Communications

Behaviourally induced changes in extracellular levels of brain glutamate monitored at 1 s resolution with an implanted biosensor

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A biosensor for L-glutamic acid (Glu), constructed by immobilisation of L-glutamate oxidase onto lipid-coated 60 μm radius Pt wire with poly(*o*-phenylenediamine), was used amperometrically in attempts to detect glutamate in brain extracellular fluid (ECF) in the awake, freely moving rat at 1 s intervals. An implanted carbon paste electrode served to monitor simultaneous changes in brain ascorbate (AA). There were spontaneous, parallel changes in both signals, depending on the behavioural state of the animal, giving rise to a significant positive correlation coefficient between the two variables. The application of a mild stressor (10 s tail pinch) caused an immediate increase in both signals. The effects of inhibiting energy metabolism, together with an existing hypothesis linking Glu and AA in the ECF, were used to conclude that AA does not interfere electrochemically with the biosensor *in vivo*, that there are parallel changes in extracellular Glu and AA concentrations under normal physiological conditions, and that sub-micromolar physiologically related changes in brain Glu could be detected in the ECF several days after implantation of the electrodes.

L-Glutamate (Glu) in brain extracellular fluid (ECF) has been the focus of much analytical interest recently with the development of a variety of new approaches to its detection and quantification.[1–6] The fascination with brain Glu is well justified; this amino acid is the most widespread intercellular chemical messenger (neurotransmitter) within the mammalian central nervous system, playing a major role in a wide range of brain functions, and has been implicated in a number of brain disorders.[7]

Many of these new techniques rely on the use of permselective dialysis membranes, either to collect perfusate,[5] convey perfusate to an on-line analyser[4,6] or to house a sensing electrode.[1,3] While time resolutions of the order of 1 min have been reported for dialysis-based techniques,[4,6] either the need to collect a minimum volume for analysis, dead volume in the flow system or the diffusion of analyte to the internal sensing electrode limits the time resolution of these approaches.

We recently reported the development of a sensitive and selective amperometric biosensor based on glutamate oxidase (GluOx) immobilised onto Pt wire with electrosynthesised non-conducting polymer and designed for detection of Glu directly in brain ECF.[8] The problem of interference by endogenous electroactive reducing agents, especially ascorbic acid (AA), was resolved by electrosynthesising poly(*o*-phenylenediamine) (PPD) onto a lipid layer containing adsorbed enzyme. Although these Pt/PPD/GluOx sensors showed excellent sensitivity and selectivity *in vitro*,[8] it remained to be seen whether they would have sufficient sensitivity, selectivity and stability *in vivo* to detect brain Glu. We report here preliminary data that suggest that Pt/PPD/GluOx electrodes are able to monitor small (sub-micromolar) physiologically-induced changes in ECF Glu levels at 1 s intervals, even several days after sensor implantation.

Materials and methods

Reagents and solutions

The enzyme L-glutamate oxidase (GluOx from *Streptomyces* sp. X-119-6, EC 1.4.3.11, 200 U ml^{-1} in 20 mmol l^{-1} potassium phosphate buffer, pH 7.4) was obtained as a generous gift from Yamasa, Chiba, Japan and stored at −20 °C. The lipid phosphatidylethanolamine (PEA, type II-S) and bovine serum albumin (BSA, fraction V) were obtained from Sigma Chemical (St. Louis, MO, USA). All chemicals, including *o*-phenylenediamine (PD, Sigma), L-glutamic acid (Glu, Sigma) and L-ascorbic acid (AA, Aldrich, Milwaukee, WI, USA), were used as supplied.

Solutions of PD monomer (300 mmol l^{-1}) were made up in 10 ml of a phosphate-buffered saline solution (PBS). Stock solutions of 100 mmol l^{-1} Glu and AA were prepared in doubly distilled water and 0.01 mol l^{-1} HCl, respectively. Experiments *in vitro* were carried out in PBS (pH 7.4) that consisted of 0.15 mol l^{-1} NaCl (Merck, Poole, UK), 0.04 mol l^{-1} NaH_2PO_4 (Merck) and 0.04 mol l^{-1} NaOH (Merck). Solutions were kept refrigerated at 4 °C when not in use.

Instrumentation and software

Experiments were microcomputer controlled with in-house software as described before.[9] Although raw data was sampled at 1000 Hz, the program, as constructed at present for on-line screen plots over several hours, was constrained to a time resolution of 1 s for recorded data. The linear and non-linear regression analyses were performed using the graphical software package Prism (GraphPad Software, San Diego, CA, USA). Calibration experiments were carried out using a standard three-electrode set-up with a saturated calomel electrode (SCE) as the reference and a silver wire in a glass sheath as the auxiliary electrode.

Preparation of the working electrodes

The biosensors, based on Pt cylinders (60 μm radius), were prepared as described recently[8] by cutting strips of Teflon-coated platinum wire, sliding the Teflon along the wire to expose about 1 mm of metal. The exposed metal was coated with PEA (to enhance AA blocking properties) before being dip coated with GluOx. The enzyme-coated wire was introduced into PBS containing the monomer (300 mmol l^{-1} PD) and 5 mg ml^{-1} of the non-enzyme protein BSA,[10] and electropolymerisation carried out at 0.7 V *vs*. SCE for 15 min.

Calibrations were performed amperometrically at 0.7 V *vs.* SCE in quiescent PBS for Glu (0–10 μmol l^{-1}) and AA (0–1 mmol l^{-1}) before implantation of the electrodes. Calibrations of the biosensor after several days of implantation were not possible because of disruption of the polymer–enzyme coating

upon removal from the tissue; *in vitro* and *in vivo* data indicate, however, that neither lipids,[9] proteins[11] nor prolonged exposure to brain tissue[9,12] have an appreciable effect on PPD/enzyme-based electrodes.

Carbon paste electrodes (CPEs) for the detection of AA were made from Teflon-coated silver wire (125 μm internal diameter, 160 μm external diameter, Clark Electromedical Instruments, Reading, UK) as described previously.[9]

Electrode implantation

Either one Pt/PPD/GluOx sensor and one CPE, or two CPEs, were implanted bilaterally in the striatum of male Sprague Dawley rats (about 250 g initial weight) as described in detail previously.[9,12] The co-ordinates, with the head level between bregma and lambda, were AP 1.0, L 2.5 (from bregma) and DV 5.4 (from skull) for the Pt/PPD/GluOx sensor and DV 4.8 for CPEs. Silver wires (200 μm) placed in the cortex served as reference and auxiliary electrodes. The animals were placed in their individual recording environments immediately after surgery and allowed to recover overnight before recording commenced. Amperometric data were recorded simultaneously with the CPE at 250 mV (peak potential for AA *in vivo*[9]) and with the Glu biosensor at 700 mV continuously over several days.

Results and discussion

The signals recorded from the Glu biosensor and CPE in striatum of the awake, freely moving rat showed spontaneous changes that were correlated with behaviour: when the rat was displaying activities, such as locomotion, grooming or feeding, both signals increased relative to the resting state. There are two possible explanations for the high correlation: both electrodes detect AA *in vivo*; or the biosensor detects Glu, but there are parallel changes in Glu and AA concentrations in the ECF as predicted by the Glu–AA heteroexchange hypothesis.[13] This model suggests that, when Glu is taken up from the ECF into a cell (after its release from neurones, for example), AA is released into the ECF by an energy-dependent carrier-mediated exchange of Glu and AA.[13]

Energy (glucose) metabolism was therefore disrupted by the administration of insulin to distinguish between these two possibilities. In rats implanted with two CPEs to detect striatal AA bilaterally, insulin caused a parallel decrease in the electrochemical AA signal on the two sides of the brain. In other experiments involving one Glu sensor and one CPE in each striatum, insulin caused opposite changes in the two signals (see Table 1). The effects of insulin are consistent with a breakdown in the Glu uptake system and indicate that the parallel changes in the biosensor and AA signals observed under normal physiological conditions are not due to electrochemical interference by AA with the biosensor, but to parallel changes in the concentration of the two neurochemicals in the ECF.[13]

Although AA is the main interferent in brain ECF, where its concentration is about 500 μmol l^{-1}, there are other possible sources of interference such as dopamine (DA) and its metabolite DOPAC.[14] Because this is a 'first generation' biosensor detecting H_2O_2, changes in pO_2 might also be expected to affect the sensor, although recent studies *in vitro*[15] and *in vivo*[12] have indicated that PPD/enzyme sensors are insensitive to oxygen over the physiological range of concentrations. The results from a pilot study (Table 1) on a range of drugs that affect both Glu and possible interference substances in the ECF suggest that the biosensor detects Glu without contamination by interference under these conditions.

To determine the response time of the biosensor *in vivo*, we used a mild stressor: a 10 s tail pinch applied with a forceps. Fig. 1 shows the time course at 1 s intervals for both the biosensor and AA signals. There was an immediate increase in the biosensor signal that reached a plateau by 3 s; this corresponded to an estimated change in extracellular Glu of about 0.5 μmol l^{-1}. There was a spike when the forceps was removed, followed by a fast decrease. The changes in the AA signal recorded with the contralateral CPE were in the same direction, although slightly more sluggish than the changes detected with the biosensor.

We suggest that Fig. 1 provides additional evidence (see Table 1) that the biosensor detected ECF Glu and was not affected by AA. The residual response of the biosensor to AA *in vitro* is very small (80 nA cm^{-2} $mmol^{-1}$ l) and slow with a $t_{95\%}$ of about 1 min.[8] The finding, therefore, that the biosensor signal changed more rapidly in response to tail pinch than the AA signal (recorded with the CPE) indicates that the changes recorded with the biosensor are unlikely to be due to AA. Furthermore, we reported that the response time for Glu at the biosensor was less than the mixing time (about 10 s) in the batch analysis set-up *in vitro*.[8] The data in Fig. 1 suggest that the inherent response time to substrate is of the order of 1 s as reported for other PPD-based biosensors *in vitro*.[16]

Conclusions

The preliminary results reported here suggest that Pt/PPD/GluOx biosensors are sensitive, selective and stable enough to detect physiologically induced sub-micromolar changes in the concentration of Glu in brain ECF at 1 s time resolution several days after sensor implantation, despite changes in extracellular

Table 1 Qualitative effects of systemic administration of a number of pharmacological agents on the signal recorded in rat striatum with a Pt/PPD/GluOx sensor and literature effects on both extracellular glutamate and possible interference compounds found in the ECF

Drug	Pt/PPD/GluOx signal	Effect on ECF glutamate	Interferent effect
Insulin (15 U kg^{-1} i.p.)	↑	↑ [20]	AA ↓ *
Apomorphine (2 mg kg^{-1} i.p.)	↑	↑ [21]	DOPAC ↓ [22] DA ↓ [22]
Chloral hydrate (400 mg kg^{-1} i.p.)	↓	↓ †	DOPAC ↑ [23] DA ↑ [23] O_2 ↑ [25]

* Present results. † Data not available for chloral hydrate, but several studies suggest that other general anaesthetics decrease Glu in brain ECF.[19,24]

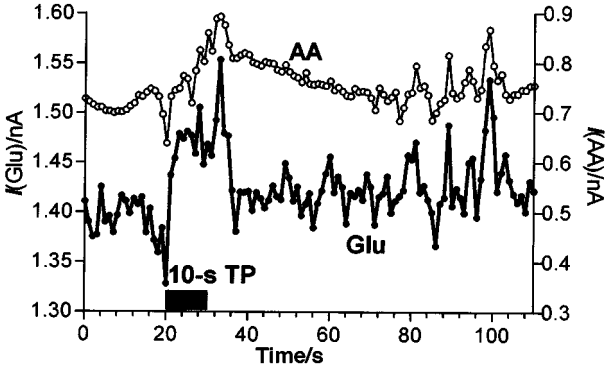

Fig. 1 Example of amperometric responses, recorded at 1 s intervals with a Pt/PPD/GluOx biosensor (Glu) and CPE (AA) placed bilaterally in rat striatum, 6 days after implantation. The period of application of a mild stressor (10 s tail pinch) is indicated by the black box. Corresponding changes in ECF Glu and AA are predicted in the Glu–AA heteroexchange model.[13]

AA concentration. This represents significant progress in the development and application of glutamate biosensors in brain systems. Reports of such applications are rare and have tended to be on acutely implanted (a few hours) electrodes in anaesthetised preparations where non-physiological stimulation, such as elevated K^+ or electrical stimulation, led to large changes in extracellular Glu.[17]

Further interference experiments, similar to the ones reported, need to be carried out with a wider range of drugs and analysed quantitatively to substantiate our conclusions. It also remains to be seen whether sub-second resolution can be achieved by reconfiguring the software, and whether ECF Glu originates from synaptic neurotransmitter release or from a metabolic pool of amino acids.[18,19]

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