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An amperometric glucose-oxidase/poly(*o*-phenylenediamine) biosensor for monitoring brain extracellular glucose: in vivo characterisation in the striatum of freely-moving rats

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Abstract

Amperometric glucose biosensors based on the immobilization of glucose oxidase (GOx) on Pt electrodes with electropolymerized *o*-phenylenediamine (PPD) were implanted in the right striatum of freely-moving rats. Carbon paste electrodes for the simultaneous monitoring of ascorbic acid (AA) and/or tissue O_2 were implanted in the left striatum. A detailed in vivo characterization of the Pt/PPD/GOx signal was carried out using various pharmacological manipulations. Confirmation that the biosensor responded to changing glucose levels in brain extracellular fluid (ECF) was obtained by intraperitoneal (i.p.) injection of insulin that caused a decrease in the Pt/PPD/GOx current, and local administion of glucose (1 mM) via an adjacent microdialysis probe that resulted in an increase in the biosensor current. An insulin induced increase in tissue O_2 in the brain was also observed. Interference studies involved administering AA and subanaesthetic doses of ketamine i.p. Both resulted in increased extracellular AA levels with ketamine also causing an increase in O_2. No significant change in the Pt/PPD/GOx current was observed in either case indicating that changes in O_2 and AA, the principal endogenous interferents, have minimal effect on the response of these first generation biosensors. Stability tests over a successive 5-day period revealed no significant change in sensitivity. These in vivo results suggest reliable glucose monitoring in brain ECF. © 1998 Elsevier Science B.V.

Keywords: Glucose biosensor; Carbon paste electrodes; Brain extracellular glucose; Ascorbic acid; Oxygen; Ketamine; Real-time monitoring; Freely-moving rats

1. Introduction

A number of biosensors have been developed for in vivo measurement of biologically important compounds which are either non-electroactive or only poorly electroactive at analytically useful potentials (Turner et al., 1987; Pfeiffer and Kerner, 1988; Pantano and Kuhr, 1995). The most thoroughly investigated is the glucose biosensor incorporating the oxidoreductase enzyme, glucose oxidase (GOx). GOx, isolated from *Aspergillus niger*, has a high substrate specificity, a high turnover rate and excellent stability, that make it ideally suited for use in a biosensor (Wilson and Turner, 1992). This enzyme catalyses the oxidation of D-glucose in the presence of O_2 producing H_2O_2:

$$\beta - D - glucose + GOx/FAD \rightarrow D - glucono - \delta - lactone + GOx/FADH_2 \quad (1)$$

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$$GOx/FADH_2 + O_2 \rightarrow GOx/FAD + H_2O_2 \quad (2)$$

where FAD is the oxidised form of the prosthetic group, flavin adenine dinucleotide.

Since the first report of a biosensor (for glucose) by Clark and Lyons (1962) there has been extensive research on the development and potential applications of biosensors, principally for subcutaneous or intravascular monitoring of glucose (Pfeiffer and Kerner, 1988). This is because of the importance of glucose monitoring in the disease diabetes mellitus and the fact that glucose determination in various body fluids, such as blood, plasma and urine, remains one of the most common analysis carried out in clinical laboratories. However, only a few such devices have been used in vivo in neurochemical studies.With the exception of a report by Boutelle et al. (1986) these have all been carried out in anaesthetized animals and have all involved amperometric enzyme-modified electrodes, predominantly for glucose (Lowry et al., 1994a; Silver and Ereciñska, 1994; Lowry and Fillenz, 1996; Netchiporouk et al., 1996; Lowry and Fillenz, 1997), with some recent reports for glutamate (Albery et al., 1992; Hu et al., 1994; Asai et al., 1996) and choline (Garguilo and Michael, 1993; Garguilo and Michael 1994). The small number of such reports reflects the numerous difficulties associated with performing direct neurochemical measurements in such a hostile and complex environment as brain extracellular fluid (ECF). In addition to the obvious immunological response to the implanted sensor, the ECF also contains surfactants (e.g. lipids), electrode poisons (e.g. proteins) and potential electrocatalysts (e.g. glutathione and ascorbic acid), all of which may affect the performance (e.g. the stability and response) of the implanted sensor (O'Neill, 1994). Other problems include restriction of mass-transport to the electrode surface by brain tissue, the biochemical effects of depletion of a chemical species from this matrix and the availability of suitable enzymes for the specific substrates of interest. Therefore, in order for a biosensor to operate reliably in brain ECF these and other factors such as size, linear calibration range and response time must be addressed in the design and development of the sensor.

Recently, the groups of Zambonin (Malitesta et al., 1990) and Yacynych (Sasso et al., 1990) have reported H_2O_2-detecting biosensors based on the immobilisation of GOx in electropolymerized poly(phenylenediamine) films on Pt electrodes with properties (e.g. interference-free, high enzyme activity and low response time) indicating ideal suitability for detecting substrates in biological fluids. Several groups, including ourselves, have now used this technology to develop and characterise biosensors for a variety of substrates (Bartlett and Birkin, 1994; Dong, 1994; Lowry et al., 1994a; Friedemann et al., 1996). We have focused on the development of a glucose sensor for applications in the mammalian brain, as glucose is the primary energy source for the brain and a constant supply is essential to maintain normal cerebral function. A detailed in vitro characterisation has been carried out; this sensor, which utilises polymeric *o*-phenylenediamine (PPD), has a fast response time, linearity over the relevant concentration range, freedom from protein and lipid fouling and minimal interference from endogenous species such as AA and O_2 over physiologically relevant concentration ranges (Lowry et al., 1994a,b; Lowry and O'Neill, 1994).

However, before any sensor can be used reliably in vivo it is necessary to perform a detailed in vivo characterisation to ensure the properties obtained in the in vitro environment are maintained in the more complex and hostile biological milieu. Thus, in the present study we have implanted the Pt/PPD/GOx biosensor in rat striatum and characterised the response in detail in awake freely-moving animals in order to provide unequivocal proof of what is being detected.

2. Methodology

2.1. Chemicals and solutions

The enzyme glucose oxidase from *Aspergillus niger* (EC 1.1.3.4, Grade I), was obtained from Boehringer Mannheim, East Sussex, UK. The *o*-phenylenediamine (free base), L-ascorbic acid (AA; A.C.S. reagent), ketamine (hydrochloride) and insulin (from bovine pancreas) were from Sigma Chemical, Dorset, UK. D(+)-glucose (AnalaR grade) was obtained from BDH Laboratory Supplies, Poole, UK. The NaCl, KCl, NaH_2PO_4, NaOH, $MgCl_2$ (all BDH, AnalaR grade) and $CaCl_2$ (Sigma) were used as supplied. Carbon paste was prepared by thoroughly mixing 2.83 g of carbon powder (UCP-1-M, Ultra Carbon, Bay City, MI) and 1.0 ml of silicone oil (Aldrich Chemical, Dorset, UK, Catalogue No. 17563-3) (O'Neill et al., 1982).

Stock solutions of 1 M glucose were prepared, left for 24 h at room temperature to allow equilibration of the anomers and then stored at 4°C. All AA solutions were prepared just before use because of their gradual decomposition. Stock solutions of 100 mM AA were prepared for in vitro calibrations. In in vivo experiments solutions of AA (2 ml, 2 g/kg), insulin (1 ml, 15 U/kg), ketamine (1 ml, 50 mg/kg) and glucose (0.36 g in 2 ml) were administered intraperitoneally (i.p.). The pH of the AA solutions was adjusted to between 6.5 and 7.0 using NaOH. Insulin solutions were prepared in a phosphate buffered saline (PBS) solution, pH 7.4 (150 mM NaCl, 40 mM NaH_2PO_4 and 40 mM NaOH) and sonicated with heating for $\approx$5 min to ensure complete dissolution. Glucose solutions used in in vivo experi-

ments were prepared in artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$ and 1 mM $MgCl_2$) (de Boer et al., 1990) for microdialysis experiments (1 mM) and in normal saline (NaCl 0.9%) for i.p. injections (1 M). All solutions were prepared using deoxygenated doubly distilled deionised water.

2.2. Working electrode preparation

Glucose biosensors were based on the immobilization of GOx on Pt electrodes with the polymer PPD. Pt/PPD/GOx disk electrodes were prepared from Teflon-coated platinum/iridium (Pt/Ir 90%/10%) wire (125 μm bare diameter, 160 μm coated diameter (5T), Advent Research Materials, Suffolk, UK) following a previously reported procedure (Lowry et al., 1994a). Pt/PPD/GOx cylinder electrodes were made from 5 cm lengths of the same 5T wire. From each end $\approx$ 5 mm of the teflon insulation was carefully cut. The tip of the wire and the end of the teflon insulation at one end were then sealed under a microscope with epoxy glue to produce a 4 mm active length of bare wire. A gold electrical contact (Semat Technical, Herts, UK) was soldered to the other end. In order to ensure good GOx adsorption the exposed 4 mm of wire was then scored repeatedly with a scalpel blade. GOx was immobilised in PPD by potentiostatic electropolymenzation of the *o*-phenylenediamine monomer (*o*-PD, 300 mM) following a modified form of a previously reported procedure (Lowry et al., 1994a). Briefly, a deoxygenated solution of the monomer was prepared in PBS, pH 7.4. Electrodes were left standing in this *o*-PD solution for 60 min and then transferred to an enzyme solution (3 mg (850 U) GOx in 5 μl *o*-PD). When the enzyme was visibly adsorbed (ca. 5–10 min, as indicated by a yellow coating on the wire), the electrodes were transferred to a plastic electrochemical cell (in house three-electrode design) containing 10 ml of a freshly prepared *o*-PD solution. The reference (saturated calomel electrode, SCE) and auxiliary electrode (large Pt wire) were then connected and the electropolymerization procedure started by applying + 650 mV to the Pt/PPD/GOx electrodes. The polymerization was terminated after 15 min and the Pt/PPD/GOx electrodes were then rinsed by immersion in PBS to remove loosely bound enzyme and unreacted monomer. When not in use all Pt/PPD/GOx electrodes were stored in PBS at 4°C. Carbon paste disk electrodes (CPEs) were also made from 5 cm lengths of 5T teflon-coated Pt/Ir wire following a previously described procedure (Lowry et al., 1997).

2.3. Microdialysis probe construction

Concentric microdialysis probes were constructed as described previously (Demestre et al., 1997). The length of the probe was 4 mm and the external diameter of the sampling region of the probe was 300 μm. Before implantation each probe was tested for leakages by perfusing aCSF at 2 μl/min using a CMA 100 microdialysis pump (CMA Microdialysis, Stockholm, Sweden).

2.4. In vitro conditioning and characterisation of Pt/PPD/GOx electrodes

Prior to implantation all Pt/PPD/GOx electrodes were preconditioned in vitro by continuous recording (see below) in 5 mM glucose over a 10 h period. Electrodes were then calibrated in vitro for both glucose (0–100 mM) and AA (0–1 mM) to ensure selectivity and linearity of response of the sensor for glucose (Lowry and O'Neill, 1994). Calibrations were carried out in a standard three-electrode glass electrochemical cell. A SCE was used as the reference electrode and a large silver wire, isolated in a compartment containing PBS, served as the auxiliary electrode. To facilitate mixing, solutions were bubbled with air (glucose; RENA 102 air pump, RENA, France) or N_2 (AA, BOC Gases, Guildford, UK) for ca. 10 s following the addition of each aliquot. The current was then measured when the solution was quiescent. The electrodes were held at a constant potential of + 700 mV, which is the value generally used for H_2O_2 detection (Lowry et al., 1994a). All calibrations were carried out at room temperature with the exception of a series of experimental measurements done at 37°C. These were made by placing the cell on a temperature regulated hot plate ($\pm$ 2°C, Sigma) and monitoring the solution temperature with a thermometer (Sigma) fixed permanently in the solution as near as possible to the biosensor. In experiments involving microdialysis, a characterized Pt/PPD/GOx electrode was glued with epoxy to a microdialysis probe ca. 1 h prior to implantation so that the 4 mm active lengths of both probe and electrode were directly aligned. Care was taken to ensure that the probe and sensor were within 1 mm of each other.

2.5. Surgical procedures

Male Sprague-Dawley rats weighing 200–300 g were anaesthetised, following published guidelines (Wolfensohn and Lloyd, 1994), with a mixture of Hypnorm (Janssen Pharmaceuticals, Oxford, UK), Hypnovel (Roche Products, Herts, UK) and sterile water, mixed 1:1:2 and injected i.p. at a volume of 3.3 ml/kg, as described previously (Fray et al., 1996; Lowry and Fillenz, 1997). Electrodes were then implanted following a previously described procedure (Lowry et al., 1997); CPEs were implanted in the left striatum (coordinates with the skull levelled between bregma and lambda, were: A/P + 1.0 from bregma, M/L − 2.5 and D/V − 5.0 from dura (Paxinos and Watson, 1986)), Pt/PPD/GOx electrodes in the right striatum (coordi-

nates: A/P +1.0 from bregma, M/L +2.5 and D/V −8.5 from dura), and in experiments involving microdialysis the combined Pt/PPD/GOx electrode and microdialysis probe (perfused with aCSF at 2 μl/min) was implanted in the right striatum (coordinates: A/P +1.0 from bregma, M/L +2.5 and D/V −8.5 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 bare Ag wire in brain tissue is very similar to that of the SCE (O'Neill, 1993). The electrodes and probe were fixed to the skull with dental screws and dental acrylate (Associated Dental Products, Swindon, UK). Surgery typically lasted 40 min and anaesthesia was reversed by an i.p. injection of naloxone (0.1 mg/kg, Sigma). Post-operative analgesia was provided in the form of a single injection (0.1 mg/kg, s.c.) of Vetergesic (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 (Morton and Griffiths, 1985) immediately after recovery from anaesthesia and at the beginning of each day. This work was carried out under licence in accordance with the Animals (Scientific Procedures) Act, 1986.

2.6. Instrumentation and software

A low-noise potentiostat (Biostat II, Electrochemical and Medical Systems, Newbury, UK) was used in all experiments. For constant potential amperometry (CPA) experiments, either a Macintosh IIx computer or a Mitac 486 PC was used with data acquisition carried out using National Instruments (NI, National Instruments, Austin, TX) 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 one data point every 2 s. For differential pulse amperometry (DPA) experiments, all data acquisition was carried out on a Mitac 486 PC with data sampling carried out at a rate of 250 determinations/pulse and averaged to give one data point/pulse (Lowry et al., 1996). A complete pulse sequence was carried out every 2 s. All experiments were carried out using in house developed software: PC (LabWindows, NI version 2.1); Macintosh (LabVIEW, NI version 2.2.1). All analysis was carried out using both in house developed software and the commercial packages Igor Pro 2.0 (WaveMetrics, OR) and Prism (GraphPad Software, CA). All data are presented as mean ± SEM. The significance of differences observed was estimated using the Student's *t*-test (two-tailed) for paired observations (StatView, Abacus Concepts, CA), or the repeated measures ANOVA with Tukey's posthoc test.

Animal motor activity was recorded using a radar based doppler shift motion detection unit (RS8960, RS Components, Northants, UK; 10.7 GHz, wavelength 2.8 cm) connected to the Mitac PC via the NI AT-MIO-16 data acquisition board. The meter was modified with a closed end metal wave guide so that ambulatory movements, grooming, feeding and drinking registered, but not lesser movements such as breathing (Berners, 1996).

2.7. Experimental conditions

Rats were housed in large plastic bowls (diameter ca. 50 cm), in a windowless room under a 12 h light/dark cycle, lights coming on at 8 a.m., with free access to water. Food was available ad libitum except for experiments involving insulin where animals were fasted for 24 h prior to administration. All experiments were carried out with the animal in its home bowl. Implanted electrodes were connected to the potentiostat 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) at least 60 min prior to the start of the first experiment each day. This arrangement allowed free movement of the animal. Once the background current for the Pt/PPD/GOx electrode had stabilised (typically 30–45 min) experiments were begun. The O_2 and AA signal from the CPE stabilised within 5–10 min. All signals were recorded at 2 s intervals. At the end of each day the animals were disconnected from the recording equipment.

2.8. Voltammetric techniques in vivo

As with in vitro experiments, CPA at +700 mV (versus implanted Ag reference electrode) was used for H_2O_2 oxidation at the Pt/PPD/GOx electrodes in vivo. Changes in O_2 and AA at implanted CPEs were monitored using either DPA or CPA. The DPA technique allows the simultaneous monitoring of both species. For O_2 reduction, two equally sized cathodic pulses were applied, the first from a resting potential at −150 to −350 mV that corresponds to the foot of the reduction wave for O_2 at lipid-modified CPEs and the second from −350 to −550 mV that corresponds to the peak of the reduction wave. Following the same criterion, equally sized anodic pulses were then applied from −150 to +50 mV and from +50 to +250 mV for AA oxidation. 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 and AA that have been shown to be interference free (Lowry et al., 1996). In experiments involving CPA, O_2 was detected by holding the implanted CPE at

the reduction potential of −550 mV (Lowry et al., 1997). The potential for AA detection was chosen as +250 mV, which is just above the peak potential for AA oxidation but below that for the onset of the oxidation of other neurochemical species at implanted CPEs (Boutelle et al., 1989). Post in vivo calibrations for AA and O_2 were carried out using the two techniques. Calibrations for dissolved O_2 were carried out in N_2 purged, air-saturated and O_2-saturated solutions where the concentrations of solution O_2 were taken as 0, 200 (Bourdillon et al., 1982; Zhang and Wilson, 1993) and 1250 μM (Bourdillon et al., 1982), respectively. AA calibrations were carried out in the range 0–1 mM.

3. Results and discussion

3.1. Effect of temperature on biosensor response

Temperature-dependence studies at GOx-modified electrodes in phosphate buffer have indicated that the glucose response may increase by between 20 (Shaolin et al., 1991) and 60% (Fortier et al., 1990) in the range 25–37°C. Thus, before performing experiments in vivo we first examined the effect of temperature on the Pt/PPD/GOx signal since all our previous in vitro experiments had been carried out at a thermostatically controlled temperature of 25.0 ± 0.5°C (Lowry et al., 1994a; Lowry and O'Neill, 1994). Glucose calibrations were therefore carried out at room (22.5 ± 0.2°C) and near physiological (37.6 ± 0.2°C) temperatures in the range 0–100 mM. The V_{max}, calculated using a modified Michaelis-Menten (Hill-type) equation (Lowry et al., 1994a), 100 mM glucose and 1 mM glucose responses were similar at both temperatures ($n = 3$): V_{max}, 127 ± 32 nA (22°C) and 127 ± 37 nA (37°C; $p > 0.97$); 100 mM, 122 ± 30 nA (22°C) and 117 ± 32 nA (37°C; $p > 0.50$); 1 mM, 4.4 ± 1.8 nA (22°C) and 6.7 ± 0.6 nA (37°C; $p > 0.42$); indicating that the change in temperature from in vitro to in vivo conditions will have minimal effect on the response of the biosensor. This property may be due to the nature of the PPD film which is a highly insulating thin (ca. 10 nm) polymer immobilising the GOx (diameter 8.6 nm) close to the electrode surface and acting as an efficient diffiusion barrier preventing glucose penetrating the film but not preventing it reaching the active site and producing H_2O_2 (Sasso et al., 1990). The detection limit, defined as the analyte (glucose) concentration yielding a signal equal to three times the standard deviation of the background current, for these 4 mm cylinder biosensors was calculated as 3.53 ± 0.48 μM ($n = 4$).

3.2. Effect of insulin on biosensor response in vivo

In order to demonstrate that Pt/PPD/GOx sensors respond to changes in glucose in brain ECF we began the in vivo characterization by examining the effect of insulin on the in vivo response. Insulin, which lowers plasma glucose levels (Chen and Steger, 1993) and has also been reported to lower brain ECF levels (Bhattacharya and Saraswati 1991), was injected i.p. (15 U/kg) into freely-moving fasted rats implanted with a glucose biosensor in the right striatum and a CPE in the left striatum. The mean baseline glucose current decreased by 0.6 ± 0.1 nA ($n = 4$; $p < 0.01$) 41 ± 11 min after injection, representing a decrease in response of 14 ± 4% (Fig. 1, top). This period of minimum glucose current lasted approximately 22 ± 10 min during which the animals were inactive but responsive to external stimuli suggesting that this dose of insulin did not produce hypoglycaemic coma. There was then a gradual return to baseline values or above during which the animals ingested food and resumed activity.

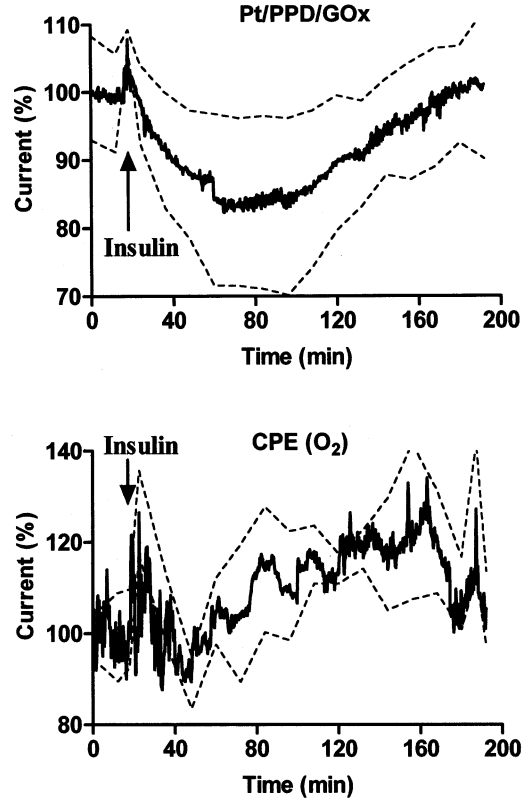

Fig. 1. The effect of intraperitoneal injection of insulin (1 ml, 15 U/kg) on the Pt/PPD/GOx (top) and carbon paste electrode (CPE) O_2 (bottom) signals recorded simultaneously with bilaterally implanted sensors in the striatum of freely-moving rats. The tissue O_2 levels at the CPEs were monitored using both differential pulse amperometry and constant potential amperometry. Data ($n = 4$) are normalised with the basal level before injection taken as 100%. The hashed lines represent the SEM which is plotted at 12 min intervals for clarity. Changes in response expressed in nanoamperes of current are given in the text. See Section 3.2.

Tissue O_2 levels were recorded simultaneously at the implanted CPEs using both DPA and CPA. Injection of insulin caused a maximum increase in cathodic current of 18 ± 7 nA ($n = 4$; $p < 0.05$) above baseline at 63 ± 22 min. We have previously shown that both these techniques can be used to monitor changes in tissue O_2 in vivo during neuronal activation (Lowry et al., 1997). However, typical baseline currents observed in vivo for the DPA and CPA O_2 signals are -748 ± 18 nA ($n = 3$) and -49 ± 4 nA ($n = 3$), respectively. It is thus not possible to calculate a percentage change in O_2 concentration from the in vivo signal. However, post in vivo calibrations of CPEs indicate that despite such background currents there is no significant difference ($p > 0.95$, $n = 5$) in the sensitivity for O_2 using the two techniques: -378 ± 136, $n = 5$ (DPA) and -371 ± 100, $n = 5$ (CPA). Therefore, using these calibrations the increase in O_2 observed with insulin corresponds to a change in concentration of 68 ± 23 μM, $n = 4$ (Fig. 1, bottom), which if one assumes a basal ECF concentration of 50 μM (Zimmerman and Wightman, 1991) corresponds to an approximate doubling of the ECF level.

These results suggest that Pt/PPD/GOx sensors respond to changing glucose levels in the ECF. A fall in brain glucose in animals given insulin has previously been reported by several groups using GOx-modified microelectrochemical sensors. Boutelle et al. (1986) observed a similar decrease of $\approx 10\%$ in awake freely-moving animals with the same dose of insulin. Silver and Ereciñska (1994) and Netchiporouk et al. (1996) however both observed decreases of ≈ 80–90% of control. This difference may be due to a combination of factors including the use of higher doses of insulin (25 and 40 U/kg, respectively) and the fact that both these reports involved acute experiments on anaesthetized animals where a decrease in the extracellular concentration of glucose would be expected (Fillenz and Lowry, 1997). An increase in brain O_2 levels accompanying an insulin induced fall in brain glucose has also been reported by Silver and Ereciñska (1994). The different time courses of changes in glucose and oxygen observed with insulin suggest that changing O_2 levels in vivo do not influence the Pt/PPD/GOx signal supporting our previously published in vitro work on O_2 interference (Lowry et al., 1994b). A more detailed investigation of this type of interference is presented below (see Section 3.4).

3.3. Effect of glucose on biosensor response in vivo

Further evidence that Pt/PPD/GOx sensors respond to glucose changes in the ECF may be obtained by increasing the extracellular concentration of glucose. This has previously been achieved using intraperitoneal injections of glucose (Silver and Ereciñska, 1994; Netchiporouk et al., 1996) or the hormone glucagon (Netchiporouk et al., 1996), which counterbalances the action of insulin in that it regulates the concentration of blood glucose by increasing the break down of glycogen stores (Stryer, 1988). However, the effect of glucagon was found to produce only a small rise in ECF glucose before triggering insulin release and a subsequent fall in glucose to below basal levels (Netchiporouk et al., 1996).

We examined the effect of injecting glucose (0.36 g in 2 ml i.p.) on the in vivo response of the implanted biosensor in awake normoglycemic animals and found no significant difference ($p > 0.46$, $n = 3$) in the signal before (12.5 ± 1.1 nA, $n = 3$) and after (12.8 ± 0.7 nA, $n = 3$) injection. This is contrary to previous reports where injection of similar doses resulted in increased glucose levels (Silver and Ereciñska, 1994; Netchiporouk et al., 1996). However, the animals used in those experiments may have been more sensitive to hyperglycemia since one of the reports involved injections during insulin induced hypoglycemia (Netchiporouk et al., 1996) and both involved anaesthetics which lower ECF glucose (Fillenz and Lowry, 1997). In the only other report involving awake freely-moving animals glucose was administered by infiusion through a cannula placed next to the implanted sensor (Boutelle et al., 1986) because i.p. injections failed to produce an increase in the extracellular concentration (Boutelle, M.G., personal communication). This and our similar observation, agree with results where elevation of plasma glucose levels from 8 to 50 mM resulted in only modest increases in CSF glucose, suggesting control and carrier saturation (DiMattio and Streitman, 1988). Therefore, in order to increase ECF glucose levels in the local environment of the Pt/PPD/GOx sensor a microdialysis probe was combined with the biosensor before implantation into the striatum. The reason for this was two-fold in that not only does it enable direct administration of glucose in the present experiments it will also allow local infusion of drugs (e.g. propranolol (Fray et al., 1996)) for future studies of brain energy metabolism. Perfusion of a 1 mM glucose solution through the dialysis probe at a flow rate of 2 μl/min produced a maximum increase in current of 2.3 ± 0.6 nA ($n = 5$; $p < 0.02$) from a mean baseline of 17.0 ± 3.7 nA. This represents a mean increase of $14 \pm 3\%$ and provides corroborating evidence that Pt/PPD/GOx sensors respond to changing glucose levels in the ECF. A typical example of the effect of such a perfusion on the current from an adjacent Pt/PPD/GOx sensor is shown in Fig. 2.

3.4. Effect of oxygen on biosensor response in vivo

Pt/PPD/GOx sensors are 'first generation' devices since they detect glucose by oxidising H_2O_2 formed

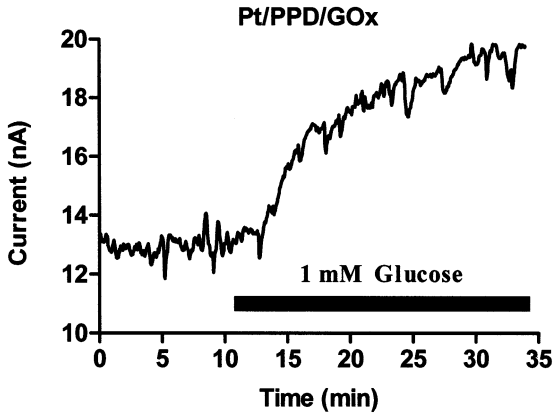

Fig. 2. A typical example of the effect of perfusing glucose (1 mM in aCSF) through a microdialysis probe on the current recorded with an adjacent Pt/PPD/GOx sensor. The combined probe and sensor were implanted in the right striatum of a freely-moving rat. The dark bar represents the period of perfusion. See Section 3.3.

from the reaction of O_2, the natural cosubstrate for GOx, with the reduced form of the enzyme (GOx/$FADH_2$) which is generated by reaction with glucose (see reactions 1 and 2 above). We have previously demonstrated in vitro that these biosensors respond to glucose in O_2-depleted (N_2-saturated) solutions with a sensitivity similar to that of air-saturated media (200 μM O_2) (Bourdillon et al., 1982; Zhang and Wilson, 1993), suggesting that in brain tissue where the range of O_2 tension is 5–50 μM (Feng et al., 1988; Zimmerman and Wightman, 1991), glucose monitoring should be free of O_2 interference (Lowry et al., 1994b).

To test the effect of changing O_2 levels on the response of the glucose biosensor in vivo we simultaneously recorded both Pt/PPD/GOx and CPE O_2 signals before and after administration of the anaesthetic ketamine. Ketamine is a non-competitive antagonist of the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor (Jantzen, 1994; Lahti et al., 1995) which causes an initial decrease in striatal tissue O_2 and regional cerebral blood flow (rCBF), followed, on recovery, by an increase in both variables to levels above basal, which is accompanied by a period of intense motor activity predominated by stereotypic head movements (Lowry, J.P., unpublished observations). A typical example of the effect of a subanaesthetic ketamine injection (50 mg/kg) on the Pt/PPD/GOx and O_2 (CPE) signals recorded simultaneously in the striatum of a freely-moving rat is shown in Fig. 3, together with a record of the motor activity for the same period. In three experiments the average O_2 current recorded using DPA increased to a maximum of 79 ± 18 nA ($n = 3$, $p < 0.05$) above baseline 3.2 ± 0.8 min following injection and was still elevated at 42 ± 6 min (77 ± 25 nA), whereas there was no significant change in the response of the glucose biosensor over the same period: 4.7 ± 0.3 nA (baseline), 4.7 ± 0.3 nA (3.2 ± 0.8 min; $p > 0.6$) and 4.5 ± 0.2 nA (42 ± 6 min; $p > 0.7$). These results support our previous in vitro O_2 interference studies (Lowry et al., 1994b) and confirm that Pt/PPD/GOx sensors are not affected by changes in O_2 over physiologically relevant concentration ranges for the brain. A similar lack of O_2 interference has also been observed by other groups; Asai et al. (1996) have used glutamate oxidase and a dialysis electrode incorporating a PPD coated Pt wire to monitor brain extracellular glutamate during acute ischemia and found that the sensor operates reliably despite the lack of O_2 around the dialysis electrode and Garguilo and Michael (1994) have found that the availability of O_2 in brain ECF is sufficient for

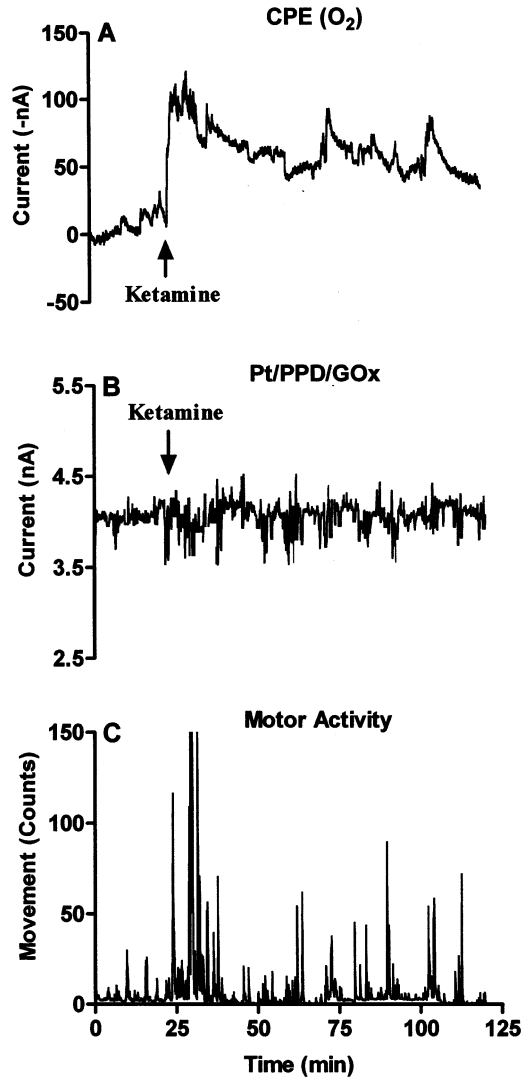

Fig. 3. An example of the effect of a subanaesthetic intraperitoneal injection of ketamine (50 mg/kg) on the carbon paste electrode (CPE) O_2 (A) and Pt/PPD/GOx (B) signals recorded simultaneously in the striatum of freely-moving rats with bilaterally implanted sensors. The tissue O_2 was monitored using differential pulse amperometry and the background current has been subtracted. Also shown is a record of the motor activity (C) recorded over the same period with a radar-based doppler shift motion detector. See Section 3.4.

reliable operation of choline sensors based on the immobilisation of choline oxidase onto carbon fiber microelectrodes.

3.5. Effect of ascorbic acid on biosensor response in vivo

The principal endogenous electroactive interferent affecting the application of electrochemical sensors in brain ECF is the reducing agent AA, which exists in anionic form at physiological pH (Youngblood, 1989) and whose extracellular concentration has been estimated at $\approx 500\ \mu$M (Miele and Fillenz, 1996). In order to determine whether AA in brain ECF would interfere with glucose detection using a Pt/PPD/GOx sensor we simultaneously monitored AA concentration in the left striatum with a CPE at a constant potential of $+250$ mV and the Pt/PPD/GOx current in the right striatum following injection of a high dose (2 g/kg, i.p.) of AA. The AA current increased from a mean baseline value of 2.4 ± 0.7 to 5.2 ± 1.0 nA ($n = 5$; $p < 0.04$) 11 ± 3 min after injection. Since techniques such as CPA which have long sampling times cause a significant depletion of extracellular AA (Rice and Nicholson, 1995), it is not possible to quantify the latter increase in current in terms of concentration, however, this increase (for i.p. injection) is substantially greater than the increases observed using CPA during normal and stimulated physiological activity (Boutelle et al., 1989). There was no overall change in the response of the Pt/PPD/GOx sensors during the same period: 3.1 ± 0.9 nA to 3.0 ± 0.9 ($n = 5$; $p > 0.25$), clearly demonstrating that the observed glucose signal is not affected by changes in AA in vivo, even those greater than observed during behavioural activation (Fig. 4).

We also monitored extracellular AA during the injection of subanaesthetic doses of ketamine using the DPA technique, which, in addition to detecting changes in O_2, also enables simultaneous monitoring of AA in vivo (Lowry et al., 1996). Injection of 50 mg/kg ketamine i.p., which produced no change in the Pt/PPD/GOx signal (see above), caused a maximum increase in AA of 6.8 ± 1.0 nA ($n = 3$; $p < 0.19$) above baseline at 8.4 ± 3.5 min following injection (see inset Fig. 4). Using post in vivo calibrations this represents an increase in AA concentration of $164 \pm 23\ \mu$M representing an $\approx 30\%$ increase in ECF levels assuming the DPA technique causes minimum depletion as it is a fast sampling technique (Lowry et al., 1997).

3.6. Stability of biosensor in vivo

The direct contact of biosensors with biological samples can lead to a decrease in sensitivity due to surface fouling by proteins and other biomolecules. This decrease in sensitivity generally varies between 20 and

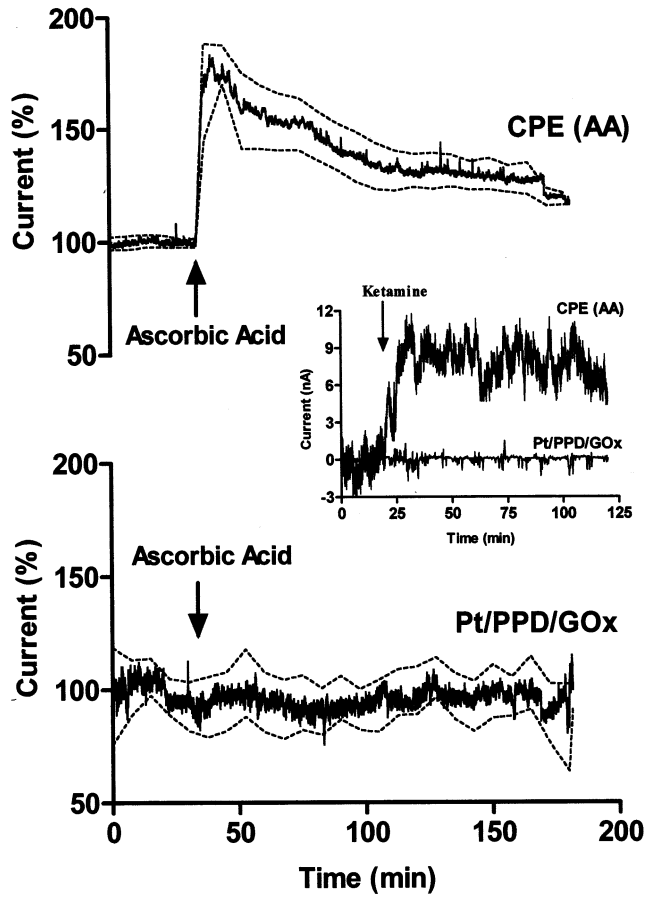

Fig. 4. The effect of intraperitoneal injection of ascorbic acid (AA; 2 ml, 2 g/kg) on the amperometric response of carbon paste (CPE; top) and Pt/PPD/GOx (bottom) sensors implanted bilaterally in the striatum of freely-moving rats. The CPEs monitored AA at a constant potential of $+250$ mV. Data ($n = 5$) are normalised with the basal level before injection taken as 100%. The hashed lines represent the SEM which is plotted at 8 min intervals for clarity. Changes in response expressed in nanoamperes of current are given in the text. Inset: Example of the effect of a subanaesthetic injection of ketamine (50 mg/kg, i.p.) on the CPE AA and Pt/PPD/GOx signals recorded simultaneously in rat striatum. AA was monitored using differential pulse amperometry. The baseline currents for both signals have been subtracted. See Section 3.5.

50% and occurs over several hours exposure to brain tissue (Garguilo and Michael, 1994; Hu et al., 1994). Although previous in vitro studies have shown that Pt/PPD/GOx sensors are virtually free from both protein and lipid fouling (Lowry and O'Neill, 1994) we carried out an additional in vitro conditioning of the sensors prior to implantation (see Section 2.4), similar to the preconditioning procedure used by Hill and coworkers to improve the stability of ferrocene-mediated glucose biosensors (Cass et al., 1984). The mean baseline current recorded with preconditioned Pt/PPD/GOx sensors implanted in the striatum of freely-moving animals showed no significant variation ($p > 0.88$, two-way ANOVA; $n = 6$) over a successive 5 day period from day 0 (ca. 1 h following recovery from surgical

anaesthesia) to day 4: 15.6 ± 3.5 nA (day 0); 16.2 ± 2.2 nA (day 1); 16.1 ± 3.6 nA (day 2); 14.0 ± 2.7 nA (day 3) and 13.2 ± 2.4 nA (day 4). This represents a statistically insignificant change in sensitivity after 5 days recording and clearly demonstrates that the combination of non-fouling properties of the PPD coating and in vitro conditioning of the enzyme and Pt surface (via prolonged enzymatic turnover and H_2O_2 oxidation) ensures minimal loss of sensitivity of these biosensors after implantation and a resultant stable glucose signal in vivo. Using glucose calibration data suggests that this baseline level corresponds to a concentration of 488 ± 84 μM ($n = 6$).

4. Conclusions

These results demonstrate that our Pt/PPD/GOx sensor responds to changing concentrations of glucose in the extracellular compartment of the brain, is unaffected by changes in the concentration of both O_2 and AA and is stable after implantation, clearly demonstrating reliable glucose monitoring in vivo in freely-moving animals.

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