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Glutamate measured by 6-s resolution brain microdialysis: capillary electrophoretic and laser-induced fluorescence detection application

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Abstract

In the present experiment the combination of brain microdialysis and CZE–LIFD permitted the measurement of glutamate in 100 nl microdialysis samples collected every 5 or 6 s. Samples were collected every 6 s, in rats anesthetized with two different anesthetic agents (ketamine and sodium thiopental). A microdialysis probe was inserted in the cortex of an anesthetized rat in the territory irrigated by the middle cerebral artery. The artery was clamped for 30 s and then released. The samples were derivatized with fluorescein isothiocyanate I (FITC) by means of a continuous-flow reactor, collected and injected into a home-made CZE–LIFD instrument. Glutamate decreased immediately after clamping the artery in ketamine anesthetized rats and increased 1 min after the onset of the ischemia in sodium thiopental anesthetized rats. In another experiment a 60 m*M* KCl solution was injected through a microdialysis probe inserted in the hippocampus of an anesthetized rat. In the first 5 s after the KCl solution reached the tissue, glutamate increased but γ-aminobutytic acid and glutamine did not. The experiments show that time resolution of brain microdialysis can be reduced to a few seconds if the analytical technique is the proper one. © 1997 Elsevier Science B.V.

Keywords: Brain microdialysis; Glutamate; Ketamine; Sodium thiopental

1. Introduction

The main limiting factor for brain microdialysis has been the lack of sensitive enough analytical techniques. The seminal work of Bito et al. [1] and Delgado et al. [2] in the sixties and seventies was hindered by the low sensitivity of the analytical techniques. The widespread use of brain microdialysis in the eighties was triggered by the high sensitivity reached by liquid chromatography and electrochemical detection (HPLC–ED) [3,4]. However, further progress in brain microdialysis has been hampered by the analytical technique. HPLC–ED requires microliter volumes and picomole to femtomole masses to detect neurotransmitters in brain dialysates. These requirements remarkably limit the time resolution of microdialysis. For instance, submicromolar concentrations of excitatory and inhib-

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itory neurotransmitters are typically found in microliter volumes of brain dialysates [5–9]. At 1 μl min^{-1} perfusion flow-rate only 100 nl of dialysate will be collected in 6 s. This amount cannot be handled by most of the HPLC syringes and injection valves. Moreover, a 0.5-μ*M* solution of glutamate contains 500 amol per nanoliter which is below the limit of detection for most of the glutamate analysis techniques. Clearly a mass sensitivity in the low attomole (10^{-18} mol) range and a technique capable of handling nanoliter volumes is required to study glutamatergic function by brain microdialysis in a subminute time frame.

Currently the most-used techniques for glutamate detection are based on liquid chromatography and either electrochemical or fluorescence detection. In a series of papers published between 1979 and 1992 the minimum volume of sample injected into an HPLC system for glutamate detection was 1 μl and the largest was 250 μl [10–18]. The mean injected volume was 40 μl. The lower volume was used with microbore columns (1 mm I.D.).

In the same papers the detection techniques have been either electrochemical, UV or fluorescent. In all of these techniques the glutamate had to be derivatized for detection. The derivatization reagents included orthophtalaldehyde (OPA), naphthalene dicarboxialdehyde (NDA), dansylchloride and diethylaminobenzene sulphonylchloride. OPA derivatization is the most-used method because of a very short reaction time. But, it produces unstable derivatives. Therefore, automatic derivatization and injection procedures are recommended. The best sensitivity reported was 4.4 femtomoles when the detection was accomplished by laser induced fluorescence [16]. The same authors reported an even better limit of mass detection when glutamate was derivatized with NDA. In this analysis a detection limit of 160 amol is reported.

Other detection methods based on enzymatic reactors and biosensor offer tantalizing second or subsecond time [19–22]. But their use has not spread because of their low sensitivity as well as their low specificity.

In previous reports we measured glutamate by means of CZE–LIFD in brain dialysate collected every 20 min [23]. In the present article we report the combination of CZE–LIFD with brain microdialysis for 6 s resolution.

2. Experimental

2.1. Subjects

Male albino rats of the Wistar strain and weighing between 250 and 300 g were individually housed with water and food ad libitum.

2.2. Surgery

First experiment: Under urethane anesthesia, a guide shaft was implanted aimed at the hippocampus according the following coordinates: 5 mm lateral to the midsaggital suture, 4 mm ventral to the surface of the skull and −5.2 mm posterior to bregma; and the microdialysis probe was inserted. Flow-rate was 1 μl min^{-1} and the system used to derivatize and collect the samples was the same as described below (see derivatization section). After a 45-min period of stabilization 5 control samples were taken every 5 s and then a tube was inserted with ACSF with 60 m*M* KCl in the perfusion line. This solution passed through the probe for 20 s and meanwhile samples were collected every 5 s.

Second experiment: Under ketamine anesthesia (100 mg kg^{-1}) or sodium thiopental (40 mg kg^{-1}) anesthesia, the rats were placed on a stereotaxic instrument. The skin of the head was opened and the parietal as well as part of the frontal bones were removed with a trephine and a gouge. The middle cerebral artery on one side was visualized by means of a dissecting microscope. A microdialysis probe was inserted at a 30° angle into the parietal lobe. A pair of fine tweezers was soldered on a 0.1 mm precision *xyz* displacer. This home-made microtweezers had a fine screw which allowed the tweezers to close and open. The middle cerebral artery was positioned between the two tips of the branches of the tweezers. Five samples were collected every 6 s before clamping the artery, then it was occluded for 1 min and 10 samples were collected and after opening the tweezers 8 more samples were collected. This experimental arrangement allowed the artery to

be clamped and released, minimizing tissue disturbance. At the end of the experiment the rat was sacrificed with an overdose of chloroform.

2.3. Microdialysis

The microdialysis probe was made of concentric fused-silica polyimide covered capillary tube (150 mm O.D.×75 mm I.D.) into a 26 gauge stainless steel tube. A cellulose hollow fiber was plugged with epoxy at one end and attached inside the 26 gauge tube. This cellulose tube has a 6000 molecular weight cut off and its permeability data has been reported elsewhere [25]. The inlet tube of the microdialysis probe was connected to a No. 10 polyethylene tube that was connected to a syringe placed on a pump. The syringe perfused the probe with artificial cerebral spinal fluid (ACSF: 136 m*M* NaCl, 3.7 m*M* KCl, 1.2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$ and 10 m*M* $NaHCO_3$ at pH 7.4) at a flow-rate of 1 $\mu l\ min^{-1}$.

2.4. Derivatization

The kinetic of derivatization was optimized by mixing a solution of 10^{-5} *M* glutamate with increasing concentrations of FITC ($1.07 \cdot 10^{-5}$ *M* to $2.5 \cdot 10^{-3}$ *M*). The mixture reacted in the dark and was analyzed as described below. The samples were derivatized in a precolumn reactor built with three borosilicate tubes heated, twisted and pulled resulting a three barreled micropipette. The first tube was connected to the microdialysis probe outlet. The second barrel was connected to a microsyringe filled with 20 m*M* carbonate buffer at pH 9.4 and placed on a precision pump. The flow-rate of the buffer was set at 2 $\mu l\ min^{-1}$. The third was connected to a microsyringe filled with a $4 \cdot 10^{-4}$ *M* fluorescein isothiocyanate I dissolved in acetone. The syringe was set in a precision pump and the flow-rate was set at 0.2 $\mu l\ min^{-1}$. The solutions flowing in the reactor were mixed at the tip of the reactor in the proportion 5:1:1 (sample–buffer–FITC). The samples were collected by capillarity into borosilicate tubes every 6 s and placed on a water-saturated chamber. A calibration curve was made derivatizing increasing concentrations of glutamate (10^{-8}–10^{-6} *M*) with a standard concentration of FITC (10^{-4} *M*). These mixtures and the ones collected from the rats were allowed to react for 16 h and diluted 10 fold with buffer. A blank solution was prepared by the same procedure described above except that the microdialysis probe was not intercalated in the reactor ACSF line. Therefore, pure ACSF was derivatized and collected by capillarity. A standard solution of glutamate was prepared by dissolving 1 mg of glutamate in carbonate buffer to obtain a 1 $mg\ ml^{-1}$ solution. The amino acid solution (100 nl) was combined with 20 nl of FITC solution and allowed to react for 16 h in the dark. Then the tubes were inserted in a polyethylene tube P10 connected to a syringe, and were immersed into another tube containing 980 nl of buffer carbonate–bicarbonate, the mixture was ejected by pushing the syringe and both liquids mixed. The mixture, diluted by a factor of 10, was injected into the CZE system.

2.5. Capillary zone electrophoresis instrument

The CZE system is a collinear instrument already described [23,24]. Briefly, an Argon ion 3 mW laser (Ion Laser Technology, Salt Lake City, UT, USA) was tuned to 488 nm and reflected by a dichroic mirror centered at 510 nm (Carl Zeiss, Caracas, Venezuela). The laser was focused by means of a 0.85 NA objective (Carl Zeiss, Caracas, Venezuela) on the window of the capillary (Polimicro Technologies, Phoenix, AZ, USA). The window was located at 20 cm of the anodic end of a 30 cm long, 20 μm bore fused-silica capillary filled with a 20 m*M* carbonate buffer at pH 9.4. The fluorescence was collected by the same objective and stray radiation was attenuated by a high pass filter centered at 520 nm (Carl Zeiss, Caracas, Venezuela) and a notch filter centered at 488 nm (Andover, Salem, NH, USA). Then the fluorescence was focused on a R1477 multialkali PMT (Hamamatsu, Bridgewater, NJ, USA) driven by a HC-123 miniaturized high voltage power supply (Hamamatsu, Bridgewater, NJ, USA). The current of the PMT was converted to voltage by a home-made voltage follower and fed to a computer. The electropherograms were acquired

and analyzed by means of a 386 computer and a Maxima software (Waters, Milford, MA, USA)

2.6. Capillary electrophoresis

The electrophoretic run consisted of injecting a plug of the test solution or the mixture with the sample by the hydrodynamic method. A pulse of −19 p.s.i. was applied for 0.3 s at the cathodic end of the capillary while the anodic end was immersed in the mixture reservoir. The anodic end was withdrawn from the mixture reservoir and immersed in a buffer reservoir. Both the cathode and the anode were made of platinum–iridium wire. A high voltage power supply (Bertan, Hicksville, NY, USA, model 30R) applied 20 KV between the anode and the cathode for 10 min. After each run the capillary was rinsed with 0.1 *M* sodium hydroxide solution for 2 min followed by water for 2 min and 20 m*M* carbonate buffer for 3 min.

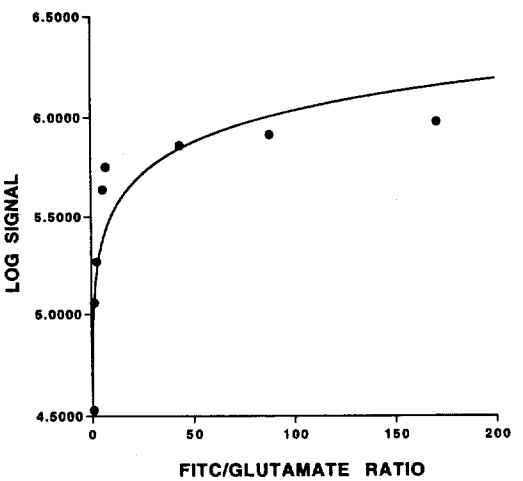

Fig. 1. Optimization of glutamate peak height in a solution 10^{-5} *M* glutamate derivatized with increasing concentrations of FITC. The logarithmic curve of glutamate signal height versus FITC excess in moles shows that a 50 molar excess with respect to glutamate is needed to obtain maximum labeling of glutamate.

3. Results

3.1. Kinetic of derivatization

Fig. 1 shows the correlation between the increase in moles of FITC and the increase in the glutamate signal made by the minimum square method (y= 0.530*log$x$+4.975; r=0.897). At the sixth point, which represents a 43.8 molecules in excess of FITC, the curve reached an asymptotic level. This means that 50 molecules in excess are needed to obtain maximum labeling of a 10^{-5} *M* glutamate solution.

3.2. Calibration curve

Fig. 2 shows a linear relationship in the 10^{-8}–10^{-6} *M* range of glutamate standards derivatized with 10^{-4} *M* FITC for 16 h (y=143 610x−1821y; r=0.99). There was a 8:1 signal-to-noise ratio for 10^{-8} *M* glutamate solution. This indicates that it is possible to have an acceptable signal-to-noise ratio for detecting glutamate in the submicromolar range.

The dialysates collected every 6 s showed electropherograms with many peaks corresponding to primary amine compounds labeled with FITC plus several unidentified FITC peaks (see Fig. 3). Some of these ghost peaks were identified by means of the blank solution. The glutamate peak in the dialysate was identified by migration time and by spiking with the standard solution. (see Fig. 4). A glutamate peak with a signal-to-noise ratio greater than 30:1 was identified in the dialysates.

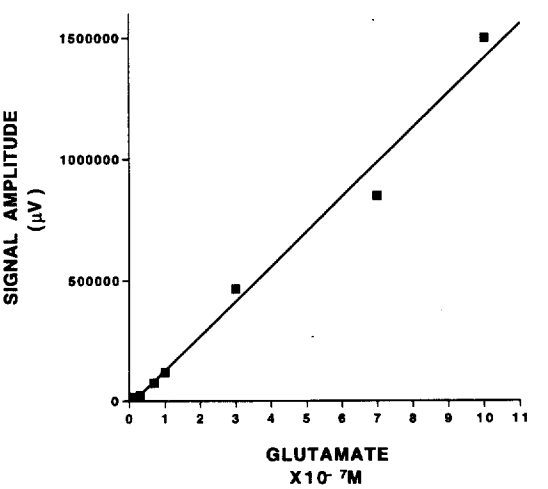

Fig. 2. Glutamate calibration graph: linear regression of the glutamate concentration versus the signal size.

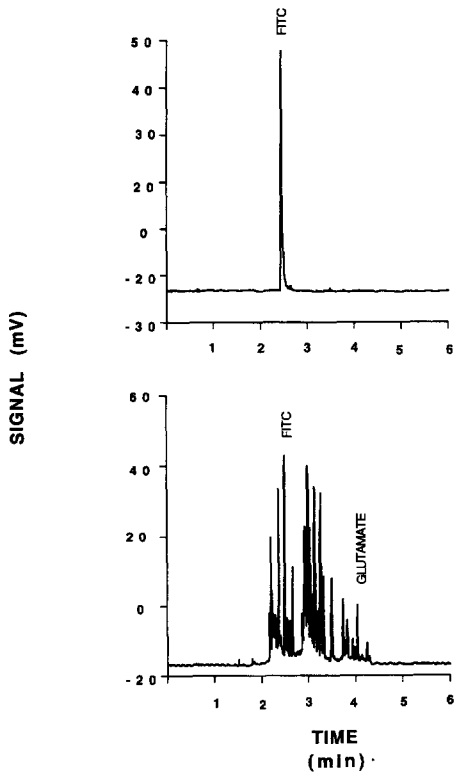

Fig. 3. Electropherograms of the blank which contains ACSF derivatized with FITC (top) and the electropherogram of a brain dialysate (bottom). In the first electropherogram there is only one peak which corresponds to FITC. The second electropherogram shows the peak of the FITC but in addition other peaks that correspond to primary amines, including amino acids and peptides. The peak shown with a star corresponds to glutamate.

3.3. First experiment

When ACSF with 60 m*M* KCl passed through the probe, glutamate and aspartate signal showed a significant increase while other peaks like glutamine and γ-aminobutyric acid did not change significantly. When the perfusion liquid was changed to normal ACSF, glutamate and aspartate levels returned to basal levels. (see Fig. 5)

3.4. Second experiment

In ketamine anesthetized rats, glutamate peaks became smaller immediately after the onset of the ischemia and then started to gradually increase when

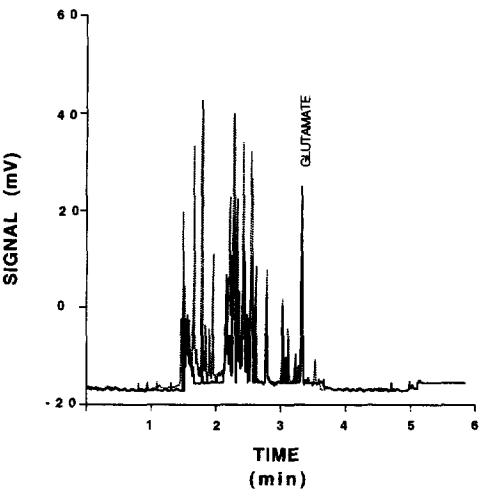

Fig. 4. Electropherogram of a parietal cortex dialysate overlaid with the same dialysate mixed with the standard solution of glutamate (spiking), the electropherograms show that there is an increase of glutamate peak while other peaks decrease.

the ischemia was over. In sodium thiopental anesthetized rats glutamate levels increased significantly 1 min after the onset of the ischemia, when the artery

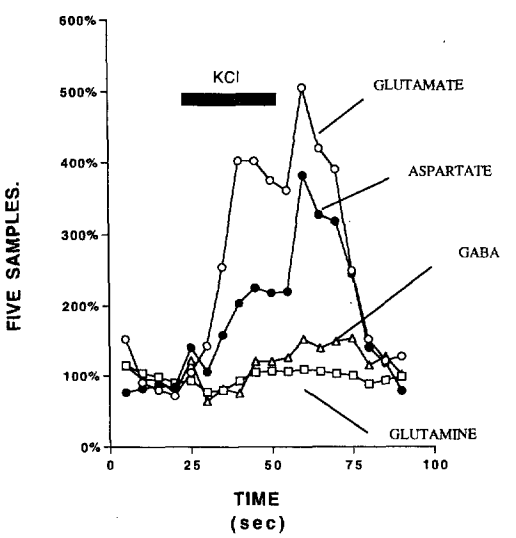

Fig. 5. The increase of glutamate and aspartate levels after perfusing the probe with ACSF containing KCl 60 m*M*, while other amino acids as glutamine and γ-aminobutyric acid did not change significantly.

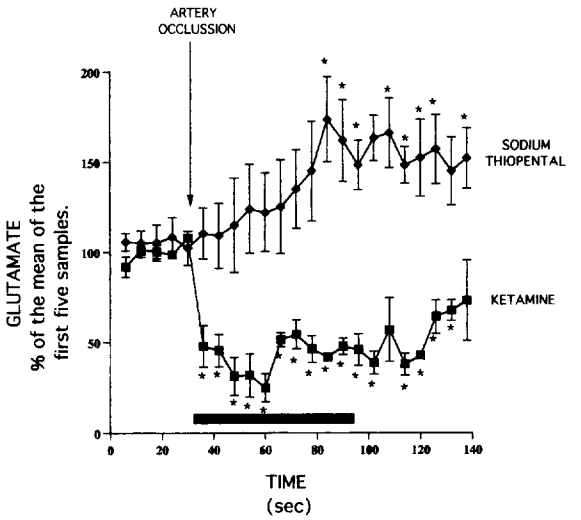

Fig. 6. The curve that represents the variation of glutamate levels during artery clamping (hatched bar) *=$P<0.0001$.

was released. Other amino acids, such as glutamine and γ-aminobutyric acid, did not change during ischemia in any case. (see Fig. 6).

4. Discussion

The present experiment proves that it is feasible to measure glutamate in a few seconds by combining brain microdialysis with CZE–LIFD. Since only a small fraction of the final sample volume (1 nl out of 1400 nl) is needed for the analysis, at least in theory it is possible to measure glutamate in fractions of a second with the experimental tools described here.

The small volume needed for analysis might contribute to lowering the perfusion flow-rate keeping an acceptable time resolution. Several experiments suggest that perfusion flow-rates greater than 100 nl min^{-1} cause significant neurotransmitter depletion in the neighborhood of the dialysis probe [26,27]. However, perfusion flow-rates have to be kept above 1 μl min^{-1} to provide sufficient sample for the majority of the analytical techniques used in conjunction with brain microdialysis. The coupling of brain microdialysis with CZE–LIFD reported here only needs a few nanoliters of sample which might better fulfil the demands of low perfusion flow-rates.

The response time of the dialysis probe was proved in the first experiment and it was shown that this response time is very fast because the increase of glutamate and aspartate levels can be seen in the first 5 s of stimulation of the tissue with KCl. The decrease of glutamate immediately after clamping can be excluded because the decrease is not observed in sodium thiopental anesthetized rats. However, we have shown (Rada et al., in preparation) that 60% of the glutamate collected with the microdialysis probe is suppressed by adding tetrodotoxin to the perfusion fluid or 30% by perfusing the probe with Ca^{2+}-free ACSF. These experiments suggest that the glutamate detected in the present report is partly of synaptic origin rather than of blood or glial origin and this warrants further research using this technology.

Many reports show that glutamate increases after hypoxia [5,28,29]. These results do not contradict the present results. In most of those experiments, collection times longer than 2 min and drastic blood-flow reductions are common. For instance, very often, simultaneous occlusion of vertebral as well as carotid arteries are used [30,31]. When local ischemia is used, the middle cerebral artery is occluded close to its branching from the carotid artery. As a consequence massive infarction is obtained. In most of the experiments, glutamate has been monitored in the basal ganglia or in rather deep structures such as the hippocampus [32]. By contrast, in the present experiment collection times of 6 s, localized infarction and cortical dialysates are used, alternatively the anesthetic agent in the present experiment (ketamine) is a blocker of glutamate receptors of the NMDA kind. This blockage might contribute to this anomalous response. These technical differences might explain the disparate results.

The glutamate peak is not the only one that decreases after artery occlusion. Some other peaks decrease too. These peaks correspond to primary amines labeled with FITC. They include several amino acids and peptides. A few of them such as aspartate have been identified. Other peaks corresponding to glutamine and γ-aminobutyric acid have also been identified and their peaks do not modify after the occlusion of the MCA. Therefore, the present combination of brain microdialysis and CZE–LIFD increases the amount of biologically active analytes that can be simultaneously studied with 6 s time resolution.

Other compounds found in brain dialysates including norepinephrine and dopamine have been studied with CZE–LIFD [33,34]. By using the 442 nm line of HeCd laser and NDA as derivatizing reagent, norepinephrine and dopamine were detected in cortical dialysates. The perfusion flow-rates was set at 200 nl min^{-1} and the collection time at 18 min. The continuous-flow reactor described in the present report might substantially reduce the collection time to a few seconds in that experimental arrangement.

In any event, the present results suggest that an improvement of the time resolution of brain microdialysis might be necessary to enhance our comprehension of the mechanisms of neurotransmitter release when in vivo monitoring methods are used. These results also suggest that the time resolution of brain microdialysis can be substantially reduced by properly choosing the analytical technique.

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