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Using carbon fibre microelectrodes to monitor the oxidative metabolism of blowfly eyes

Uporaba mikroelektrod iz ogljikovih vlaken za spremljanje oksidativnega metabolizma mušjih oči

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Abstract. The oxidative metabolism in animal tissues can be conveniently monitored by measuring tissue P_{O2} with a carbon fibre microelectrode. We have established a recording configuration in a living animal by insertion of a carbon fibre electrode (CFE) into the retina of a blowfly (*Calliphora vicina* – chalky). The current flowing over an exposed carbon disc at the tip of an insulated carbon fibre with 5 µm diameter is linearly proportional to P_{O2} when the P_{O2} was varied between 0 kPa (100% N_2) and 100 kPa (100% O_2) in the recording chamber. The slight changes in sensitivity of CFE during the recording time were corrected by calibrations performed at the start and at the end of the experiments. Exposure of the eye to bright light caused a drop in tissue P_{O2}. Hypoxia increased with the stimulation time, reaching a maximum after about 20 s (ΔP_{O2}=11.6 kPa). These results are in good agreement with direct measurements of O_2 consumption in isolated eyes.

Keywords: blowfly eye, *Calliphora vicina* – chalky, carbon fibre electrode, P_{O2} measurement, amperometry

Izvleček. Oksidativni metabolizem živalskih tkiv je možno priročno spremljati s pomočjo meritev P_{O2} v tkivu z mikroelektrodami iz ogljikovih vlaken. Pri našem delu smo uporabili merilno konfiguracijo pri živi živali, tako da smo v retino muhe (*Calliphora vicina* – chalky) vstavili elektrodo iz ogljikovih vlaken (CFE). Tok, ki je tekel čez izpostavljen disk na konici izoliranega ogljikovega vlakna premera 5 µm, je bil premo sorazmeren P_{O2}, če smo P_{O2} spreminjali med 0 kPa (100% N_2) in 100 kPa (100% O_2) v merilni kamrici. Izpostavitev očesa močni svetlobi je povzročila padec P_{O2} v tkivu. Hipoksija se je povečevala s časom osvetlitve in je dosegla maksimum pri osvetlitvah dolgih približno 20 s. Ti rezultati se dobro skladajo z neposrednimi meritvami porabe kisika izoliranih oči.

Ključne besede. mušje oko, *Calliphora vicina* – chalky, ogljikova elektroda, merjenje P_{O2}, amperometrija

Introduction

The photoreceptors of animal eyes collect optical information of the environment, contained in the incident photon flux. The phototransduction process of the photoreceptors converts the incident light into a change in the photoreceptor's membrane potential, and this signal is subsequently transmitted to the animal's central nervous system. The phototransduction process requires an ionic imbalance across the photoreceptive plasma membrane and thus metabolic energy to power the ion pumps. The necessary power is provided by ATP, which is produced by the mitochondria. The mitochondrial activity of insect photoreceptors has been shown to be tightly coupled to the process of phototransduction (Tsacopoulos & al. 1983). Although this tight coupling, which in honeybee drones even precedes the actual changes in ion gradients, has been demonstrated a while ago, its nature and mechanism remains unknown. The most likely agent is the increase in $[Ca^{2+}]_i$, following the opening of the TRP and TRPL transduction ion channels.

The oxidative metabolism of insect eyes has been studied by various methods. The most direct approaches, where the actual oxygen consumption of the tissue is measured directly, require isolation of the eyes (Hamdorf & al. 1988, Pangršič & al. 2005). The eye needs to be put into a closed container, which prevents any other experimental manipulations like electrophysiological measurements. Other approaches include monitoring the redox states of the respiratory pigments (Tinbergen & Stavenga 1986, Mojet & al. 1991, Tinbergen & Stavenga 1987, Zupančič 2003) and monitoring the tissue P_{O2} (Tsacopoulos & Poitry 1982, Widmer & al. 1990, Poitry & Widmer 1996, Poitry & al. 1996) using polarization electrodes. In ideal circumstances, when the sample geometry is simple and the oxygen diffusion can be properly modelled, the latter method allows the transformation of the P_{O2} values into O_2 consumption. However, this can only be done with perfused slices of insect eyes, which again presents some limitations for other experimental procedures.

The aim of the present study was to record changes in P_{O2} in response to illumination of the blowfly eye *in situ*. For this we used carbon fibre polarization electrodes and amperometrically measured the P_{O2} within the retinal tissue.

Material and Methods

Experiments were done on male blowflies, *Calliphora vicina*, white-eyed mutant chalky. Adult flies were kept under a 12/12 h light-dark cycle and fed sucrose. Larvae were grown on liver, to assure a high rhodopsin content of the photoreceptors. We used flies between one and three weeks of age. Preparation was done under white light. The legs were removed and the animals were attached to a copper holder with a thin yoke around the neck of the animal in order to immobilize the head. The abdomen and mouth apparatus were glued to the holder using a 5:1 mix of bee wax and colophony. This mounting procedure allowed immobilizing the animal while keeping the tracheal openings unobstructed. The copper holder was placed at a cork support, attached to a microscope slide. The support also had a socket for attaching the reference electrode, made of a chlorinated silver wire. The reference electrode was manually inserted in the eye margin. For the insertion of the carbon fibre electrode we made a triangular incision in the same eye, thus removing ~10–20 facets from the cornea. The entire support, with the fly, was placed inside a plastic chamber, which allowed changes of the atmosphere surrounding the animal with a rapid gas-exchange system (Zupančič 2003). The chamber had a small window through which the carbon fibre electrode could be entered and subsequently inserted into the eye. The recording chamber was placed at the stage of a modified Leitz Orthoplan microscope, below the microscope objective (Leitz Plan Fl 4, 0.14 NA), so that the eye of the fly was in the focal plane of the objective (Fig. 1). The light beam of a 900 W xenon arc lamp (Osram, Germany) filtered by a blue interference filter (476 ± 10 nm; Schott, Germany) delivered the stimulus via the epi-illumination pathway. The final light intensity was adjusted using neutral density filters. Light was turned on and off by a mechanical shutter (Compur, Germany), controlled by the Spike 2 sequencer program (CED, Cambridge, UK) and a CED1401 plus (CED, Cambridge, UK) A/D

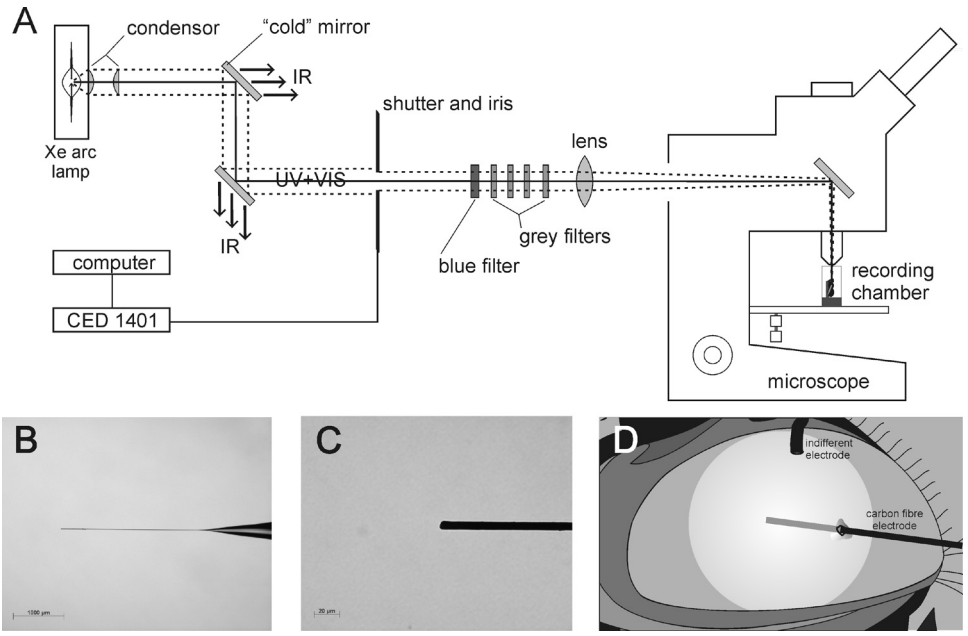

Fig. 1: **Diagram of the experimental apparatus, the preparation and the electrodes used. A –** All experiments were done on a modified Leitz Orthoplan microscope using a 900 W Xe arc lamp. Light was filtered with a 473 ± 10 nm blue filter. The recording chamber, placed under the microscope, allowed rapid gas exchange. **B** – We used electropainted, 5 µm diameter carbon fibre electrodes for measuring the P_{O2}. **C** – The insulated carbon fibre was cut with a scalpel blade to expose a disc-shaped, electroactive surface area. **D** – Positions of the carbon fibre and the reference Ag/AgCl electrode in the eye. The carbon fibre electrode was inserted through a pre-cut opening.

Slika 1: **Shema sistema za osvetljevanje, položaj ogljikove in referenčne elektrode v očesu in slika ogljikove elektrode. A** – vsi poskusi so bili izvedeni na modificiranem mikroskopu Leitz Orthoplan s pomočjo 900 W Xe obločne žarnice. Svetlobo smo filtrirali z modrim filtrom 473 ± 10 nm. Mehanski zaklop je vklapljal in izklapljal svetlobo. Merilno kamrico, ki je omogočala hitro zamenjavo plinov smo postavili pod mikroskop. **B** – Za meritve P_{O2} smo uporabili ogljikovo vlakno preseka 5 µm izolirano z elektrodepozitno barvo. **C** – Izolirano ogljikovo vlakno smo pred poskusom vsakič prerezali s pomočjo skalpela, da smo izpostavili diskasto aktivno površino. **D** – Poziciji ogljikove in referenčne Ag/AgCl elektrode v očesu. Ogljikovo elektrodo smo vstavili skozi poprej izrezano odprtino.

converter. The duration of the light stimuli was 0.03, 0.06, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 s. The interval between the light pulses was chosen manually to allow the response to each stimulus to decay back to the initial level before the next stimulus.

For amperometric P_{O2} measurements we used electropainted, 5 µm diameter carbon fibre electrodes. The electrodes were manufactured according to the procedure of Schulte and Chow (Schulte & Chow 1996, Chow & Rüden 1995). Briefly, a single carbon fibre was attached to the stripped end of a copper wire using silver conductive paste (Bison, Netherlands). The fibre was inserted into a borosilicate tubing, from which a microelectrode was then produced with a microelectrode puller. The gap between the glass tubing and the carbon fibre was sealed with silicone coating (Dow Corning Corporation, USA). The carbon fibre was isolated using anodic electrodeposition paint (Glassphor ZQ 84-3122; BASF, Germany) by applying a 2.5 V voltage for 3 min between the carbon fibre electrode and a platinum wire. Prior to each experiment, the tip of the elec-

trode was cut with a scalpel blade, thus exposing a disc-shaped electro-active surface area.

During recording the carbon fibre electrode was held at a polarisation voltage of –600 mV (relative to the reference electrode). The majority of current at this voltage is attributable to the reduction of oxygen (Mojet & al. 1997). The electrode current was measured with a home-made current to voltage converter feeding into a CyberAmp 380 (Axon Instruments, UK) amplifier. The signal was filtered below 6 Hz and sampled at 100 Hz.

Carbon fibre calibration procedure

In order to verify the basic assumption of linearity between the amperometric current and P_{O2} we tested the response properties of the carbon fibre electrode within the eye tissue. We examined this relationship using dead blowflies that were killed by hyperthermia (exposure to 50 °C for 2 min) to avoid problems due to the oxidative metabolism of active, live tissue (Fig. 2). We changed the P_{O2} in the recording chamber with a Cole-Parmer (USA) mixing flow meter in steps from 0 to 100 kPa and measured the resulting cur-

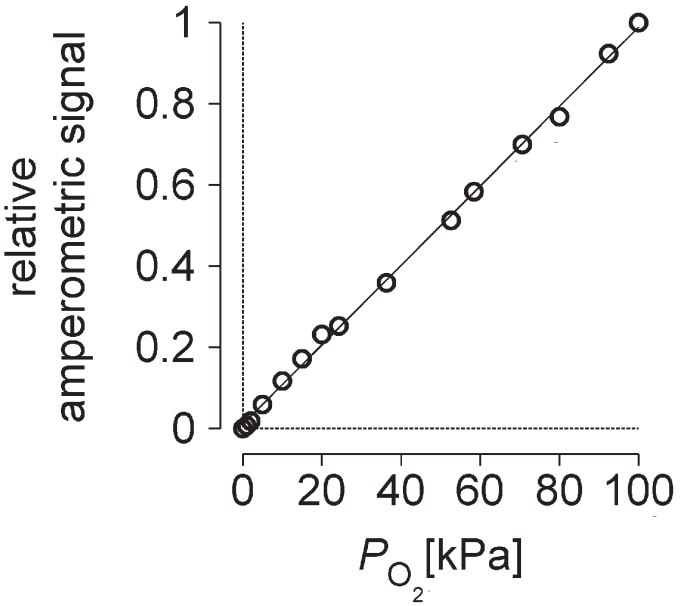

Fig. 2: **The relationship between P_{O2} and the relative amperometric signal (relative to maximal current in pure O_2) from the carbon fibre electrode.** Four blowflies killed by hyperthermia were exposed to different preset levels of environmental P_{O2} determined by a mixing flow-meter and verified with an electrochemical sensor. Since the sensitivity of four electrodes slightly differed, the values from each recording were normalized to the maximal current recorded at 100 kPa O_2. The ± 1 s.d. limits are in all cases smaller than the diameter of the circles indicating means. The relationship between the P_{O2} and relative amperometric signal was fitted with a linear function.

Slika 2: **Odvisnost med P_{O2} in relativnim amperometričnim signalom (relativnim glede na maksimalni tok v čistem O_2), izmerjenim z ogljikovo elektrodo.** Štiri muhe, usmrčene s hipertermijo, smo izpostavili različnim vrednostim P_{O2}, določenimi z mešalnim flow-metrom in preverjenimi z elektrokemičnim senzorjem. Ker se je občutljivost štirih uporabljenih elektrod nekoliko razlikovala, smo normalizirali vrednosti glede na makasimalni tok izmerjen pri 100 kPa O_2, ki je dal relativni amperometrični signal vrednosti 1. Meje ± 1 s.d. so v vseh primerih manjše od premera krogov, ki označujejo povprečja. Odvisnosti med P_{O2} in relativnim amperometričnim signalom je možno prilagoditi tudi linearno funkcijo.

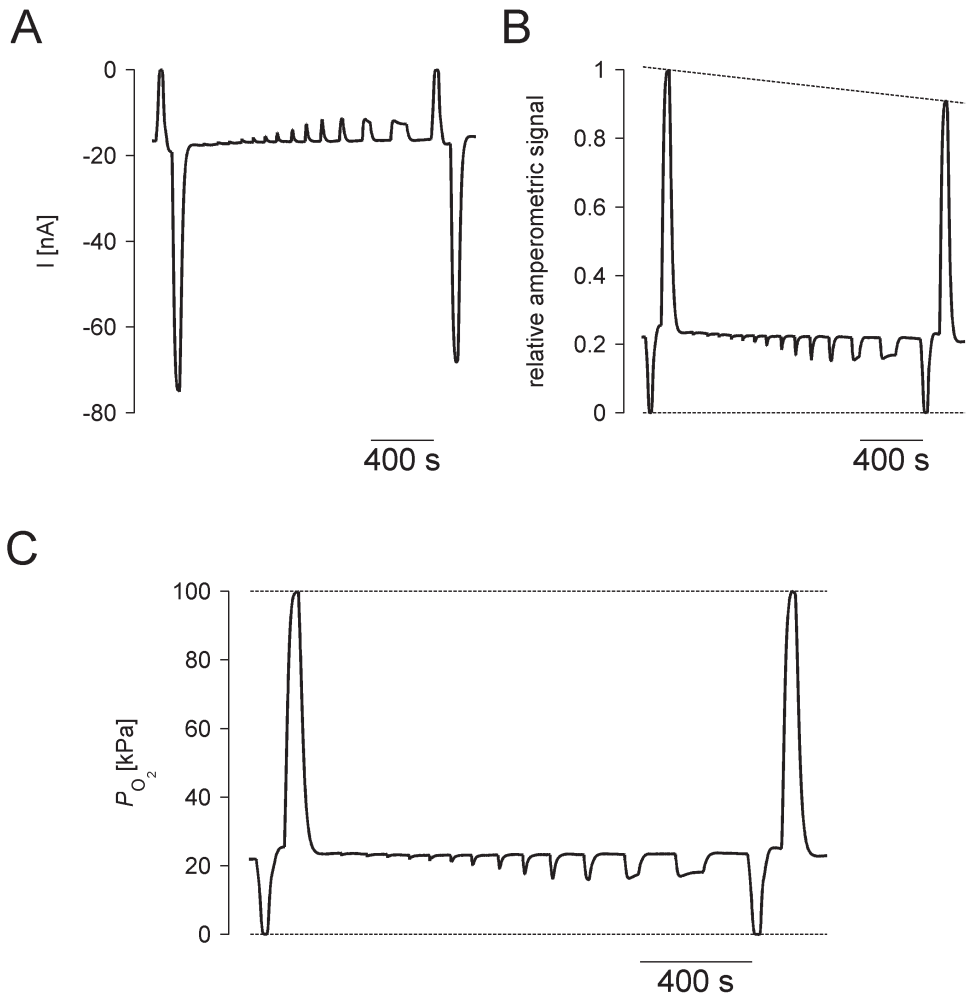

Fig. 3: **The calibration procedure and the correction for changes in sensitivity.** The measuring protocol consisted of: exposure to N_2 and O_2 calibrating pulses at the beginning, a series of illuminations, and a second exposure to N_2 and O_2 exposure at the end. The current in the electrode (**A**) was normalized with respect to the maximal current recorded in pure O_2 at the start of the recording (**B**). Because the electrode sensitivity changed during the experiment (**B**), a linear interpolation between the calibrating points in N_2 and O_2 was made, and the relative signal was transformed into P_{O2} values (**C**).

Slika 3: **Postopek kalibracije in korekcije zaradi sprememb v občutljivosti elektrode.** Merilni protokol so sestavljali izpostavitev kalibracijskim pulzom N_2 in O_2 na začetku, serija osvetlitev in druga izpostavitev N_2 in O_2 na koncu. Tok iz elektrode (**A**) smo normalizirali glede na maksimalni tok v čistem O_2 na začetku poskusa (**B**). Ker se občutljivost elektrode spreminja med poskusom (**B**), smo uporabili linearno interpolacijo med kalibracijskimi točkami v N_2 in O_2 in nato transformirali relativni signal v vrednoti P_{O2}. (**C**).

rent in the carbon fibre electrode. The P_{O2} values were verified independently with an electrochemical P_{O2} sensor (ECHO, Slovenia). Data from four blowflies are shown in Fig. 2. The sensitivity of electrodes used slightly varied, and therefore the values from each recording were normalized to the maximal current recorded at 100 kPa O_2 in order for the results to be comparable. The relationship between current and P_{O2} in the eye of a dead blowfly appeared to be linear, in accordance with Faraday's law describing the linear relationship of the number of reacting molecules and the total charge transferred. Having demonstrated the linearity of the carbon fibre electrode current with P_{O2}, we calibrated the electrodes prior to each experiment at two points: the signal at 0 kPa O_2 was given the value 0 (kPa) and that at 100 kPa O_2 obtained the value 100 (kPa). Intermediate values (>0 kPa and <100 kPa) were then calculated by linear interpolation (Fig. 3c).

The actual measurement protocol consisted of the following steps: at the beginning of an experiment, the preparation was exposed to pure N_2 and pure O_2, and subsequently a series of light pulses was applied; at the end of the protocol, the preparation was again exposed to pure N_2 and pure O_2. The last two calibration points allowed the correction for drift in the properties of the carbon fibre electrodes, which most likely are due to contamination of the active surface (Fig. 3b, c). The correction was made by linear interpolation between the calibration points before and after the experiment.

Results

The P_{O2} in the blowfly eye tissue drops upon illumination. The decrease in amplitude depends on stimulus duration and intensity (Fig. 4a). The largest decrease observed, with 20–50 s bright light illumination, was 11.6 kPa. On average, however, with intermediate light intensities (the photon flux averaged over the entire surface of the eye was 10^{16} photons s^{-1} m^{-2}) the decrease in P_{O2} with 20 s light pulses was 9.6 ± 0.7 kPa (mean

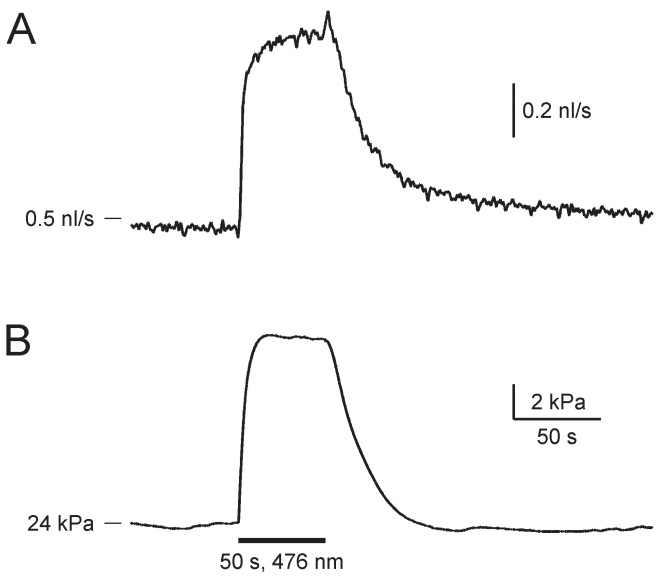

Fig. 4: Comparison of the time courses of the increase in O_2 consumption (A; adapted from Pangršič & al. 2005) and the absolute change in tissue P_{O2} (B).

Slika 4: Primerjava med časovnim potekom povečanja porabe O_2 (Pangršič & al. 2005) in časovni potek spremembe P_{O2} v tkivu.

± SEM, n=5). In such conditions the actual time course of the absolute change in P_{O2} was very similar to the increase in the O_2 consumption, elicited with comparable stimulation parameters, measured directly using a magnetic diver balance (Pangršič & al. 2005, Fig. 4b).

The results were similar with shorter light pulses, the only difference being that shorter light pulses produced smaller changes in P_{O2}. Fig. 5a shows an example of responses to a series of light pulses of different durations, from 30 ms to 50 s. Here it has to be noted that the measured drop in P_{O2} strongly depended on the position of the electrode within the eye, i.e. the depth of insertion and the position with respect to the centre of the illuminated area. In our case we achieved reproducible values with the electrode positioning in a series of experiments with intermediate light intensities comparable to the range between 10^{17} and 10^{18} photons s^{-1} m^{-2} as recorded with the magnetic diver balance (Pangršič & al. 2005). The comparison is only approximate since the entire eye was not uniformly illuminated, as was the case with the diver balance. In the present experiments the light flux in the centre of the illuminated area was quite different from that at the eye periphery. Nevertheless, another qualitative similarity with the measurements of O_2 consumption is the relationship between the stimulus duration and ΔP_{O2} (Fig. 5b). The relationship shown is very similar to the one recorded with direct respirometry. It covers three log units of stimulus durations and it saturates with durations longer than 20 s.

Discussion

Monitoring the mitochondrial activity within living tissue or cells is a prerequisite for any research dealing with questions concerning the role of mitochondria within active cells. Insect and especially blowfly eyes have in the past been the object of this kind of research using direct and indirect methods. Indirect approaches involved measurement of the absorption (Tinbergen & Stavenga 1986, Smits et al. 1995, Stavenga 1995, Zupančič 2003) or fluorescence (Stavenga & Tinbergen 1983, Tinbergen & Stavenga 1986, Tinbergen & Stavenga 1987, Mojet et al. 1991) of the mitochondrial respiratory pigments, but they suffer from the problem how to quantita-

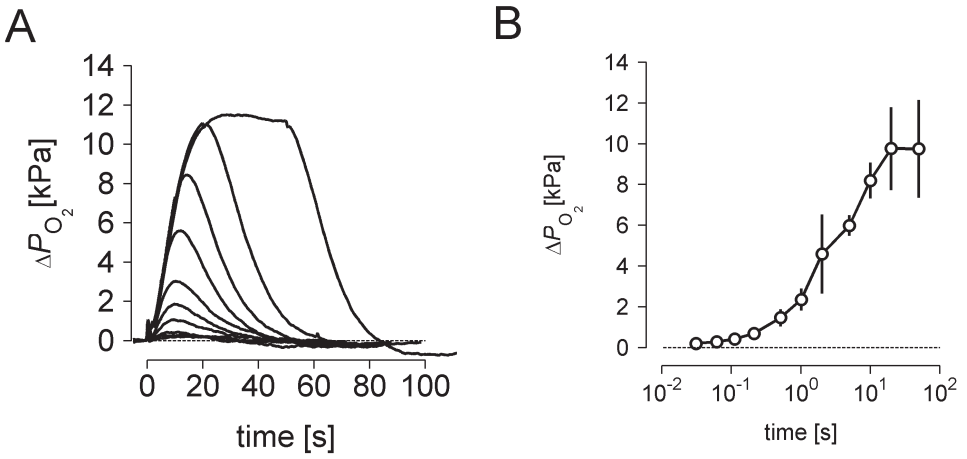

Fig. 5: **Responses to a series of light pulses of increasing duration. A –** Superimposed changes of P_{O2} in response to different stimulus duration. **B –** Dependence between light stimulus duration and changes in P_{O2}. Maximal changes are reached with 20 s light pulses.

Slika 5: **Odziv na serijo različno dolgih svetlobnih pulzov. A** – Superpozicija sprememb P_{O2} v odgovor na dražljaje različnega trajanja. **B** – Odvisnost med dolžino svetlobnih dražljajev in spremembami P_{O2}. Maksimalne spremembe so bile dosežene z 20 sekundnimi pulzi.

tively relate the measured parameters to the actual energy consumption. On the other hand, the direct measurement of the consumed O_2 (Hamdorf & al. 1988, Pangršič & al. 2005) is experimentally very demanding and in most cases prohibits any other manipulations of the preparation. We therefore have developed the measurement of the P_{O2} within the blowfly eye tissue with the carbon fibre electrodes, which are normally used for amperometric monitoring of secretion of oxidizable compounds (Mojet & al. 1997). We were able to successfully adapt the electrodes and the recording apparatus to measure the tissue P_{O2}, and we were able to at least qualitatively relate the recorded changes in P_{O2} to direct recordings of time courses of O_2 consumption using a magnetic diver balance (Pangršič & al. 2005). We found that both the time courses of the changes in P_{O2} as well as the relationship between stimulus duration and the consequent change in P_{O2} were comparable to the results obtained by direct measurements of the increase in O_2 consumption measured in isolated eyes.

Our main findings were that the carbon fibre electrodes are a good tool for measuring the P_{O2} within the tissue, providing some caveats are observed:

1. Small diameter carbon fibre electrodes of the type we used are prone to changes of their sensitivity when used within the tissue, either due to contamination of the surface area or to damage of their insulation. Often neither can be avoided, so a means of calibrating each recording before and after the experimental procedure must be assured. Our experimental animals, the flies, have a very high hypoxic tolerance, and also have a tracheal system that allows rapid exchange of gases deep within the tissue. It thus was a simple case of exposing the animal to pure N_2 and pure O_2 at the beginning and at the end of each experiment. Linear interpolation of the measured data between these two time points allowed adequate corrections for changes in sensitivity. This method is therefore very suitable for use in insects, provided they can tolerate an extremely low and extremely high P_{O2} for any length of time.
2. The carbon fibre electrode only records the P_{O2} locally. If the O_2 consumption varies within the tissue, the recorded values will reflect this. For the purpose of comparability, the recording sites therefore have to be as much standardized as possible.
3. Also it has to be noted that the P_{O2} represents the balance between the O_2 consumption and O_2 delivery. Normally we would like to correlate the P_{O2} to O_2 consumption, but this is only true when the rate of delivery does not change. However, even in insects this is not entirely true. Large loads on O_2 consumption are bound to trigger homeostatic mechanisms, which increase O_2 delivery, like opening of stigmata and ventilation movements, which will show up in the P_{O2} records. A possible example of this can be seen in figures 3 and 5, where P_{O2} actually increases with long illumination times of 50 or 60 s.

In conclusion, we have shown that carbon fibre electrodes can be used successfully to monitor P_{O2} within live tissue, especially in the eyes of flies and presumably also in other insects. The method has some limitations, which can easily be dealt with by proper design and execution of the experiments.

Povzetek

Fotoreceptorji posredujejo svetlobno informacijo iz okolja v živčni sistem živali. V tem procesu se svetlobna energija pretvori v električni odziv receptorske celice. Za proces fototransdukcije je nujno vzdrževanje ionskih gredientov prek celičnih membran, ki pa zahteva precej energije, zato morata procesa fototransdukcije in aktivacije mitohondrijev biti tesno povezana. Te procese so v preteklosti študirali pri žuželkah. Na tesno povezavo kaže starejše poročilo (Tsacopoulos & al. 1983), saj pride do povečanega delovanja mitohondrijev v retini čebeljih trotov pred spremembami ionskih gradientov, najverjetneje na račun povečanja $[Ca^{2+}]_i$ zaradi odprtja transdukcijskih ionskih kanalčkov TRP in TRPL. Najbolj natančne podatke o delovanju mitohondrijev v mušjih očeh so dale neposredne meritve porabe kisika (Hamdorf & al. 1988, Pangršič & al. 2005). Resna pomanjkljivost teh metod je, da potekajo na izoliranih očeh, zaprtih v drobno kamrico, ki preprečuje dostop za opravljanje dodatnih sočasnih meritev, na primer elektroretinografije. Vendar lahko energijski metabolizem spremljamo

tudi prek sprememb P_{O2} v tkivu. Uvedli smo merjenje P_{O2} prek izpostavljene površine 5 µm ogljikovega vlakna. Šlo je za amperometrične meritve, pri polarizacijski napetosti –600 mV, kjer večino večino toka prispeva redukcija kisika. Elektrode z majhnim presekom pa imajo pomanjkljivost – njihova občutljivost se tekom poskusa spreminja, bodisi zaradi kontaminacije aktivne površine ali zaradi poškodb izolacije. Umeritev elektrode pred vsakim poskusom je torej nujna. Žuželke, in predvsem muhe, imajo kot poskusne živali v tem primeru dve veliki prednosti. Trahealni sistem omogoča hitro izmenjavo plinov globoko v tkivu, živali pa so izjemno odporne na anoksijo. Umeritve smo izvedli tako, da smo pred in po vsakem poskusu izpostavili živali čistemu O_2 in čistemu N_2. Z linearno interpolacijo med temi umeritvenimi točkami smo korigirali spreminjanje občutljivosti elektrod. Upoštevati moramo tudi, da z ogljikovimi elektrodami merimo spremembe P_{O2} zelo lokalno. Če poraba O_2 v tkivu ni enakomerna, bo to odsevalo tudi pri meritvah P_{O2}. Položaj elektrode mora biti zato kar najbolj standardiziran. P_{O2} v tkivu in poraba O_2 sta povezana, vseeno pa ju ne gre povsem enačiti. P_{O2} namreč predstavlja ravnotežje med porabo in dostavo O_2. Veliko metabolno breme tako povroči še druge homeostatske mehanizme, ki povečajo dostavo O_2, kot so povečana frekvenca dihalnih gibov in odprtje stigem. Kljub temu pa so časovni poteki sprememb P_{O2} v tkivu ob osvetlitvi zelo podobni časovnim potekom sprememb porabe O_2 (Pangršič & al. 2005).

Acknowledgments

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