Sensors **2005**, *5*, 473-487

sensors

ISSN 1424-8220

http://www.mdpi.org/sensors

Brain Tissue Oxygen: *In Vivo* Monitoring with Carbon Paste Electrodes

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Received: 28 April 2005 / Accepted: 1 July 2005 / Published: 16 November 2005

Abstract: In this communication we review selected experiments involving the use of carbon paste electrodes (CPEs) to monitor and measure brain tissue O_2 levels in awake freely-moving animals. Simultaneous measurements of rCBF were performed using the H_2 clearance technique. Voltammetric techniques used include both differential pulse (O_2) and constant potential amperometry (rCBF). Mild hypoxia and hyperoxia produced rapid changes (decrease and increase respectively) in the *in vivo* O_2 signal. Neuronal activation (tail pinch and stimulated grooming) produced similar increases in both O_2 and rCBF indicating that CPE O_2 currents provide an index of increases in rCBF when such increases exceed O_2 utilization. Saline injection produced a transient increase in the O_2 signal while chloral hydrate produced slower more long-lasting changes that accompanied the behavioral changes associated with anaesthesia. Acetazolamide increased O_2 levels through an increase in rCBF.

Keywords: *In-vivo* voltammetry; cerebral blood flow; hypoxia and hyperoxia; neuronal activation; chloral hydrate anaesthesia; acetazolamide

Introduction

Molecular O_2 was one of the first substances detected voltammetrically *in vivo*, both in brain [1,2] and peripheral tissues [3], using noble metal and carbon electrodes that continue to be the electrode materials of choice for this analyte [4-7]. Brain tissue O_2 is one of the most important substrate for energy metabolism delivered by the blood and responds to a range of perturbations

including electrical stimulation [8] and neuromediator release [9], with more rapid turnover in cerebral cortex compared with other brain areas [10]. The tissue concentration is determined by the balance between supply and utilization and the anatomical distribution of concentrations reported within the brain is wide, depending on the depth of penetration of the sensor into the tissue [11] and the heterogeneity of the tissue [12,13]. The spread of mean concentrations reported ranges from 40 μM [12], through 50 μM [10] and 60 μM [14], to 80 μM [8,15].

The majority of such measurements have generally been obtained amperometrically using constant potential amperometry (CPA) at a noble metal microelectrode where the measured current is taken to be proportional to the dissolved O_2 [16]. The use of carbon-based electrodes as cathodes for O_2 reduction, instead of the more commonly used Pt, has recently been reported by several groups [17-20]. Carbon electrodes have the advantage that they are less prone to surface poisoning and as such do not require the use of protecting membranes which are a characteristic of metal-based O_2 electrodes, including the most commonly used Clark electrode. Carbon electrodes are also more stable and in the case of carbon paste electodes (CPEs) have been found to be stable over several months *in vivo* [21]. Recently, a new method for the measurement of dissolved O_2 *in vivo* involving Nafion™-modified carbon fibre disk electrodes (CFEs) and fast cyclic voltammetry has been reported by Wightman's group [18,20,22]. As a fast-scan technique this method also has advantages associated with subsecond time resolution. However, because of the small dimensions of CFEs (typically 10 μm) the concentration of O_2 observed varies depending on the orientation of the electrode relative to the blood vessels and metabolically active sites. Since the dimension (typically 125-μm active internal diameter) of CPEs is greater than the scale of a capillary zone (< 100 μm) [23], an average tissue O_2 level should be detected, thus offering a practical advantage over the use of CFEs for O_2 measurements. In this communication we present a review of selected experiments involving the use of CPEs to successfully monitor and measure brain tissue O_2 levels in awake freely-moving animals.

Experimental

Reagents

The acetazolamide (Diamox) and chloral hydrate were obtained from Sigma Chemical Co. (Dorset, UK) and BDH Laboratory Supplies (Poole, UK) respectively. Solutions of normal saline (0.5 mL, 0.9% NaCl), chloral hydrate (350 mg/kg), and Diamox (50 mg/kg) were administered intraperitoneally (i.p.). All solutions were prepared using doubly distilled deionised water.

Working Electrode Preparation

CPEs were made from 5T (125-μm bare diameter, 160-μm coated diameter) Teflon-coated silver wire (Advent Research Materials, Suffolk, UK). The Teflon insulation was slid along the wire to create an approximately 2-mm deep cavity which was packed with carbon paste using a bare silver wire as plunger. Carbon paste was prepared by thoroughly mixing 2.83 g of carbon powder (UCP-1-M, Ultra Carbon Corp., Bay City, MI, USA) and 1.0 mL of silicone oil (Aldrich, Catalogue No. 17,563-3) [24]. The H_2 detection electrodes for rCBF measurements (see *Voltammetric Techniques*) were made

from 2T (50-µm bare diameter, 75-µm coated diameter) Teflon-coated Pt/Ir wire as reported previously [25]. All electrodes were soldered to gold connectors, which were cemented into a six-pin plastic socket (both from Plastics One, Roanoke, VA, USA).

Voltammetric Techniques

Changes in O_2 at CPEs were monitored using differential pulse amperometry (DPA). For DPA O_2 reduction, two equally sized cathodic pulses were applied, the first from a resting potential at -150 mV to -350 mV that corresponds to the foot of the reduction wave for O_2 at lipid-modified CPEs, and the second from -350 mV to -550 mV that corresponds to the peak of the reduction wave. The difference in the current (ΔI) sampled during these respective pulse pairs was calculated, and changes in ΔI used as a measure of changes in O_2 that have been shown to be interference free [19]. Because the CPE DPA signal has a significant capacitance (background) current (mean value: -244.8 ± 59.7 nA, n = 23) [26] changes in the signal cannot be calculated as a percentage [25].

The CPEs were calibrated for O_2 *in vitro* after removal from the brain because implantation affects the sensitivity of these paste electrodes [27,28]. All *in vitro* experiments were carried out in a standard three-electrode glass electrochemical cell (capacity 25 mL) in phosphate buffered saline (PBS) solution (20 mL), pH 7.4 (150 mM NaCl, 40 mM NaH_2PO_4 and 40 mM NaOH). A saturated calomel electrode (SCE) was used as the reference electrode, and a large silver wire, isolated in a compartment containing PBS, served as the auxiliary electrode. Calibrations were performed in N_2-purged, air-saturated and O_2-saturated solutions where the concentrations of solution O_2 were taken as 0, 200 [29,30] and 1,250 µM [29], respectively.

Measurements of rCBF were performed using the H_2 clearance technique. CPA, where the detecting electrode is held at a constant potential sufficient to detect the oxidation or reduction of the target substance, was performed by holding the CPE at the peak potential for H_2 oxidation (+250 mV). The methods of H_2 administration and analysis used in these experiments have been described in detail in a previous publication [25].

Surgical Procedures

Male Sprague-Dawley rats weighing 200-300 g were anaesthetized, following published guidelines [31], with a mixture of Hypnorm (Fentanyl citrate/Fluanisone, Janssen Pharmaceuticals Ltd., Oxford, UK), Hypnovel (Midazolam, Roche Products Ltd., Herts, UK), and sterile water, mixed 1:1:2 and injected i.p. at a volume of 3.3 mL/kg, as described previously [32,33]. Once surgical anesthesia was established animals were placed in a stereotaxic frame and the electrodes implanted following a previously described procedure [32]. Animals were implanted with either one of two combinations of sensors: (i) carbon paste electrodes, for monitoring tissue O_2, one in the right and one in the left striatum (coordinates with the skull leveled between bregma and lambda, were: A/P +1.0 from bregma, M/L ±2.5, and D/V -5.0 from dura [32]; (ii) a 2T Pt/Ir electrode, for monitoring rCBF by the H_2 clearance method, was implanted in the right striatum (coordinates: A/P +1.0 from bregma, M/L +2.5, and D/V -8.5 from dura), and a carbon paste electrode, for monitoring tissue O_2, in the left striatum (coordinates: A/P +1.0 from bregma, M/L -2.5, and D/V -5.0 from dura). A reference electrode (8T Ag

wire, 200-µm bare diameter; Advent Research Materials) was placed in the cortex, an auxiliary electrode (8T Ag wire) placed between the skull and dura, and an earth wire (8T Ag wire) attached to one of the support screws. The reference potential provided by the Ag wire in brain tissue is very similar to that of the SCE [27]. The electrodes and probe were fixed to the skull with dental screws and dental acrylate (Associated Dental Products Ltd., Swindon, UK). Surgery typically lasted 40 min and anesthesia was reversed by an i.p. injection of naloxone (0.1 mg/kg, Sigma Chemical Co.). Post-operative analgesia was provided in the form of a single injection (0.1 mg/kg, s.c.) of Vetergesic (Buprenorphine hydrochloride, Reckitt and Colman Pharmaceuticals, Hull, UK) given immediately following the surgery. Animals were allowed to recover after surgery and were assessed for good health according to published guidelines [34] immediately after recovery from anesthesia and at the beginning of each day. This work was carried out under license in accordance with the Animals (Scientific Procedures) Act, 1986.

Experimental Conditions

Rats were housed in large plastic bowls (diameter ca. 50 cm), in a windowless room under a 12 h light, 12 h dark cycle, lights coming on at 8 a.m., with free access to water. Food was available *ad libitum*. All experiments were carried out with the animal in its home bowl. Implanted electrodes were connected to the potentiostat at least 60 min prior to the start of each experiment, through a six-pin Teflon socket, and a flexible screened six core cable which was mounted through a swivel above the rat's head (Semat Technical Ltd.). This arrangement allowed free movement of the animal. Once the background currents for the various sensors had stabilized (typically 5-10 min for the O_2 CPEs, and 30 min for the rCBF Pt/Ir electrodes) experiments were begun. All signals were recorded at 2-s intervals (see *Instrumentation and Software*).

Mild hypoxia and hyperoxia were produced by the administration of O_2/air and N_2/air mixtures; plastic tubing, connected to the respective gas cylinder (British Oxygen Co. (BOC), Guildford, UK) was held *ca*. 2-3 cm from the animal's snout for 3-min periods. A flow rate of ca. 150 mL/min was used. This procedure resulted in the inhalation of an air/gas mixture.

Instrumentation and Software

A low-noise potentiostat (Biostat II, Electrochemical and Medical Systems, Newbury, UK) was used in all experiments. For CPA experiments, either a Macintosh IIx computer or a Mitac 486 PC was used with data acquisition performed using National Instruments (NI, National Instruments Corp., Austin, TX, USA) multifunction data acquisition boards: NB-MIO-16X (Macintosh) and AT-MIO-16 (PC). Further noise reduction was achieved by averaging 75 (Mac) and 100 (PC) determinations to give 1 data point every 2 s. For DPA experiments, all data acquisition was performed on a Mitac 486 PC with data sampling performed at a rate of 250 determinations/pulse and averaged to give 1 data point/pulse [19]. A complete pulse sequence was performed every 2 s. All experiments were performed using in house developed software: PC (LabWindows, NI version 2.1); Macintosh (LabVIEW, NI version 2.2.1). All analysis was performed using both in house developed software and the commercial package Prism (version 4.03, GraphPad Software Inc., CA, USA).

The significance of differences observed was estimated using the Student's *t*-test for paired observations (StatView, Abacus Concepts, Inc., CA, USA). Two-tailed levels of significance were used and $p < 0.05$ was considered to be significant. All data are presented as mean ± S.E.M.

Results and Discussion

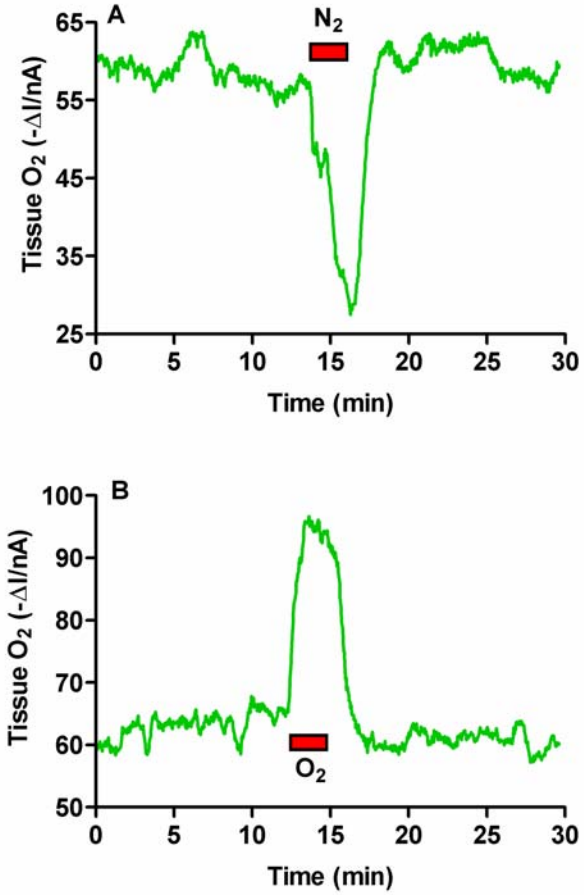

Figure 1. Typical raw data examples of the effects of mild hypoxia (**A**) and hyperoxia (**B**) on brain tissue O_2 levels monitored using a carbon paste electrode implanted in the striatum of a freely-moving rat.

Hypoxia and Hyperoxia

We have previously used mild hypoxia and hyperoxia to study brain extracellular glucose metabolism *in vivo* [35]. Both these conditions are ideally suited to investigating and verifying the response of CPEs to changes in brain tissue O_2 *in vivo*. A 3-min period of hypoxia caused a decrease in the O_2 current (ΔI) of 27.6 ± 2.8 nA, while a 3-min period of hyperoxia caused an increase in the O_2 current (ΔI) of 27.2 ± 4.2 nA, corresponding to mean percentage changes of 57 ± 5% and 43 ± 6% respectively ($n = 4$, see Figure 1A and 1B). Changes in both cases were immediate and on cessation of inhalation the signals quickly returned to baseline levels indicating a rapid return to normoxic conditions. These results confirmed that CPEs implanted in freely-moving animals respond rapidly to changes in cerebral tissue O_2 concentrations.

Neuronal Activation

Two forms of physiologically stimulated neuronal activation were used; these were tail pinch and induced grooming. In the first, a paper clip was attached ca. 3 cm from the tip of the rat's tail for 5 min; this produces a well characterised behaviour pattern which consists of gnawing, licking, eating and a general increase in the level of motor activity [36]. In the second, water was dropped from a plastic dropper onto the rat's head until the animal engaged in a period (usually between 5 to 10 min) of active grooming.

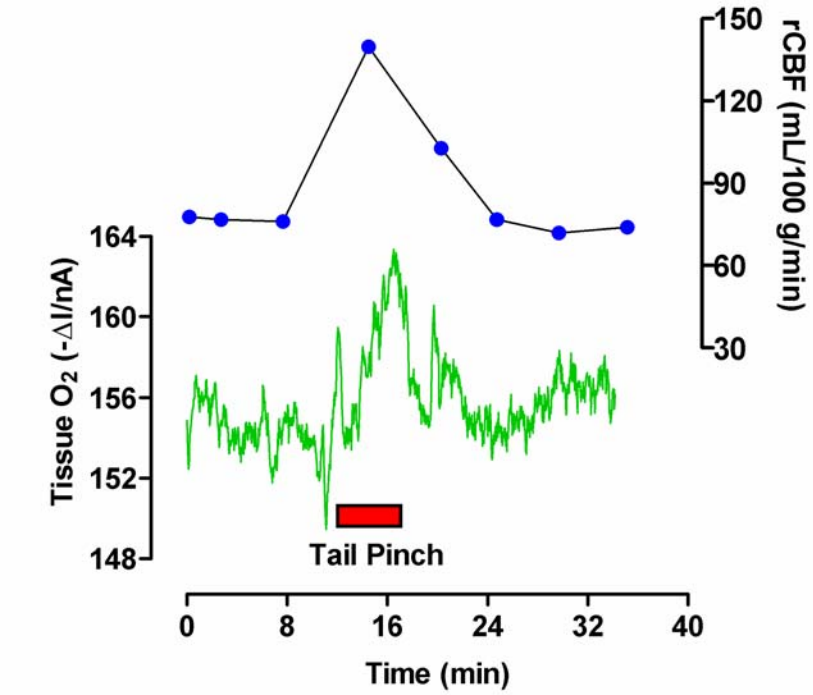

Figure 2. Typical examples of raw data obtained for the measurement of tissue O_2 and blood flow (rCBF), monitored simultaneously with bilaterally implanted carbon paste (O_2) and Pt/Ir (H_2 clearance) electrodes in the striatum of a freely-moving rat, in response to behavioral stimulation (5-min tail pinch).

The application of a 5 min tail pinch resulted in an immediate and rapid increase in the O_2 current (ΔI) recorded at the CPEs by 10.8 ± 1.9 nA from a mean baseline of -200 ± 24 nA ($p < 0.001$, eight tail pinches in two rats). On removal of the stimulus the current immediately began to fall and returned to baseline within ca. 10 min. The rCBF, calculated from the H_2 clearance at the Pt/Ir electrodes, also showed an immediate increase from a basal value of 89 ± 8 mL (100 g)$^{-1}$ min^{-1} to a maximum of 158 ± 13 mL (100 g)$^{-1}$ min^{-1} ($p < 0.001$), and returned to prestimulus levels ca. 16 min after the end of the stimulus. These basal and stimulated flow values are in good agreement with previous estimations made in awake animals: basal, 93 mL (100 g)$^{-1}$ min^{-1} [37] and 105 mL (100 g)$^{-1}$

min^{-1} [38]; stimulated, 160 mL $(100\ g)^{-1}\ min^{-1}$ [37]. A typical example of the effects of a 5 min tail pinch on both O_2 and blood flow is shown in Figure 2.

As observed with tail pinch, stimulated grooming (see Figure 3) resulted in an immediate increase in striatal O_2 levels by 6.2 ± 1.1 nA from a mean baseline of -160 ± 10 nA ($p < 0.001$, eight measurements in two rats). The signal rapidly returned to baseline (typically within 5 min) when the animal stopped grooming. Values for rCBF rose from a mean basal level of 81 ± 7 mL $(100\ g)^{-1}\ min^{-1}$ to a maximum of 112 ± 10 mL $(100\ g)^{-1}\ min^{-1}$ ($p < 0.001$). On cessation of activity values also returned to basal levels (data not shown) [25].

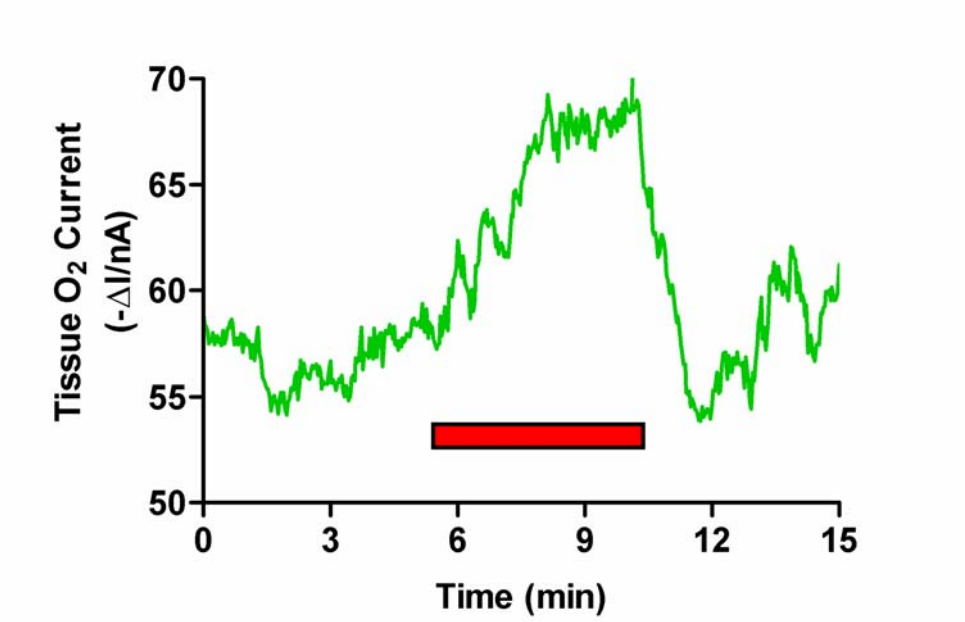

Figure 3. Typical example of the effect of induced grooming (red bar) on striatal O_2 levels monitored with an implanted carbon paste electrode and differential pulse amperometry.

The mean percentage increase in rCBF in response to tail pinch was 83 ± 13%, similar to the value of 98 ± 16% previously reported for tail pinch by Fellows and Boutelle [37]. The maximum percentage increase during induced grooming was 37 ± 4%. Whereas absolute rCBF can be calculated from the H_2 clearance values, the DPA reduction current at the CPE includes a large background signal typical of implanted CPEs. This is due to increased surface wetability resulting from removal of the insulating silicone oil by lipids, which constitute approximately 40% of the dry mass of brain tissue [19,39]. However, using *in vitro* calibrations (see *Voltammetric Techniques*) the increases in O_2 for tail pinch and grooming were converted to concentrations: 44 ± 8 µmol/L (tail pinch) and 25 ± 4 µmol/L (grooming). The O_2 concentration in the extracellular fluid (ECF) is a dynamic balance between supply of O_2 via blood flow in capillaries and O_2 consumption associated with metabolism in cells. The range measured with implanted microelectrodes is from 5 to 50 µmol/L [18,40]. Assuming a maximum ECF concentration of 50 µmol/L the above concentrations represent mean percentage increases of 88 ± 15% and 50 ± 9% for tail pinch and grooming respectively. Although O_2 utilization increases during stimulated neuronal activity, the present results confirm previous findings that the rise in rCBF exceeds the increase in O_2 utilization resulting in a net increase in O_2 [22,41,42]. For this

reason O_2 currents provide an index of increases in rCBF only when such increases exceed O_2 utilization [25].

Saline Injection and Chloral Hydrate Anaesthesia

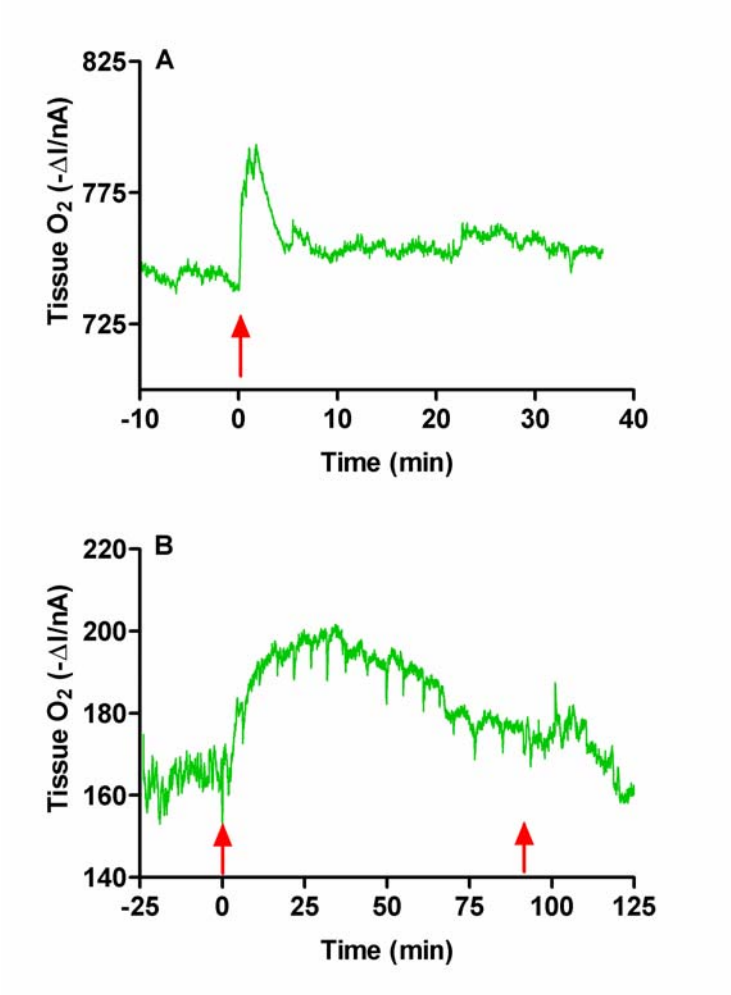

Figure 4. Typical examples of the effects of intraperitoneal injection of normal saline (**A**, 0.5 mL, NaCl 0.9%) and chloral hydrate (**B**, 350 mg/kg) on tissue O_2 monitored in rat striatum with carbon paste electrodes. Arrows indicate the points of injection (A & B), and recovery (B) from the anaesthetic.

Chloral hydrate is a commonly used non-volatile anaesthetic agent for animal surgery. It causes general CNS depression by rapidly metabolizing into trichloroethanol and trichloroacetic acid [43]. Trichloroethanol is suspected to be the active substance for inducing anaesthesia [44] because, similar to barbiturates, it has the ability to prolong chloride influx induced by exogenous GABA [45].

Since the anesthetic was administered by i.p. injection we first examined the effect of i.p. injection of normal saline (NaCl 0.9%). A typical example of changes in tissue O_2 is shown in Figure 4A. There was an immediate increase in O_2 following injection. The O_2 signal (ΔI) increased maximally by 13.5 ± 3.7 nA at 2.5 ± 0.4 min and had returned to baseline by 4.1 ± 3.7 min (n = 4). This increase in current corresponds to an increase in concentration of 50 ± 15 μM based on calculations from *in vitro* calibration curves. Similar initial and brief injection effects have also been

observed during the injection of anaesthetic agents, with a comparable return to baseline levels occurring before the effect of the anaesthetic takes place and the animal becomes anaesthetized [46]. The stress of the i.p. injection stimulates neuronal activation [47], increasing rCBF and thus O_2, with the supply of the latter exceeding utilization.

The i.p injection of chloral hydrate produced the same initial response as the i.p. saline injection; a brief increase in O_2. This was followed by slower more long-lasting changes which accompanied the behavioral changes associated with anaesthesia (*see* Figure 4B). The current (ΔI) increased to a maximum of 18.7 ± 3.1 nA ($p < 0.03$, $n = 10$) at 11.6 ± 1.9 min. This corresponds to an increase in concentration of 69 ± 9 μM. The duration of anesthesia, as measured by reflex responses (palpebral, corneal and withdrawal reflexes) and spontaneous movements of the rats, was 92 ± 6 min ($n = 10$).

Several studies have previously been carried out comparing experiments performed in conscious and anaesthetized rats indicating significant differences resulting from the effects of the different anaesthetics investigated. These have focused on dopamine, DOPAC, 5-HT and HVA and have primarily involved the use of microdialysis techniques [44,48-50], although some voltammetric studies of dopamine have been made with carbon paste [51] and carbon fiber electrodes [52]. Recently, several groups have used *in vivo* voltammetry with 'first generation' amperometric enzyme-based biosensors to monitor brain extracellular levels of several non-electroactive analytes under anaesthesia in acute experiments [53-66]. A problem which is often overlooked is the effect of anaesthesia on the levels of enzymatic mediators, which are intrinsic to the design and operation of these biosensors; oxidoreductase enzymes use molecular O_2 as a mediator to produce the signal generating H_2O_2:

$$\text{Enzyme/FAD} + \text{Substrate} \rightarrow \text{Enzyme/FADH}_2 + \text{Product}$$

$$\text{Enzyme/FADH}_2 + O_2 \rightarrow \text{Enzyme/FAD} + H_2O_2$$

where FAD and $FADH_2$ are the oxidized and reduced states of the redox active prosthetic group, flavin adenine dinucleotide. It is clear from this reaction scheme that changing O_2 levels can affect accurate substrate measurement. Thus, in developing such devices it is important to consider mediator interference as well as the direct heterogeneous interference from endogenous electroactive species such as ascorbic acid, which has been the traditional focus of interference studies. The findings communicated here suggest that caution must be exercised in extrapolating results from acute experiments to the conscious state. This is especially true for estimations of basal concentrations, as extracellular levels of enzymatic substrates [26] and mediators are clearly altered in a complex manner by anaesthesia.

The Effect of Acetazolamide

The carbonic anhydrase inhibitor acetazolamide (Diamox) administered systemically has been shown to increase brain pO_2 in the ECF [2]. It is an amide derivative belonging to the sulfonamide family and

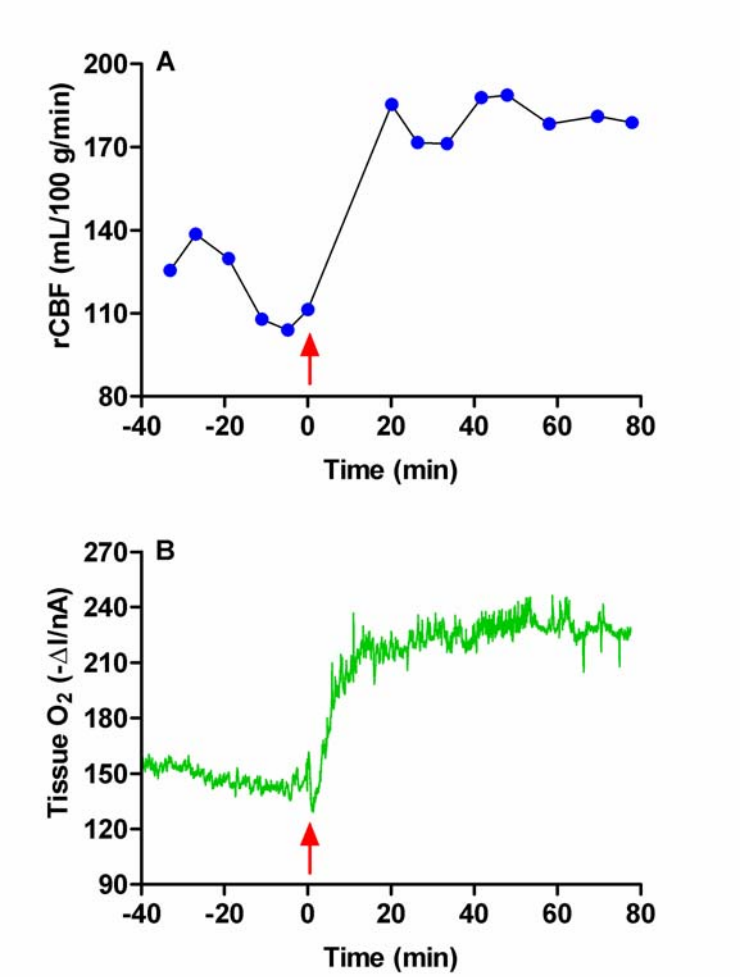

Figure 5. The effects of systemic administration of the carbonic anhydrase inhibitor acetazolamide (Diamox, 50 mg/kg, i.p.) on the tissue O_2 and blood flow (rCBF) levels monitored simultaneously with bilaterally implanted carbon paste (O_2) and Pt/Ir (H_2 clearance) electrodes in the striatum of a freely-moving rat.

and by inhibiting carbonic anhydrase reduces HCO_3^-. The resulting increase in PCO_2 is accompanied by a pH reduction which causes dilation of cerebral blood vessels. Figure 5 shows the effect of this drug (50 mg/kg, i.p.) on the O_2 signal and rCBF recorded simultaneously in rat striatum. As expected, acetazolamide increased O_2 levels in the ECF; the O_2 current (ΔI) recorded at the CPEs increased by 43 ± 10 nA. Calibration of the CPEs recovered after the experiments allowed us to estimate the increase as 40 ± 12 μM (n = 5, $p < 0.03$) 10 min after the injection, which is similar to the increase observed following mild stress [19]. Values for rCBF rose from a mean basal level of 118 ± 4 mL (100 g)$^{-1}$ min^{-1} to a maximum of 182 ± 2 mL (100 g)$^{-1}$ min^{-1} ($p < 0.001$). These results confirm that acetazolamide increases brain tissue O_2 levels through an increase in rCBF. This increase would appear to be sustained for a prolonged period after administration (see Figure 5). Such characteristics make this drug ideally suited for testing the *in vivo* O_2 sensitivity of 'first generation' amperometric enzyme-based biosensors [67].

Conclusions

Mild hypoxia and hyperoxia produced rapid changes (decrease and increase respectively) in the *in vivo* O_2 signal confirming that CPEs implanted in freely-moving animals respond rapidly to changes in cerebral tissue O_2 concentrations. Neuronal activation (tail pinch and stimulated grooming) produced similar increases in both O_2 and rCBF indicating that CPE O_2 currents provide an index of increases in rCBF when such increases exceed O_2 utilization. Saline injection produced a transient increase in the O_2 signal while chloral hydrate produced slower more long-lasting changes that accompanied the behavioral changes associated with anesthesia. Acetazolamide increased O_2 levels through an increase in rCBF. While the latter confirms that acetazolamide is ideally suited for testing the *in vivo* O_2 sensitivity of 'first generation' amperometric enzyme-based biosensors, the former suggests that caution must be exercised in extrapolating results from acute experiments to the conscious state.

Acknowledgements

We gratefully acknowledge financial support from the Health Research Board (HRB) Ireland (EQN12/2000 & RP/14/2002), Science Foundation Ireland (Investigator Programme Grant 03/IN3/B376), Enterprise Ireland (SC/2003/331E), the RSC & EPSRC UK (03/F/018), and NUI, Maynooth (New Researcher Award 2000 & Research Enhancement Fund 2003).

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