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Monitoring excitatory amino acid release *in vivo* by microdialysis with capillary electrophoresis–electrochemistry

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ABSTRACT

Capillary electrophoresis (CE) with electrochemical detection (ED) was used to determine extracellular levels of aspartate, glutamate and alanine in samples from the frontoparietal cortex of the rat which were obtained by microdialysis. The method was used to monitor the effect on the overflow of the excitatory amino acids aspartate and glutamate of an influx of high concentrations of potassium ion. Samples were derivatized with naphthalenedialdehyde–cyanide prior to analysis. Detection limits for aspartate and glutamate were 80 and 100 n*M*, respectively. CE–ED is extremely useful for the analysis of microdialysis samples because of the very small sample volumes required by this analytical technique. The use of ED provides the requisite sensitivity and allows verification of peak purity by voltammetry.

INTRODUCTION

Electrochemical detection (ED) has been shown to be a sensitive method for use with capillary electrophoresis (CE) [1–3]. The combination of an extremely high-resolution separation technique with a high-sensitivity detector results in a powerful analytical tool. The small sample volume requirement of CE is an added advantage when coupled to microdialysis sampling, in which collected volumes are typically a few microliters. Such volume-limited samples and the ability to easily reduce the volume to achieve greater temporal resolution using CE should prove to be an ideal combination of both techniques.

Microdialysis, which has recently been reviewed [4,5], is accomplished by implantation of a small probe into the tissue of interest. The probe, composed of a short length of dialysis tubing, is perfused with an isotonic saline solution. The use of a precisely controlled flow-rate allows chemicals to be predictably removed from or introduced into the extracellular space by establishment of a steady-state flux across the membrane wall. This type of sampling is advantageous over conventional methods for several reasons. First, continuous sampling can be achieved without fluid loss. Second, samples collected are relatively clean, and thus amenable to direct injection without the necessity of sample cleanup procedures prior to analysis. Furthermore,

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post-sampling enzymatic reactions are eliminated since enzymes are prevented from crossing the dialysis membrane.

Many analytes of biological interest, such as amino acids, lack properties for direct determination at physiologically relevant levels. To circumvent this limitation, chemical derivatization is generally employed to enhance detection sensitivity. Naphthalene-2,3-carboxaldehyde (NDA) reacts with primary amines in the presence of cyanide to produce cyano[*f*]benzoisoindole (CBI) derivatives which are both fluorescent and electroactive [6,7]. The analysis of NDA-labeled amino acids by liquid chromatography (LC) with ED has been reported previously [7,8]. We have recently developed a procedure for the determination of the major NDA-labeled amino acids involved in neurotransmission using CE with UV detection [9]. The detection limits obtained with this technique were sufficient for the analysis of a brain homogenate; however, a more sensitive technique is required for the detection of the levels of amino acids found in brain tissue.

In this study, the combination of microdialysis sampling and CE–ED is demonstrated for the continuous monitoring of amino acids in the brain. The ability of the probe to sample a tissue as well as to deliver a test compound to the tissue is shown by the K^+-induced stimulation of excitatory amino acid release.

EXPERIMENTAL

Reagents

All amino acids were purchased from Sigma (St. Louis, MO, USA) and used as received. NDA was supplied by Oread Labs. (Lawrence, KS, USA). Sodium cyanide and sodium borate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Solutions were prepared in Nanopure water (Sybron-Barnstead, Boston, MA, USA) and passed through a membrane filter (0.2 μm pore size) before use.

Stock solutions

A 10 m*M* sodium borate solution, amino acid stock solutions (5 m*M*) and potassium cyanide (10 m*M*) were all prepared in Nanopure water. Stock solutions of NDA (5 m*M*) were prepared in acetonitrile. The Ringer's solution consisted of 147 m*M* NaCl, 4.0 m*M* KCl and 2.3 m*M* $CaCl_2$. For potassium-evoked overflow, the perfusate was changed to 51 m*M* NaCl, 2.3 m*M* $CaCl_2$ and 100 m*M* KCl.

Capillary electrophoresis system

The CE–ED system has been described previously [1]. Briefly, the electrochemical detector is isolated from the applied electrical field using a Nafion joint on the capillary column. This joint is positioned in the cathodic buffer resevoir and permits ion movement but not bulk electrolyte flow. This allows ED to be achieved without adverse effects from the electrical field. Detection was performed in the amperometric mode using a 33-μm-diameter carbon fiber microelectrode with an exposed length of 200–250 μm which was inserted into the end of the capillary column. A three-electrode cell configuration was used with the electrode connections made to a BAS LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA). The low currents generated at the microelectrode necessitated the use of a Faraday cage to shield the electrochemical cell from external electrical noise. Electrochemical pretreatment of the microelectrode was accomplished using a function generator (Exact Electronics, Hillsboro, OR, USA) connected to the external input of the BAS LC-4C. An oscilloscope was used to monitor the applied waveform, which involved the application of a 50-Hz square-wave of 2 V amplitude to the microelectrode for 1 min. The pretreatment was performed while the microelectrode was inserted in the capillary column and buffer was flowing past its surface.

A separation voltage of 30 kV and a column length of 1 m were used for all separations. The capillary columns (50 μm I.D., 360 μm O.D.) were obtained from Polymicro Technologies. The operating buffer was 0.02 *M* borate (pH 9.0). The detection potential was operated at +800 mV *vs.* Ag/AgCl.

Cyclic voltammetry

Cyclic voltammetry experiments were conducted in a three-electrode cell configuration using a Model CySy-1 computerized electrochemical analyzer (Cypress Systems, Lawrence, KS, USA). For these studies, excess amino acid was employed in the derivatization step to prevent interference from any impurities or side-reactions.

Microdialysis system: apparatus

Microdialysis sampling was performed using a CMA/100 syringe pump coupled to a BAS/Carnegie Medicine CMA-12 4-mm dialysis probe (Bioanalytical Systems) with a MW cut-off of 20 000. Perfusion was carried out with Ringer's solution at 1 μl/min for all experiments.

Derivatization procedure

A 1.8-μl volume of sodium borate and 0.9 μl of potassium cyanide were added to 3 μl of dialysate sample containing 1.5 μl of the α-aminoadipic acid (internal standard, varying between 2.5 and 22.5 μM final concentration, depending on the anticipated concentration of aspartate and glutamate) followed by 1.8 μl of NDA. The reaction was allowed to proceed for 30 min.

Microdialysis probe characterization

In order to determine the *in vivo* concentration of amino acids giving rise to the concentrations detected in the perfusion medium, it was necessary to calculate the recovery of the dialysis probe. Determination of the recovery was performed by placing the dialysis probes in standard concentrations of amino acids. The probes were perfused at 1 μl/min and samples of the perfusate were collected and analyzed. Recovery is then expressed as the ratio of the concentration of amino acid in the perfusate to the concentration of the standard. The average recovery of alanine, glutamine and aspartate was 24.3 ± 3.7, 20.8 ± 4.3 and 21.1 ± 3.7%, respectively (n = 3). The recovery was independent of concentration and was determined both before and after implantation. This never differed by more than 6%.

In vivo experiments

Male Sprague–Dawley rats six months or older (400–700 g) were used. Rats were initially anesthetized with the inhalation anesthetic isoflurane to simplify weighing and administration of chloral hydrate C-IV. Between 0.5 and 1.0 ml of 400 mg/ml chloral hydrate in isotonic saline was administrated intraperitoneally to anesthetize rats for surgery. Anesthesia was maintained during the experiment by infusion of 1 μl of 200 or 400 mg/ml chloral hydrate in saline per min via a catheter in the right jugular vein. In some cases, boosts of isoflurane were needed during surgery. Caution must be exercised when using this combination of anesthetics, as it can cause respiratory depression.

The rat was positioned in the stereotaxic apparatus, its scalp was shaved and a sagittal incision was made from just in front of the ears to just behind the eyes. The scalp and underlying soft tissues were scraped back to expose the skull. A hole less than 0.5 mm in diameter and 2.5 mm forward and 2.5 mm to the right of bregma was drilled just through the skull. A 25-gauge hypodermic needle in the probe carrier of the stereotaxic device was lowered until it just touched the membrane covering the brain. The hole for the dialysis probe (2.5 mm deep) was created with the needle since this causes minimal damage to the tissue. The needle was then withdrawn and the dialysis probe inserted, thus centering it in the frontoparietal cortex [10]. The scalp was then closed with tissue staples and the wound covered with paper soaked with isotonic saline to prevent drying.

Experiments were performed by perfusing the implanted probe with a normal Ringer's solution at a perfusion rate of 1 μl/min. Samples were continuously collected over 5-min intervals. Initial samples were collected for at least 1 h following insertion of the microdialysis probe and discarded. Subsequent dialysis samples were derivatized as described above and analyzed by CE–ED to establish basal extracellular amino acid concentrations. The perfusion fluid was then switched to the hyperpotassium Ringer's solution using the liquid switch. Dialysis samples were collected for an additional hour during continuous perfusion with hyperpotassium Ringer's solution. These samples were also derivatized and analyzed by CE–ED.

RESULTS AND DISCUSSION

Electrochemistry

Initial studies concerned the investigation of the electrochemical behavior of NDA-labeled amino acids. Fig. 1A shows the voltammetric response for glutamate at an untreated microelectrode. A broad peak response was obtained with an E_p of *ca.* +680 mV. It has been recognized that the pretreatment of carbon fiber microelectrodes has a pronounced effect on the electron transfer properties of many solution species, in particular, enhancement of the electrochemical response [11–13]. Following elec-

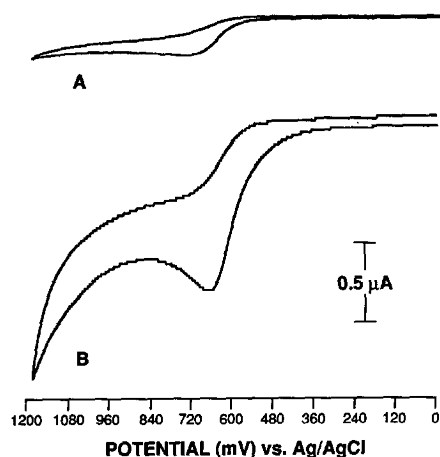

Fig. 1. Cyclic voltammograms of $5 \cdot 10^{-4}$ *M* NDA-labeled glutamate (excess glutamate was used in the derivatization procedure) in 0.1 *M* borate buffer (pH 9.0). Scan-rate, 100 mV/s. (A) Response at untreated microelectrode. (B) Response at electrochemically pretreated microelectrode.

trochemical pretreatment (described in the Experimental section), a better-defined peak shape and nearly five-fold increase in peak current were obtained with a cathodic shift in the E_p value to +650 mV (Fig. 1B). The other NDA-labeled amino acids studied exhibited similar voltammetric behaviors.

Electrochemical pretreatment was found to be necessary between each CE run, as the amino acid derivatives appeared to foul the microelectrode surface. A diminution of *ca.* 10–20% in peak height between successive injections was observed. A similar effect was reported by Oates and Jorgenson [8], who employed carbon fiber microelectrodes for the detection of NDA-labeled amino acids following open tubular chromatography. However, using the electrochemical pretreatment regime between each injection, a reproducible response was obtained with a relative standard deviation of 1.2% (n = 7). Hydrodynamic voltammetry was then investigated, and $E_{1/2}$ values for several NDA-labeled amino acids, including γ-aminobutyric acid (GABA), alanine, glycine, glutamine, aspartate and glutamate, were found to be 600, 610, 626, 635, 640 and 650 mV, respectively. These values are in agreement with those given in a report by Nussbaum *et al.* [14], which indicated that acidic derivatives were more difficult to oxidize than the basic derivatives. On the basis of these studies, an applied potential of +800 mV was selected for subsequent investigations.

Detection limit

Fig. 2 shows the separation of alanine, glutamate, aspartate and the internal standard, α-aminoadipic acid. Quantitation was achieved using response factors which were based on peak areas and were obtained in the range $2 \cdot 10^{-7}$ to $1 \cdot 10^{-4}$ *M*. The limit of detection (signal-to-noise ratio = 3) for all the NDA-labeled amino acids was extrapolated from the electropherograms at $2 \cdot 10^{-7}$ *M* levels. For alanine, glutamate and aspartate, the limits of detection calculated were $4.9 \cdot 10^{-8}$, $1.1 \cdot 10^{-7}$ and $7.9 \cdot 10^{-8}$ *M*, respectively. Using 8.0 nl as the injection volume, the corresponding mass detection limits were 0.4, 1 and 0.8 fmol, respectively. These detection limits were based on derivatizing the actual dilute solution, resulting in apparent detection limits which are higher than those previously reported, where high concentrations of amino acids were derivatized and then diluted [15]. In the latter case, many of the interferences resulting from reagent impurities and side-reactions are diluted along with the analyte.

Based on the recoveries of the amino acids with the dialysis probe, the limits of detection *in vivo* for alanine, glutamate and aspartate were $8.2 \cdot 10^{-8}$,

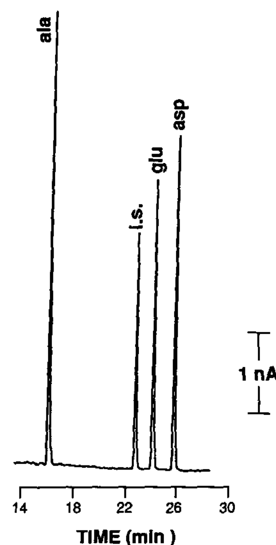

Fig. 2. Capillary electrophoretic separation of $1 \cdot 10^{-4}$ *M* standards of alanine, α-aminoadipic acid (internal standard, i.s.), glutamate and aspartate. Separation conditions: 0.02 *M* borate buffer (pH 9.0); voltage, 30 kV; column length, 1 m; detection potential, +800 mV *vs.* Ag/AgCl.

$4.8 \cdot 10^{-7}$ and $3.7 \cdot 10^{-7}$ *M*, respectively. Standards of the amino acid derivatives were analyzed periodically between dialysate samples to ensure that the detector response remained reproducible over the course of the study.

Voltammetric analysis

A typical electropherogram of a derivatized brain dialysate obtained by *in vivo* microdialysis sampling is shown in Fig. 3A. In addition to co-migration, the identity and purity of the amino acids were confirmed by voltammetric characterization. This identification is based on the $E_{1/2}$ value and the shape of the current–voltage curve, which together give a characteristic unique to each compound. It has been demonstrated previously that the entire voltammogram is not required; the comparison of current response in the region where most change occurs is sufficient [16]. The use of current ratios for compound identification has been previously reported [14,17,18].

The current ratios were calculated by measurement of the responses in the region where the current is most dependent on potential (450 and 650 mV) and ratioed to the response where the current is no longer dependent on potential, *i.e.*, the mass transport-limited value (800 mV). The current ratios recorded are given in Table I. The ratios for standards of alanine, glutamate and aspartate agreed well with those of the dialysate components eluting at the same time, confirming peak identification and purity. However, peak a, tentatively identified as GABA based on its migration time, differed significantly from the voltammetric behavior of the GABA standard.

TABLE I

VOLTAMMETRIC CHARACTERIZATION

Component	Retention time (min)	Current response	
		+0.45 V/0.80 V	+0.65 V/0.80 V
Peak a	15.9	0.21	0.72
GABA	15.7	0.12	0.65
Peak b	16.2	0.12	0.64
Alanine	16.0	0.10	0.62
Peak d	24.4	0.04	0.55
Glutamate	24.1	0.03	0.55
Peak e	25.7	0.03	0.54
Aspartate	25.9	0.03	0.56

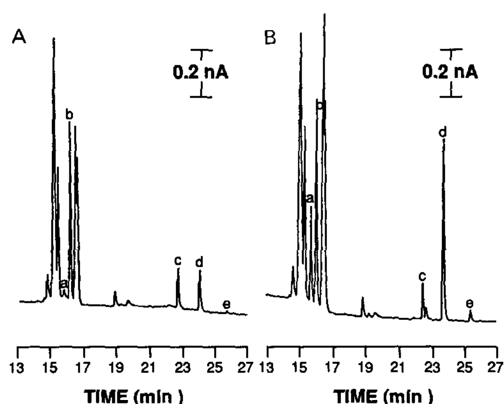

Fig. 3. Derivatized brain dialysate samples obtained by perfusion with (A) normal Ringer's solution and (B) high-K^+ Ringer's solution. Peaks: a = GABA; b = alanine; c = internal standard; d = glutamate; e = aspartate. Separation conditions as outlined in Fig. 2.

Potassium-evoked amino acid overflow analysis

High K^+ stimulus of brain tissue is known to increase the overflow of several amino acids [19,20]. Fig. 3A shows the electropherogram obtained from a typical NDA-labeled brain dialysate sample using normal Ringer's solution. Upon increasing the level of KCl from 4.0 to 100 m*M* (while still retaining the correct osmolality), the electropherogram (Fig. 3B) showed a substantial increase in the impure GABA (peak a), glutamate (peak d) and aspartate (peak e). These increased levels are indicative of their roles as neurotransmitters. The levels of alanine (peak b) remained unaffected by the K^+ stimulus, which was expected as it is thought only to have a role in metabolic functions. These results are in agreement with the findings of Tossman *et al.* [19].

Fig. 4 shows the concentration–time curves obtained for alanine, glutamate and aspartate. After 30 min of collecting dialysate samples of the basal amino acid concentration levels, the K^+ stimulus was applied. For both glutamate and aspartate, a maximum increase over the basal concentration levels of nearly four-fold was observed. After an initial sharp increase, the levels of both amino acids decreased to a steady-state level approximately 30 min after the stimulus was introduced. The curve ob-

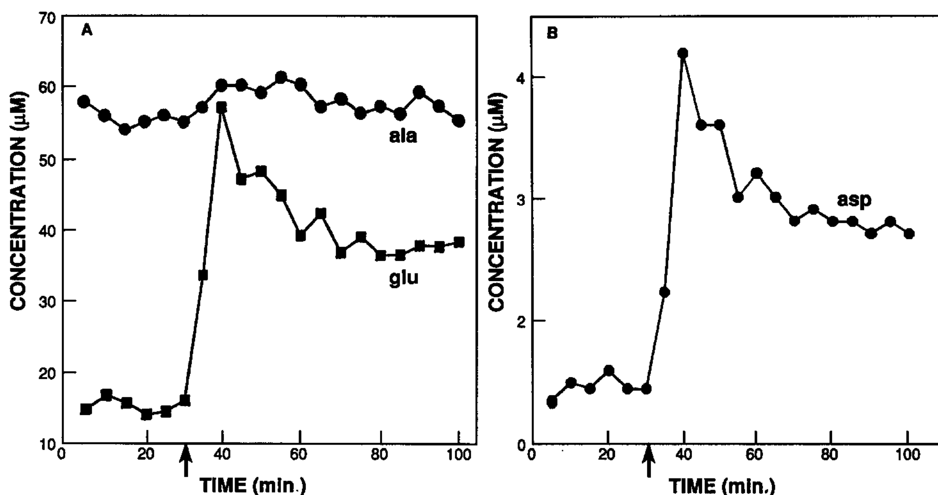

Fig. 4. Concentration–time curves of (A) alanine and glutamate and (B) aspartate in rat brain. Arrow indicates application of potassium stimulation.

tained for alanine fluctuated between $5.5 \cdot 10^{-5}$ and $6.2 \cdot 10^{-5}$ M and did not show any response to the K^+ stimulus. The previous studies of K^+-invoked overflow of amino acids in the brain [19,20] did not present a concentration–time curve; they reported only two concentration levels, a basal level and the level obtained after a 10-min stimulation.

CONCLUSIONS

One of the main advantages of CE as an analytical tool for microdialysis sample analysis over the more commonly used LC is that it is more amenable to small sample volumes. In this study, 3-μl samples were derivatized; however, if microderivatization procedures [21] were employed, much greater temporal resolution could be achieved. Additionally, because of the small injection volumes (typically of the order of a few nanoliters), multiple analyses can be performed on a single sample.

Another purpose of this study was to demonstrate the high sensitivity of ED for CE and the possibility of analyzing a wider range of analytes using derivatization chemistry. Curry *et al.* [3] have reported the high sensitivity obtainable using CE–ED for the determination of several electroactive neurotransmitters. Microdialysis sampling combined with CE–ED should prove to be a very powerful tool for future *in vivo* neurochemical studies.

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