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Coupling on-line brain microdialysis, precolumn derivatization and capillary electrophoresis for routine minute sampling of *O*-phosphoethanolamine and excitatory amino acids

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Abstract

In previous papers, we described the analysis of excitatory amino acids (EAAs) and catecholamines in microdialysis samples using capillary electrophoresis with laser-induced fluorescence detection (CE–LIFD). In the present paper, we report that an automated analysis of such samples can be easily achieved by on-line coupling of the microdialysis probe with a continuous flow derivatization system and a commercially available CE–LIFD apparatus. Because of the short analysis time (less than 2 min) and high separation efficiency (100–200 000 theoretical plates), high temporal resolution of microdialysis (minute range) is preserved as compared to off-line systems, while both EAAs and *O*-phosphoethanolamine (PEA) can be simultaneously detected. This new method has been applied to the measurement of these compounds in microdialysis samples from hippocampal slice cultures and striatum of anesthetized rats. Extracellular concentrations of EAAs, but not PEA, increased during perfusion of a solution containing high K^+ or a glutamate uptake inhibitor. However, after in vitro ischemia on hippocampal slices, both EAAs and PEA concentrations increased, but with different temporal patterns.

Keywords: Microdialysis; Phosphoethanolamine; Amino acids; Neurotransmitters; Aminoadipic acid

1. Introduction

Neurotransmitters, but also compounds which are not directly involved in neurotransmission, regulate brain functioning during physiological or pathological phenomena. The excitatory amino acids (EAAs) glutamic acid (Glu) and aspartic acid (Asp) are responsible for most of the fast excitatory transmission in mammalian central nervous system. Also, they have been shown to participate in brain damage like neurodegeneration, stroke and ischemia [1]. A phosphoglyceride precursor, *O*-phosphoethanolamine (PEA), has been shown to be involved in osmoregulation in the mammalian brain [2], while its extracellular concentration may be a sensitive indicator of membrane disorder after brain insult [3]. It is therefore of particular interest to simultaneously monitor EAAs and PEA levels in brain.

Brain microdialysis has been shown to be a powerful technique for neurochemical investigations in living animals [4]. Capillary electrophoresis with laser-induced fluorescence detection (CE–LIFD) has been introduced as an appropriate technique for neurotransmitter determinations in microdialysates, especially when a high microdialysis sampling rate is needed [5–10]. Since dialysates are protein-free, on-

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line coupling between microdialysis and laboratory-made CE–LIFD has been described to prevent sample loss and evaporation: using various interfaces, 45-s to 3-min sampling rates were reported for the in vivo monitoring of EAAs [11,12] and/or some non-neurotransmitter compounds [12,13].

The aim of the present work was to develop on-line coupling of brain microdialysis with CE–LIFD in order to simultaneously determine EAAs and PEA with routine minute sampling rate. A simple liquid interface was connected to a continuous flow derivatization device to achieve routine on-line coupling between the microdialysis probe and a commercially available CE–LIFD apparatus. Glu, Asp and PEA were monitored in brain culture slices and in striatum of living rats. Pharmacological or pathological stimulations are reported to demonstrate the performance of this system.

2. Experimental

2.1. Reagents and solutions

All chemicals were analytical grade and purchased from Sigma (St. Louis, MO, USA). naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from Fluka (Buchs, Switzerland) and L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) from Tocris Cookson (Bristol, UK).

Sodium borate buffer (500 m*M*, pH 8.7 used as derivatization buffer) was obtained by mixing separate solutions of boric acid (500 m*M*) and sodium tetraborate (125 m*M*). Sodium borate buffer (100 m*M*, pH 9.2 used as amino acid separation buffer) was prepared by mixing boric acid (100 m*M*) with sodium hydroxide (5 *M*).

The 2.5-m*M* NDA solutions were prepared in acetonitrile–water (50:50, v/v) bi-weekly and stored at 4°C.

The sodium cyanide solution was prepared by mixing (40:100, v/v) sodium cyanide solution in water (43 m*M*) with sodium borate buffer (500 m*M*, pH 8.7). The mixtures were prepared daily and stored at 4°C.

Artificial cerebrospinal fluid (aCSF) used for probe perfusion had the following composition (in m*M*): NaCl 145.0, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 2.5 (1.2 for in vivo studies), NaH_2PO_4 0.45, Na_2HPO_4 1.55 (pH 7.4).

Electrophysiological medium used for slice perfusion had the following composition (in m*M*): NaCl 124.0, KCl 1.6, $MgCl_2$ 1.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 24.0, glucose 10.0, ascorbic acid 2.0 (pH 7.4).

The 1 m*M* stock solutions of Glu, Asp, PEA and the internal standard (I.S.) α-aminoadipic acid (AAD) were prepared in 0.1 *M* perchloric acid and stored at −20°C by aliquots of 1 ml. The required working solutions were obtained by further dilution with aCSF (endogenous amino acids) or 0.1 *M* perchloric acid (AAD).

2.2. Microdialysis

Side-by-side microdialysis probes were made from fused-silica capillary tubing (105 μm O.D.×40 μm I.D.; Polymicro Technologies, Phoenix, AZ, USA) and cellulose dialysis tubing (225 μm O.D.). A 2-mm active dialysis length was used for studies on brain slices, whereas a 3-mm probe was used for striatal microdialysis in living animals. The probes were perfused with aCSF (1 μl/min unless otherwise specified). The in vitro recoveries of 2- and 3-mm probes were found to be 17 and 22%, respectively, for Glu.

In vivo microdialysis experiments were carried out on male Sprague–Dawley Oncins France souche A (OFA) rats (280–320 g; IFFA Credo, L'Arbresle, France) anaesthetized with 1.4 g/kg i.p. urethane (Sigma). The probes were implanted in the striatum at the following coordinates (relative to bregma): anterior 0 mm, lateral 3.5 mm, ventral 6.5 mm below the brain surface.

In vitro microdialysis experiments on brain slices were carried out on hippocampal organotypic slice cultures placed under visual control in a Physiocard system perfused with the electrophysiological medium [8]. A probe was delicately put down onto the surface of a slice. Because of the transparency of this device, the probe can be positioned in the desired area of hippocampus, between CA_1 and CA_3 areas, in the apical dendritic field of CA_1 (i.e., in a synaptic region).

The collection of basal samples was initiated 1–2 h after microdialysis probe positioning. The inlet of

the microdialysis probes was connected via fused-silica capillary tubing to the perfusion pump while the outlet was connected to the continuous-flow derivatization system (see below).

2.3. Capillary electrophoresis

2.3.1. Apparatus

CE was performed on a SpectraPhoresis 100 module purchased from ThermoSeparation Products (Les Ulis, France) equipped with a LIF detector from Zeta Technology (Toulouse, France). The LIF detector is directly derived from the instrument described by Hernandez et al. [14], with some differences in lens position and in the presence of a ball lens [15], allowing better mechanical tolerance for capillary adjustment and stability. The excitation was performed by an Omnichrome (Chino, CA, USA) helium–cadmium laser (model 4056-30M) with a power of 7–10 mW at the wavelength of 442 nm. The emission intensity was measured at the wavelength of 490 nm filtered by a band pass filter, and a notch filter was used to attenuate background radiations. Fluorescence was detected by a photomultiplier tube. Data were acquired (100 points/s) with a Borwin data acquisition system (JMBS Developments, Grenoble, France).

2.3.2. Separation procedure

Separations were carried out with a fused-silica capillary (Polymicro Technologies) of 50 μm I.D.× 375 μm O.D. having a total length of 60 cm and an effective length of 19 cm. On-column LIFD was carried out through a 5-mm wide window opened by removing the polyimide cover of the capillary. The injection end was placed in a grounded laboratory-made injection interface (see below) while the detection end was held at a negative potential. Capillary was sequentially flushed (3×10 min) with 1 and 0.1 *M* sodium hydroxide solutions and water prior to use. The separation buffer used was 100 m*M* borate buffer (pH 9.2) and running voltages were 24 or 30 kV (generated currents, 24 or 30 μA, respectively). Peak identification was based on absolute migration time.

Off-line analyses were performed for the characterization of PEA detection in in vivo microdialysates using similar derivatization and separation conditions (30 kV).

2.3.3. Derivatization procedure

An on-line derivatization device with very low dead volume was used. This device has been previously described [7,8,16], and only minor modifications were made to limit clogging of the system for a routine use. Briefly, it consisted in three 5 cm×150 μm O.D.×75 μm I.D. fused-silica capillary tubings (Polymicro Technologies) inserted into a 1 cm×700 μm O.D.×300 μm I.D. polyethylene tubing (PE 10). The ends of the three silica tubings were glued to the PE 10 with a 1-mm interval between the extremity of each of them. The first capillary was connected to the outlet of the microdialysis probe. A Y-connector was placed on the second inlet to connect two Hamilton syringes containing the sodium cyanide solution in borate buffer and the I.S. solution (10 μ*M* AAD), respectively. The last inlet was connected to an Hamilton syringe containing NDA. The perfusion flow-rates were 1 μl/min (microdialysis probe), 0.2 μl/min (sodium cyanide in borate buffer) and 0.1 μl/min (I.S. and NDA). The I.S. was used to account for sample-to-sample variability in derivatization efficacy and injection.

2.4. On-line coupling between a microdialysis probe, the derivatization device and the CE–LIFD apparatus

A schematic representation of the entire on-line microdialysis system is shown on Fig. 1. The outlet of the derivatization system (PE 10) was connected to an automatic injection valve (Valco, Schenkon, Switzerland) equipped with an external 0.63-μl sample loop (14 cm×75 μm I.D. fused-silica capillary; Polymicro Technologies) via a 4 cm×150 μm I.D. fused-silica capillary, so as to keep free 2 mm of PE 10 as reaction chamber. An on-line microdialysis–CE system was constructed according to previous reports and filled with the separation buffer [11,17]. CE separation buffer was delivered to the interface at a flow-rate of 10 μl/min using a 12 cm×150 μm I.D. capillary, either directly (during the separation time) or via the sample loop in order to send the derivatized sample loaded to the separation capillary (during the injection time). A sepa-

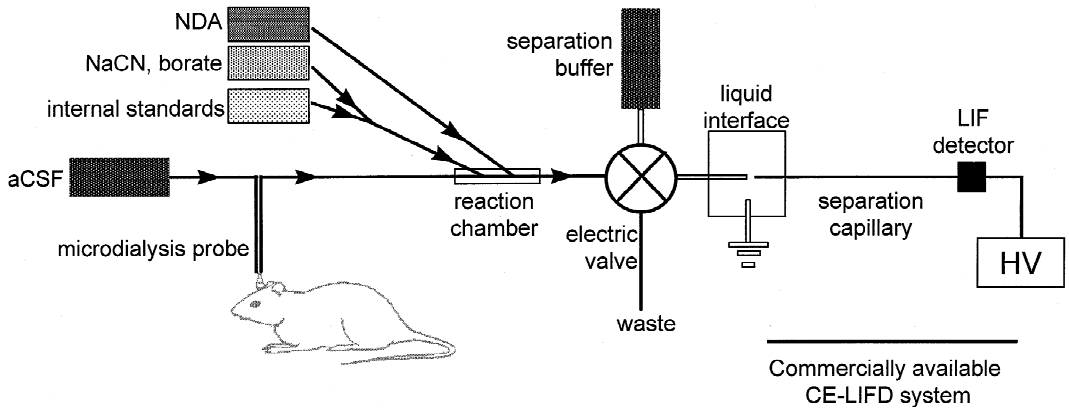

Fig. 1. Schematic diagram of the experimental microdialysis set-up with a continuous-flow derivatization system of microdialysis samples and on-line CE–LIFD analysis. Note that this system allows rapid derivatization and automated injection of minute microdialysis samples using a commercially available CE and LIF detector apparatus. The total delay time between the step change at the probe and the response of the entire system is 4.5 min.

ration gap of 200 μm was kept between the capillary coming from the valve and the separation capillary. The running voltage was applied continuously and electrokinetic injections were performed by commutating the injection valve for 2 s every 2 min (or 1 min) at +1.1 min (or +0.1 min) of the acquisition time of the preceding analysis using the Borwin software, so that the peaks corresponding to this injection appeared on the next electropherogram.

2.5. Expression of the results

Time presented in the figures corresponds to real time of collection of the microdialysates. Administration of drugs was timed to consider the dead volume of the entire system.

Data are given as means±S.E.M. values and are expressed as percent as baseline levels.

3. Results and discussion

Previous data [8], using the same commercially available CE–LIFD system, has shown the potential of this analytical technique for the off-line neurotransmitter detection in microdialysis samples. High temporal resolution of microdialysis (up to 30 s) was achieved while the simultaneous determination of catecholamines and EAAs (Glu and Asp) was possible. This was largely due to the use of a continuous-flow derivatization system allowing to perform the derivatization step on very low sample volumes, that matched very well with the CE injection requirements [7,16]. In the present work, we demonstrate that an automated analysis of microdialysates can be easily achieved by on-line coupling of probes, derivatization device and a commercially available CE–LIFD apparatus. Such a system allows the simultaneous determination of Glu, Asp and PEA, without hampering the temporal resolution of the microdialysis sampling (minute range), as compared to off-line systems.

3.1. In vitro testing

In our on-line system, the temporal resolution of the microdialysis sampling is limited by the separation times of CE. In the present work, by using a 50-μm I.D. capillary with a total length of 60 cm, an effective length of 19 cm and an applied voltage of 30 kV, a 1.6-min separation time with approximately 105 000 theoretical plates per meter for Glu and AAD was obtained. Under such analytical conditions, electropherograms could be obtained every 1 min (Fig. 2A): since no peaks appeared in the first 45 s, injections were performed at 6 s of run time of the preceding analysis and corresponding peaks were obtained on the next electropherogram. Also, by

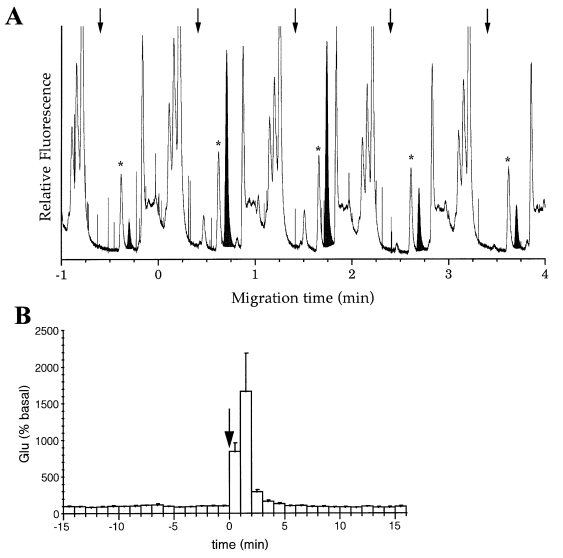

Fig. 2. In vitro microdialysis response to a step change of 1–2 s in the external concentration. (A) Series of electropherograms illustrating the rapid analysis time of CE–LIFD for high microdialysis sampling rate. Fluctuations of the external medium produced significant responses of the probe. Arrows indicate injection times, asterisks AAD peaks, while Glu peaks are in grey. Time zero corresponds to the step change, after correction by the dead volume of the system. (B) Averaged response ($n=7$). The arrows indicate the time when a 2-mm probe was transferred from a 1 μ*M* Glu solution to a 100 μ*M* Glu solution. On-line derivatized samples were injected every 1 min by switching the injection valve for 2 s. Note that 1–2-s fluctuations of the external medium produced significant responses of the system.

decreasing the applied voltage to 24 kV, separation efficiency was increased to reach approximately 210 000 theoretical plates per meter for Glu and AAD while separation time was increased to 2.6 min: this leads to a decrease in microdialysis sampling rate from 1 to 2 min.

Inter-assay reproducibility was good: when samples obtained from a probe placed in a 10 μ*M* Glu were injected either every 2 or 1 min, the relative standard deviations for migration times and peak area (after correction with the I.S.), were 1.5 and 6.9% ($n=10$), respectively. We wish to emphasize that the use of an I.S. is required since standard deviations for peak areas rose to 10.8% if no correction with the internal standard was made. This indicates sample-to-sample variability in derivatization efficacy and injection, inherent to the manipulation of very low sample volumes and the electrokinetic mode of injection. On-line detection limit was 40 n*M* for Glu, and calibration curves for Glu and Asp were linear up to 100 μ*M*.

In order to test the ability of the on-line microdialysis–CE–LIFD system to follow changes that occur in the external medium in which the microdialysis probe is placed, the in vitro response to rapid step changes was studied with a 1-min sampling rate. For that purpose, the probe was manually placed during 1–2 s in a concentrated external medium where Glu concentration was 100-fold higher than the basal values. A maximum response was observed in the +4 and +5 min dialysate fractions following the step change in the medium. A maximum increase of +1700% was observed (Fig. 2B). This result indicates that rapid fluctuations (less than 5 s) of the external medium produced significant responses of the system. A quantitative and specific automated determination of rapid variations in the external medium could thus be achieved with the present experimental conditions.

The total delay in the response of the on-line system to a biochemical event is a sum of the following: (1) the time it takes for the sample to be collected, derivatized and transferred to the sample loop; (2) the time the sample spends into the loop; (3) the time the derivatized sample spends traveling from the injection valve to the injection interface; and (4) the CE separation time. In our case: (1) was 75 s; (2) was 30 s; (3) was 20 s; and (4) was 2.6 min for brain dialysate analysis. Thus, the total delay time was ~4.5 min which represents an improvement over previously reported systems [11].

3.2. In vivo testing

To illustrate the capabilities of this technique in in vivo or ex vivo experiments, EAAs and PEA were monitored in the striatum of anesthetized rats and in hippocampal slices. Unlike EAAs, the monitoring of PEA in brain microdialysis samples using CE–LIFD has not been previously described. Thus, in a first stage of this study, we performed electrophoretic validation of PEA determination using an off-line analysis procedure. The electropherogram of a striatal microdialysis sample showed a peak with a migration time corresponding to the one of PEA in

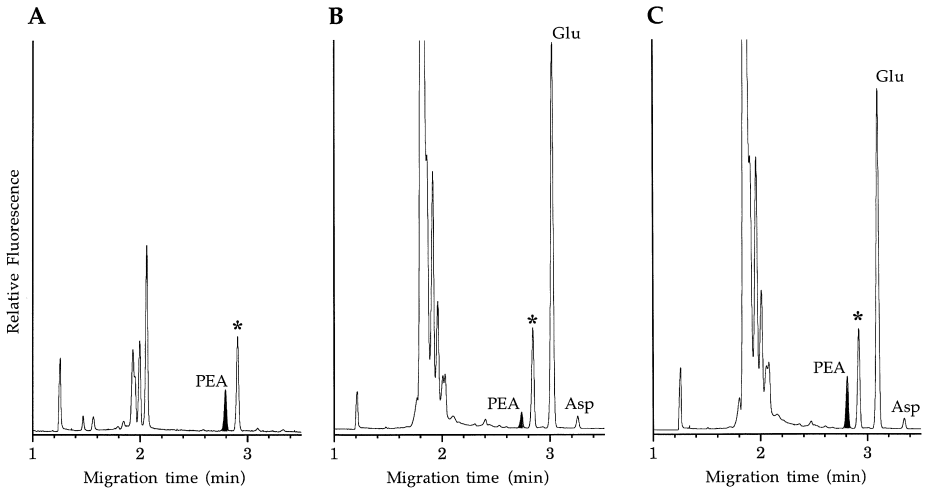

Fig. 3. Electrophoretic characterization of the PEA peak in microdialysis samples using an off-line analysis procedure. (A) Standard solution of 0.1 μ*M* PEA; (B) microdialysis sample from rat striatum; (C) same microdialysis sample after addition of exogenous derivatized PEA (final concentration 123 n*M*). Asterisk indicates AAD peak. Basal striatal concentrations are: PEA, 33 n*M*; Glu 4.4 μ*M*; Asp 0.21 μ*M*.

standard solution (Fig. 3A,B). The height of this endogenous peak increased when exogenous PEA was coinjected with the microdialysis sample, whereas no additional peak appeared (Fig. 3C). Moreover, PEA was separated not only from AAD but also from amino acids or primary amines potentially present in microdialysis samples. Indeed, the injection of a mixture of 26 amino acids and primary amines, each at a concentration of 25 μ*M*, showed that none of these compounds comigrated with PEA (data not shown; the compounds tested were as follows: alanine, β-alanine, α-aminoadipic acid, α-amino-γ-butyric acid, β-aminoisobutyric acid, asparagine, Asp, citrulline, cystathionine, cystine, Glu, glycine, hydroxyproline, isoleucine, leucine, methionine, phenylalanine, *O*-phosphoserine, proline, sarcosine, serine, taurine, threonine, tyrosine, urea, and valine). Unfortunately, PEA and AAD were not well resolved using 30 kV as applied voltage: this necessitates performing analyses with a 24-kV voltage. Under these conditions, the migration time for PEA was 2.6 min which leads to a 2-min sampling rate for in vivo microdialysis studies.

Basal concentrations of Glu and PEA in hippocampus slice cultures and striatum of anaesthetized rats were stable throughout the experiment, and were found to be, respectively, 0.12±0.02 μ*M* (*n*=6) and 2.7±0.9 μ*M* (*n*=5) for Glu, and 51±8 n*M* (*n*=6) and 0.65±0.10 μ*M* (*n*=5) for PEA, which is similar to those previously reported [3,8,18]. Basal Asp levels in striatal dialysates of living rats were found to be 0.25±0.05 μ*M* (*n*=5), but the extracellular basal levels of this EAAS were below detection in hippocampal slice dialysates during control conditions. Using such separation protocol, EAAs and PEA can be used as indexes of excitatory transmission and brain metabolism, respectively.

A 10-min application, through the dialysis probe, of aCSF containing 0.5 m*M* of the Glu uptake inhibitor PDC induced an increase (200–300% of basal values) in extracellular Glu concentrations from hippocampal slices (Fig. 4A) and striatum of anaesthetized rats (Fig. 5A). This effect is of the same magnitude as those previously reported in the same brain regions [18–20]. On the other hand, a 10-min perfusion of a high K^+ aCSF caused an increase of 680% in striatal Glu levels (Fig. 5A). Striatal Asp levels showed similar variations after PDC or high K^+ perfusion, with maximal increases of about +1000% (Fig. 5B). In contrast, PEA levels showed no variation after PDC or high K^+, either in hippocampal or striatal dialysates (Fig. 4B, Fig. 5C), which is in agreement with the non-neurotransmitter nature of this compound.

Ischemia induced on hippocampal slice cultures by metabolic inhibition [21] showed a two-step increase

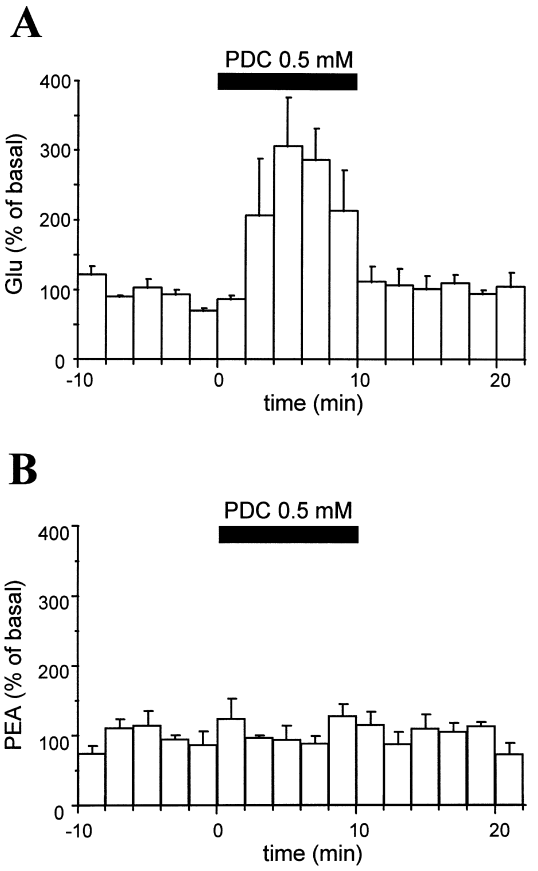

Fig. 4. Effect of PDC administration (0.5 m*M* for 10 min through the probe) on Glu (A) and PEA (B) concentrations in 2-min fractions of hippocampus dialysates from culture slices ($n=3$). Means±S.E.M. of the baseline values: Glu, 0.12±0.01 μ*M*; PEA, 53±8 n*M*.

in Glu levels (short plateau from +2 to +8 min at +180% followed by a larger increase from +8 to +28 min, with a maximum of +815% at +16 min) while a delayed and massive increase of extracellular PEA starting at the end of the ischemic period was observed to reach a plateau from +10 to +28 min (~900% of basal values) before returning close to baseline values (Fig. 6). This is in agreement with previous reports [3,22]. This temporal shift emphasizes the potential for determining simultaneously EAAs and PEA with a high sampling rate.

These improvements now allow 2-min sampling to be performed on a routine basis, with no sample manipulation for the derivatization and injection

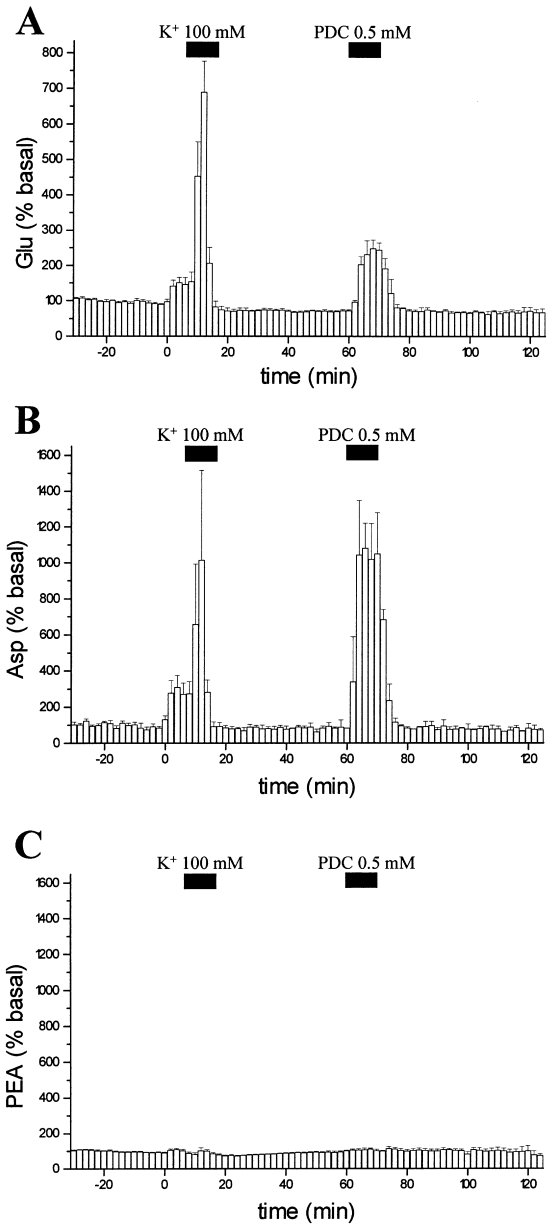

Fig. 5. Effect of high-K^+ (100 m*M*) perfusion and PDC administration (0.5 m*M*) for 10 min through the probe on Glu (A), Asp (B) and PEA (C) concentrations in 2-min fractions of striatum dialysates from anaesthetized rats ($n=5$). Means±S.E.M. of the baseline values: Glu, 2.7±0.1 μ*M*; Asp, 0.25±0.05 μ*M*; PEA, 0.65±0.10 μ*M*.

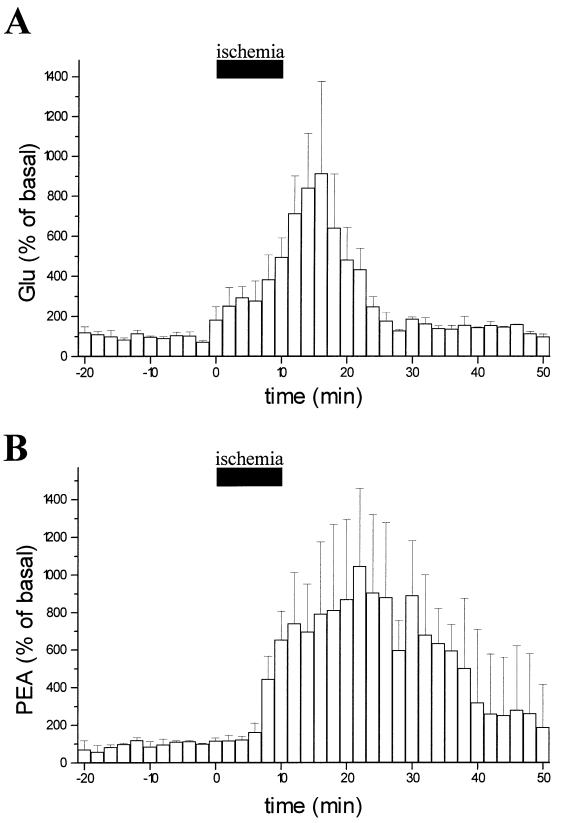

Fig. 6. Effect of ischemia on Glu (A) and PEA (B) concentrations in 2-min fractions of hippocampus dialysates from culture slices ($n=3$). Ischemia was induced by perfusing a glucose-free, potassium cyanide (1 m*M*) and 2-deoxyglucose (5 m*M*) containing medium for 10 min through the perfusion chamber of the Physiocard. Means±S.E.M. of the baseline values: Glu, 0.13±0.02 μ*M*; PEA, 48±9 n*M*.

steps. Unfortunately, a 1-min sampling rate could not be used under the present conditions for brain dialysates since AAD was not resolved from PEA using 30 kV as applied voltage.

Finally, we would like to emphasize that this study reports for the first time the on-line coupling of a microdialysis probe with CE–LIFD analysis using a commercially available CE–LIFD system. Continuous-flow derivatization system and liquid interface can easily be made in the laboratory to achieve routine and sensitive analyses in microdialysis samples. This technique allows 2-min sampling to be performed, with no sample manipulation for the derivatization and injection steps. Using these conditions, the system allows to monitor EAAs and PEA concentrations during 6–7 h (i.e., 200 analyses). This paper also shows the performance of the system for studies on brain slices or on living animals. Additionally, an electrophysiological approach can be simultaneously performed on slices, allowing a global monitoring of the neuronal functioning with a fine time resolution, which can be used as a complement to conventional in vivo microdialysis studies [8].

In summary, the use of a CE–LIFD on-line system for automated EAAs and PEA detection has improved temporal resolution of microdialysis sampling through a routine and manipulation-free method. In vivo or in vitro microdialysis can be easily performed to study rapid changes induced by various pharmacological or physiological treatments on extracellular levels of both EAAs and the non-neurotransmitter compound PEA. This allows to have representative indexes of excitatory transmission and brain metabolism since they are determined with a high temporal resolution. Further decrease in sampling time will be obtained by optimizing separation conditions, while other neurotransmitters or non-neurotransmitter compounds could be detected using this on-line system.

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