, and surrounding blood vessels. We report the detection of a population of type I cells that are heterogeneous with respect to hypoxic calcium response and spontaneous burstlike potential changes lasting ~1.5 s. In contrast, homogeneous 0.02-Hz membrane potential changes cease in response to hypoxia-induced depolarization. We conclude that only studies of type I cells inside the intact CB can reveal the cellular complexity of the oxygen-sensing process. Furthermore, the multifocal microscopy method described here is applicable to future cellular studies inside the intact CB in vivo.

MATERIALS AND METHODS

Ethical approval. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and
the guidelines of the National Institutes of Health regarding the care and use of animals for experimental procedures. The study protocol was approved by the institutional animal ethics committee of the Universität Klinikum Essen, University of Duisburg-Essen.

Procedure. Female C57BL/6J mice, 8 to 12 mo old, were used for the experiments. Animals were euthanized by inhalation of isoflurane, a method that minimizes the deleterious effects of anesthesia on the oxygen-sensing properties of type I cells (3). Immediately thereafter, the bifurcations of both common carotid arteries were dissected and stored in an ice-cold phosphate-buffered saline (PBS) solution until being subjected to the following experimental steps. First, fine preparation of CBs from the bifurcation was done under a stereomicroscope (American Optical, Southbridge, MA). For this purpose the carotid bifurcation was carefully freed from connective tissue with tweezers until the nerve bundle between the CB (11) and the superior cervical ganglion became visible. Transillumination of the bundle visualizes the CB as an opaque oval organ which can be easily separated from the arterial wall by blunt preparation. Second, the CBs were stained with 1.2-bis-(o-aminophenoxethane- N,N,N’,N’- tetraacetic acid tetra(ace-toxymethyl) ester (Oregon Green 488 BAPTA-1 AM, 15 μM, Invitrogen, Karlsruhe, Germany) for 40 min in ice-cold PBS for cellular calcium measurements. Third, CBs were stained with aminonaphthylethenylpyridinium dye with a propylsulfonate head group (di-4-ANEPPDHQ, 15 μM, Invitrogen) for 40 min in ice-cold PBS for cellular potential measurements. For cellular oxygen measurements, two CBs were incubated with the oxygen-sensitive dye PEPP0 for 24 h. For this purpose 2.5 μl of a stock solution (0.8 mM PEPP0 dissolved in dimethylsulfoxide, DMSO) were added to 197.5 μl Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) under tissue culture conditions resulting in a final concentration of 10 μM PEPP0 and 1.25% DMSO. Osteosarcoma cells stained with PEPP0 by means of the same method showed no sign of cell death after 24 h (not shown). The PEPP0 probe is based on the cell-penetrating peptide bactenecin 7, with properties similar to those of previously described Pt(II)-coproporphyrin conjugates (6).

Multifocal microscopy. CBs covered by a small layer of DMEM culture medium for optimizing a stable position were placed in a previously described tissue culture chamber (28) mounted on the stage of an Axiovert 200M inverted microscope (Zeiss, Oberkochen, Germany). The gas environment inside the chamber was controlled by a gas-mixing device (MGC647C; MKS, Berlin, Germany). A Nipkow disk (QLC100; Visitech International, Sunderland, UK) equipped with a charge-coupled device (CCD) camera (ORCA-ER; Hamamatsu, Herrsching, Germany) and mounted on the head of the Axiovert microscope was used for multifocal imaging and was operated by a custom-designed computer program. Nipkow Spinning disk multifocal microscopy utilizes multiple pinholes to project some hundred parallel excitation light beams onto the specimen in a multiplexed pattern. Subsequent detection of fluorescence emission is achieved through the same pinholes. The technique is highly useful for high-speed imaging of living cells stained with calcium- or potential-sensitive dyes. Photobleaching and phototoxicity are significantly reduced with spinning disk microscopy (5). Calcium- and potential-sensitive dyes were excited by a 532-nm diode-pumped solid-state (DPSS) laser (CrystaLaser, Reno, NV), and fluorescence emission was selected by a 565/40-nm filter (AHF Analysentechnik, Tübingen, Germany). The emissions of the PEPP0 oxygen-sensitive probe were collected with a 660/50-nm filter after excitation with the 532-nm DPSS laser. An increase in fluorescence is induced by increases in cellular calcium concentrations or by decreases in cellular potential or oxygen concentration. All registrations were corrected for baseline drift due to photo bleaching and were normalized. In addition, some fluorescent images were obtained with a Leica TCS SP5 microscope (Wetzlar, Germany).

Immunofluorescence. CBs were washed in PBS and fixed in methanol/acetone (7:1) at −20°C for 1 h. After being washed in 0.1% Triton X-100-PBS for 30 min and blocked in 3% bovine serum albumin-BSA for 1 h, CBs were stained with the primary antibodies sheep anti-tyrosine hydroxylase (TH, diluted 1:25; Abcam, Cambridge, UK) and rat anti-CD31 (diluted 1:10; BD Pharmingen, Heidelberg, Germany) for 1 h each. CBs were then washed in PBS and incubated with the secondary antibodies CY2-conjugated donkey anti-sheep immunoglobulin G (IgG, diluted 1:100; Dianova, Hamburg, Germany) and Alexa Fluor 568-conjugated goat anti-rat IgG (diluted 1:400, Invitrogen) for 1 h. After being washed in PBS, CBs were incubated with Hoechst 33342 (1:500; Invitrogen) for 10 min. The stained CBs were washed in PBS and mounted on slides in Mowiol (Calbiochem, EMD Chemicals, Gibbstown, NY).

Analysis. Relative changes of hypoxia-induced calcium, potential, and oxygen changes are graphically presented using TechPlot as described (14). Student’s paired two-sided t-test with unequal variance was used to calculate significant differences of hypoxia-induced calcium and potential changes. The Jaccard index was used to detect significant diversity of cell populations with respect to their hypoxia-induced changes in calcium concentration or spontaneous changes of membrane potentials. The Jaccard index is a mathematical instrument commonly used for affinity analysis. In neuroimage analysis, for example, it has been set up for comparison of distinct neuron populations (21).

RESULTS

Identification of type I cells. Measuring cellular activities inside the intact CB by multifocal microscopy requires the identification of type I cells in the heterogeneous CB tissue during measurements (27). Type I cells with typical large cell nuclei assembled in clusters dominate the mouse CB (see Fig. 1 in Ref. 10). Figure 1A confirms this highly specific finding by Leica

![Image](316x147 to 568x391)

Fig. 1. Identification of type I cells. A: whole mount immunofluorescence with blood vessels stained in red (anti-CD31), type I cells in green (anti-tyrosine hydroxylase), and the corresponding cell nuclei in blue (Hoechst 33342). B: cell nuclei in blue surrounded by calcium-sensitive fluorescent dye Oregon Green 488 BAPTA-1 AM in green. C: cell nuclei in blue surrounded by membrane potential-sensitive fluorescent dye di-4-ANEPPDHQ in red. D: three-dimensional multifocal recording of potential sensitive dye-stained type I cells typically arranged in clusters with a central dark spot corresponding mostly to the cell nucleus. The white rectangle emphasizes these features used during the measurements to identify type I cells.
TCS SP5 recordings of whole mount immunofluorescence with blood vessels stained in red (anti-CD-31) as well as type I cells in green (anti-TH) with the corresponding large cell nuclei in blue (Hoechst) arranged in the typical type I cell cluster formation of the intact mouse carotid body. Figure 1B shows in high resolution (Leica TCS SP5) cell nuclei in blue of an intact living mouse CB cell cluster surrounded by calcium-sensitive fluorescent dye in green. Figure 1C shows cell nuclei in blue of cells in cluster formation surrounded by the potential-sensitive fluorescent dye in red. During the multifocal measurements, typical large cell nuclei as well as cell clustering were used for identification of type I cells. Figure 1D exemplifies this feature by a multifocal three-dimensional plot of five 1-μm-thick optical slices of mouse CB tissue stained by the potential-sensitive dye. As exemplified by the white rectangle, many clusters of three and more cells are stained at the outer part with a dark central part most likely corresponding to the cell nucleus. This feature is in excellent agreement with images from previous publications (see Fig. 1 in Ref. 10) showing mouse CB type I cell cluster with TH staining at the outer part and a dark central corresponding to the cell nucleus.

Changes in cellular oxygen and calcium concentrations. Figure 2A shows the representative hypoxic time course determined by staining with PEPP0 (dotted line), as observed in two CBs. PEPP0 is localized to the cytoplasm (23). After 5 min of control conditions (80% O2, 5% CO2, and 15% N2) inside the tissue culture chamber of the microscope for optimizing the oxygenation of the compact tissue, the gas flow was changed to 5% CO2 and 95% N2 for 2.5 min; this mixture induced an obvious and slow decrease in the oxygen concentration of CB tissue. When the gas composition was restored to control conditions, the oxygen concentration of CB tissue returned to control levels after 2.5 min and remained stable until the end of the experiment (15 min). Images of the oxygen-sensitive dye fluorescence were obtained every 30 s. Figure 2IV, shows an image obtained during tissue deoxygenation, whereas Fig. 2V, shows an image obtained after tissue reoxygenation. For the purpose of clarity, the hypoxia-induced increase in fluorescence of the oxygen-sensitive dye was inverted in Fig. 2A. Changes in oxygen concentration could be clearly correlated with changes in cell calcium concentration and potential because of the computer-controlled time schedule of the gas-mixing device.

In addition, Fig. 2A shows the hypoxia-induced calcium response of two type I cells in close proximity, marked as region of interest ROI 1 and ROI 2 in Fig. 2I. The cell in ROI
responds to hypoxia by increasing the cellular calcium concentration, whereas the cell in ROI 1 responds by decreasing the cellular calcium concentration. To determine how many cells in a cluster respond to hypoxia with either an increase or a decrease in the calcium concentration, we calculated the steepness of the fluorescence change during hypoxia and reoxygenation for each pixel. Figure 2 III demonstrates that the number of cells that respond to hypoxia with an increase in the calcium concentration (yellow-to-red mapping) is nearly equal to the number of cells that respond with a decrease (green-to-blue mapping). Figure 2 II gives an overview of the reoxygenation response, in which cells that had demonstrated yellow-to-red mapping (Fig. 2 II) now show green-to-blue mapping during reoxygenation, and vice versa. Large, distinct type I cell populations respond to hypoxia with either an increase or a decrease (Jaccard index >0.01 between cells mapped as yellow-to-red or green-to-blue in Fig. 2, II or III). Animation 1 of these recordings (see Supplemental Material, which is available online at the Journal website) provides a dynamic view of this heterogeneous hypoxic cellular calcium response within the mouse CB. Figure 2 B summarizes the calcium response of five CBs; each curve has a ROI covering one cell. Each CB can be identified by its own symbol. Bold symbols mark the hypoxia-induced increase in calcium concentrations. The dependence of cellular calcium responses to changes in the cellular oxygen concentration is plotted in Fig. 2 C, which demonstrates the various oxygen sensitivities of the CBs studied.

**Changes in cellular membrane potential.** Figure 3 A depicts the response of the cellular membrane potential within the intact mouse CB to hypoxia. Figure 3 I, depicts the four ROIs (green boxes) that were evaluated, each of which is indicated by a particular symbol. Under control conditions, cellular potential oscillates at ~0.02 Hz. Upon the onset of hypoxia (which can be recognized by tissue oxygen recordings), oscillations cease and cells depolarize. The oscillations return upon reoxygenation. Figure 3 B summarizes the results of studies of five CBs; each curve represents the mean of four ROIs covering two to four cells. In spite of small changes in membrane potential (1–2%), hypoxia-induced depolarization lasting from 5.5 min to 9 min is significantly different from the potentials of control cells before and after hypoxia (P < 0.001). Figure 3 C correlates the mean changes in calcium concentration with the mean changes in membrane potential. Oscillations of mem-

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**Fig. 3. Changes in cellular membrane potential.** A: time course of CB tissue hypoxia (right y-axis, oxygen, arbitrary units). Dotted line represents cytosolic oxygen concentration. Time-dependent response in cellular membrane potential (left y-axis, arbitrary units) to tissue hypoxia (right y-axis, arbitrary units) within the intact mouse CB with 4 ROIs, as shown in I (ROI 1, circle; ROI 2, square; ROI 3, rhombus; ROI 4, inverted triangle). B: time-dependent (x-axis, minutes) cellular membrane potential response (left y-axis) of 5 CBs to tissue hypoxia (right y-axis, arbitrary units). Each normalized potential curve represents the mean of 4 ROIs composed of 2 to 4 cells each. The bar indicates a 2% change in fluorescence intensity of the potential-sensitive dye. C: correlation of mean changes in calcium concentration (y-axis, arbitrary units) with mean changes in membrane potential (x-axis, arbitrary units). Arrows indicate the course of hypoxia and reoxygenation. D: burstlike spontaneous increases or decreases in membrane potential (y-axis, arbitrary units) over time as observed in 3 CBs. Burst peaks have been normalized to time 0. The cellular membrane potential of cells in ROI 1 (green box in II) decreases (bold circles in D) and that of cells in ROI 2 (green box in II) increases (regular circles in D). The steepness from control to peak (III) and from peak to control (IV) was calculated. Cells with decreases in membrane potential are mapped as yellow-to-red, and cells with increases in membrane potential are mapped as green-to-blue.
brane potential under control conditions are not correlated with cellular calcium concentrations. Upon hypoxia, however, cellular calcium concentrations either increase or decrease with depolarization. Upon reoxygenation, both variables return to control levels, thereby forming a hysteresis loop (Fig. 3C).

These relatively uniform potential kinetics are in contrast to spontaneous cellular burstlike changes in membrane potential, which were observed with higher time resolution (one image per second) in three CBs. Figure 3D shows that each CB undergoes spontaneous decreases or increases in membrane potential with a duration of \( \pm 1.5 \) s. Corresponding CB curves are marked with identical symbols; depolarization is shown in bold. Figure 3III uses green boxes to depict the selected ROIs of the CB marked by circles. Upon the onset of a potential burst, the cellular membrane potential of cells in ROI 1 decreases, and that of cells within ROI 2 increases, as can be seen in Fig. 3D. The diversity of type I cell populations (Jaccard index \(< 0.01\)) is shown in Fig. 3III, from control to peak, and in Fig. 3IV, from peak to control; cells mapped in yellow-to-red exhibit decreases in membrane potential, and cells in green-to-blue demonstrate increases in membrane potential. The dynamics of this remarkable heterogeneity of burstlike membrane potential changes in CB cells can be observed in animation 2.

**DISCUSSION**

An increase in the cytosolic calcium concentration is generally accepted as a basic requirement for excitatory transmitter release from type I cells under hypoxic conditions (26). We confirmed these calcium increases in our experiments; however, we also found cells that responded with a decrease in the cytosolic calcium concentration as reported in literature (8). All of these cells were identified as type I cells in accordance with the literature because of cluster formation and typical rim staining pattern with large dark central parts (see Fig. 1 in Ref. 10). Other means of identifying type I cells, such as TH-green fluorescent protein (GFP) staining, cannot be used because of the overlapping excitation and emission spectra of GFP and the dyes used (unpublished observation). Cells in which the calcium concentration either increased or decreased were imaged in close proximity as also shown for isolated type I cells in previous work by others (see Fig. 8 in Ref. 8).

The question whether hypoxia-induced cell volume changes may induce erroneous fluorescence signals by apparent changes in dye concentration is very critical. As already described by Donnelly and Kholawadala (8), we found no indication for type I volume changes under hypoxia (not shown). In case of minor changes that we cannot detect, those changes should affect the apparent concentration of all three intracellular dyes used in our experiments in a similar way. However, only hypoxia-induced calcium changes showed the heterogeneity described for isolated type I cells (1, 8), whereas oxygen and potential measurements with an homogeneous response were in full accordance with the literature (17). We therefore believe that it is justified to exclude significant hypoxia-induced volume changes in our experimental approach.

BAPTA calcium chelators show insensitivity toward intracellular pH change and a fast release of calcium as indicated by data sheets provided by the supplier (Invitrogen). BAPTA chelators have calcium dissociation constants covering the biologically significant range from \( 10^{-7} \) to \( 10^{-2} \) M and are therefore very well suited for intracellular measurements. The concentration (15 \( \mu \)M) of Oregon Green 488 BAPTA-1 AM as well as the incubation time used in the experiments were in the upper range as recommended by the supplier (Invitrogen) for monolayer cells depending on cell type (1–10 \( \mu \)M up to 1 h).

We are confident therefore that calcium binding necessary for proper BAPTA function has a negligible influence on our measurements. We used the upper range because pilot measurements did not provide a sufficient signal-to-noise ratio of the calcium signal when using lower concentrations or shorter incubation times. Pang and Elzayaguirre (18) showed for rat type I cells isolated as clusters a decrease as well as an increase of intracellular pH under hypoxia. We hypothesize that these intercellular pH variations are one possible reason for the observed calcium variations due to pH-dependent intracellular protein binding or release of calcium. The hypoxia-induced calcium decrease might be furthermore due to calcium binding to the vesicle releasing machinery or mitochondrial uptake triggered by a putative oxygen sensitivity of the described calcium sensor (20, 24) with subsequent closing of calcium-activated potassium channels (22) and a further potential decrease. Obviously, we have to take into account that type II or endothelial cells are also stained by the dyes, but due to their very small size and the dominance of type I cells (10), these cells seem to contribute little to the recorded calcium and potential signal. Furthermore, for type Ip cells, identification ROIs for fluorescence changes were set to include only bright fluorescence close to the cell nucleus.

In the current setup it is difficult to precisely quantify the oxygen curves. But from our previous experiments on the superfused rat CB (14, 25) we know that \( \text{O}_2 \) during hypoxia never reaches zero Torr since there is always some oxygen leakage in the experimental setup. The CBs in our experiments are therefore challenged by mild to severe hypoxia but not anoxia. Cellular calcium increase as well as calcium decrease gradually follow the slow changes of tissue oxygen under mild hypoxia as can be seen from Fig. 2, B and C. During the total hypoxic period of 5 min, the severely hypoxic period peaks for about 10 subsequent seconds only without any abnormal calcium changes. One needs longer severely hypoxic periods of about 1 min and more to see a deterioration of CB function with a decrease of the sinus nerve discharge and a complete reduction of the cytochromes (14, 25). We therefore think that our ex vivo preparation can well cope with the very short period of severe hypoxia and is not driven into a too exaggerated nonphysiological situation causing the observed heterogeneous calcium response. In addition, the more uniform hypoxia-induced potential decrease of all CBs studied here is in accordance with hypoxia-induced potential changes of isolated rat type I cell cluster (17) and favors an intact CB function. Figure 3C shows that the “on” reaction of the hypoxic calcium-membrane potential relationship is faster than the “off” reaction, meaning that the oxygen-sensing system might have a fast regulatory adaptation to hypoxia and needs longer time to recover. This is in line with our observation with a fast nervous discharge increase and mitochondrial reduction within 1–2 min during on the “on” reaction and a long lasting depression of nervous discharge and mitochondrial activity during the “off” reaction within 30 or more minutes as seen with the superfused rat carotid body (14, 25).
The uniform depolarization indicates that all cells should experience an influx of calcium because of the opening of the corresponding channels. On the other hand, cells in close proximity either hyperpolarize or depolarize spontaneously under control conditions as can be seen from the green and yellow color in Fig. 3, III and IV. This again demonstrates for the first time type I cell heterogeneity with respect to their electrical activity perhaps due to differences in their ion channel or receptor equipment as well as intercellular communication (17).

We therefore propose that preservation of the CB tissue structure during experiments is a basic requirement for revealing the physiologically heterogeneous pattern of oxygen-sensing properties and mechanisms of type I cells. It is remarkable that the observed spontaneous burstlike changes in potential support the interpretation of type I cell heterogeneity. Figure 3 and animation 2 demonstrate the obvious differences between cells in close proximity, in which the membrane potential can either spontaneously increase or decrease. The additional sensing properties of the CB in detecting changes in pH, CO2 and glucose concentrations, tonicity, or temperature (26), the diversity of molecules triggering the oxygen sensitivity of potassium channels as mentioned above, or developmental heterogeneity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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