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Short communication

Detection of basal acetylcholine in rat brain microdialysate

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Abstract

A liquid chromatography–electrochemistry (LC–EC) method is described for the determination of basal acetylcholine (ACh) in microdialysate from the striatum of freely moving rats. This method is based on the separation of ACh and choline (Ch) by microbore liquid chromatography followed by passage of the effluent through a post-column immobilized enzyme reactor (IMER), containing acetylcholinesterase (AChE) and choline oxidase (ChO), and then the electrochemical detection of the hydrogen peroxide produced. Instead of the conventional platinum electrode generally used for the anodic detection of hydrogen peroxide, a peroxidase-redox polymer modified glassy carbon electrode operated at + 100 mV vs. Ag/AgCl has been used to detect the reduction of hydrogen peroxide. With this method, a detection limit of 10 fmol (injected) for ACh ($S/N = 3{:}1$) was obtained and the basal ACh concentration in striatal microdialysate was determined without using esterase inhibitors.

1. Introduction

Microdialysis sampling coupled with liquid chromatography–electrochemistry (LC–EC) has proven to be a very efficient method to measure the release of acetylcholine (ACh) in the brain tissue of freely moving rats [1,2]. The detection of acetylcholine and choline (Ch) using the LC–EC approach was first reported by Potter et al. [3]. In their method, Ch and ACh were separated by reversed-phase LC and post-column allowed to mix/react with a solution containing acetylcholinesterase (AChE) (EC 3.1.1.7) and choline oxidase (ChO) (EC 1.1.3.17) to generate hydrogen peroxide. The resulting hydrogen peroxide was detected in a thin-layer amperometric cell equipped with a platinum working electrode. Damsma et al. [4,5] extended this method by covalent immobilization of AChE and ChO in a post-column reactor. The immobilization of AChE and ChO significantly increased the stability and efficiency of the original approach. Continuing efforts have been made to improve the separation and detection of ACh and Ch by reducing the column size [6], optimizing the column packing and enzyme immobilization procedure [7], and modifying the electrochemical detector [8].

The extracellular concentration of ACh in rat brain is typically very low due to its rapid conversion to Ch by the action of AChE. Many investigators have used esterase inhibitors to restrain this conversion so that the ACh con-

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centration in the rat brain tissue could be measurable. These esterase inhibitors may affect the physiology of the system and thus the accuracy of data interpretation. A low detection limit for ACh is required to allow the investigator to determine basal ACh in the rat brain without using an esterase inhibitor. Damsma et al. [9] achieved a detection limit of 50 fmol ($S/N = 2$) for ACh and Ch, and detected 250 fmol of ACh in a 100-μl microdialysate sample. The large sample size required a long microdialysis sampling time and resulted in an average value of the in vivo ACh. This is obviously not desirable for researchers whose focus is on the dynamics of the neurosystem. Xu et al. [2] and Greaney et al. [8] have obtained a detection limit of 20 fmol for ACh, and observed basal ACh release in 20- and 10-μl microdialysate samples, respectively. From their chromatograms, however, the ratio of the basal ACh signals to the noise is not very satisfactory. Overall, the measurement of basal ACh release in microdialysate in the absence of an esterase inhibitor is still quite challenging.

In all the previous determinations of ACh and Ch using the LC–EC approach, Pt was used as the working electrode to detect the hydrogen peroxide from the AChE–ChO immobilized enzyme reactor (IMER). The Pt electrode has the disadvantages of long equilibration time and lack of stability at the high amplification required for basal ACh determinations. Vreeke et al. [10] recently developed a redox polymer "wired" peroxidase (EC 1.11.1.7) electrode for the detection of hydrogen peroxide. The authors coated the surface of a glassy carbon electrode with a redox polymer film containing peroxidase and measured the reduction current of hydrogen peroxide with this modified electrode. We utilized a similar peroxidase electrode in a flowing system for the determination of oxidase substrates with LC and an appropriate post-column IMER. Compared with the conventional Pt electrode, the peroxidase-modified electrode exhibited significant improvements in sensitivity, detection limit, and operational stability [11]. In the present study, the peroxidase electrode was used with microbore LC coupled with an AChE–ChO IMER for the determination of basal ACh release in rat brain microdialysates.

2. Experimental

2.1. Materials

Horseradish peroxidase (Type VI, EC 1.11.1.7) was purchased from Sigma (St. Louis, MO, USA). The Os-poly(vinylpyridine) redox polymer (Os(PVP)) was synthesized as described by Gregg and Heller [12]. Acetylcholine chloride, choline chloride, and neostigmine bromide were obtained from Sigma. All other chemicals were analytical reagent grade, from Sigma or Aldrich (Milwaukee, WI, USA). Ringer's solution was prepared as follows: 147 mM Na^+, 2.3 mM Ca^{2+}, 4 mM K^+, 155 mM Cl^-, pH 6.0.

2.2. Microdialysis sampling

Sprague-Dawley rats were anesthetized with an intraperitoneal injection of 1 ml/kg of KX [10 ml ketamine (100 mg/ml) + 1 ml xylazine (100 mg/ml)], after which a guide cannula (MF-5429, BAS, West Lafayette, IN, USA) was stereotaxically implanted. The animals were allowed to recover from surgery for approximately 4 days. On the day of the experiment, the guide cannula dummy stylette was removed and replaced with a microdialysis probe (CMA/12 4 mm, CMA, Stockholm, Sweden). The probe was perfused with Ringer's solution at 2 μl/min via a syringe pump (MF-5102, BAS). After 5 h of perfusion with Ringer's solution, as a comparison experiment, the probe in the same rat was perfused with Ringer's solution containing 10 μM neostigmine. In both cases, the dialysate fractions were automatically collected (CMA/142 fraction collector, CMA) into small plastic vials and the collection time for each sample was 10 min. Microdialysis samples (5 μl) were then injected directly onto the LC system. During microdialysis sampling, the awake unrestrained rat was housed in the BAS "Beekeeper Rodent Residence" containment and swivel system

(MD-1575, BAS). All the experimental protocols were approved by the BAS Animal Care and Use Committee.

2.3. Chromatography

The chromatography was performed using a BAS 480 liquid chromatography system (PM-80 pump, LC-4C amperometric detector, CC-5 flow cell and column compartment, DA-5 data acquisition interface, and ChromGraph software for data collection and analysis). The peroxidase-redox polymer coated glassy carbon electrode was prepared as described previously by Vreeke et al. [10] and operated at +100 mV vs. Ag/AgCl. The analytical column was a microbore cation exchange column (530 × 1 mm I.D., 10 μm) (MF-8904, BAS). The enzyme reactor column (50 × 1 mm I.D., 10 μm) (MF-8903, BAS) containing AChE and ChO was inserted between the analytical column and the electrochemical detector. The back pressure of this system was about 3000 PSI. The mobile phase contained 50 m*M* Na_2HPO_4 and 5 ml/l Kathon CG reagent (CF-2150, BAS), pH 8.0. The flow-rate was 140 μl/min. A vacuum degassing unit (MF-8500, BAS) was used to partially degas the mobile phase. All experiments were carried out at room temperature.

3. Results and discussion

Fig. 1 shows typical chromatograms for ACh/Ch standards. These chromatograms demonstrate that the separation of ACh and Ch with the present microbore LC–IMER system is quite satisfactory. Injections of 10 fmol ACh–Ch consistently gave signal-to-noise ratios above 3 as shown in Fig. 1B. Therefore, 10 fmol is determined as the practical detection limit for ACh in this study. The very good proportionality of the ACh/Ch responses to their injected amounts can be seen in Figs. 1B and 1C. The response was found to be linear with the amount of ACh injected over at least two orders of magnitude. The r^2 value from the linear regression of these

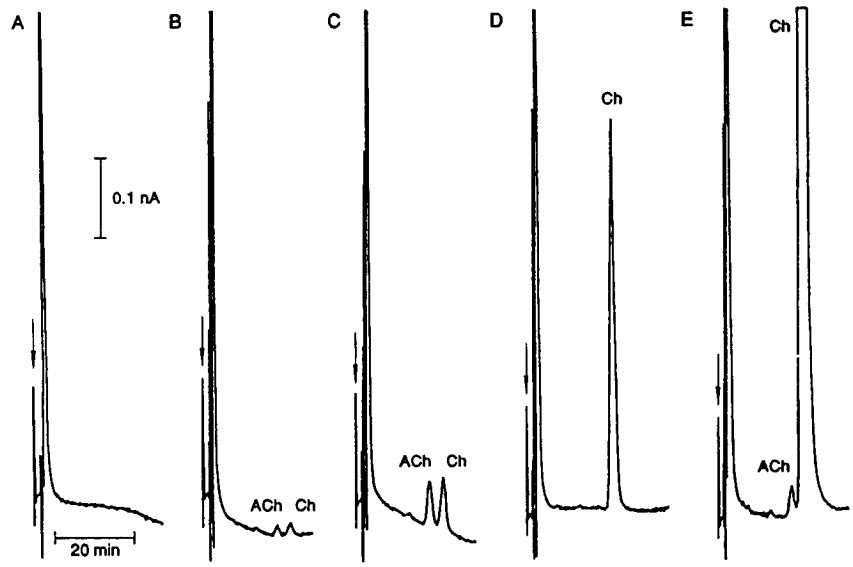

Fig. 1. Chromatograms of: (A) Ringer's solution; (B) 10 fmol ACh/Ch standard; (C) 50 fmol ACh/Ch standard; (D) 500 fmol Ch standard; (E) rat striatal microdialysate. The sample size was 5 μl. The electrode was operated at +100 mV vs. Ag/AgCl. Arrows indicate the injection times.

data is 0.999, and the slope of the linear regression line is $9.01 \cdot 10^{-4}$ nA/fmol. With this LC–IMER–EC system, the linear range of the response could be limited either by the reaction kinetics at the electrode or by the enzymatic reaction kinetics in the IMER. A previous study [11] using the wired peroxidase electrode suggested that the reaction kinetics of the IMER is most likely the determining factor for the linear range of the response.

Fig. 1E shows the chromatogram of a rat brain microdialysate sample collected without the use of any esterase inhibitors. The basal striatal concentration of ACh in the dialysate is determined to be 31 ± 5 (mean $\pm$ S.D., $n = 6$) fmol/5 μl. The concentration of Ch can be seen to be much higher and off-scale in the chromatogram. During the 5 h of perfusion with Ringer's solution without any esterase inhibitors, the concentration of ACh in the rat dialysate samples was relatively stable. After the 5 h of perfusion with Ringer's solution, the perfusion solution was changed from Ringer's solution to Ringer's solution containing 10 μM neostigmine. The concentration of ACh in the dialysates increased by 10 times during the first hour of perfusion with the neostigmine-containing solution, and increased further with lengthened perfusion. Meanwhile, the rats appeared to become increasingly active during the neostigmine perfusion, indicating an effect of the esterase inhibitor on the physiology of the animal.

The above results indicate that the present LC–EC system provides a sufficiently low detection limit for basal ACh determination in a very small sample (5 μl). This high sensitivity is attributable to the use of both the peroxidase electrode and the microbore LC column. Compared with a normal bore column (3–5 mm), the microbore column can reduce the dispersion of the sample and thus increase the sensitivity of the system. Our previous basic study of the peroxidase electrode [11] demonstrated that it was four times as sensitive as the conventional Pt electrode for the detection of hydrogen peroxide. Therefore, the use of the peroxidase electrode in this study distinguishes the present LC–EC method in sensitivity from the previous LC–EC methods, where the conventional Pt electrode was used.

In addition to its high sensitivity and low detection limit for ACh, the present LC–EC system has also shown satisfactory operational stability. For this LC–EC system, there are two sources of instability. These are the AChE–ChO IMER and the enzyme-modified electrode, both of which are limited by enzyme stability. We found that the AChE–ChO IMER of this system did not exhibit any decrease in response over a six-month period, during which it had been used intensively and stored at 4°C while not in use. As to the peroxidase electrode, no significant variation in electrode response was observed after one week of continuous operation. The peroxidase enzyme electrode requires less than 2 h for initial equilibration.

The peroxidase electrode operated at +100 mV vs. Ag/AgCl exhibited a background reduction current. It was found that the LC pump noise was proportional to the magnitude of this reduction current. In order to achieve a low detection limit for ACh, the noise and thus the background current need to be minimized. The background reduction current was found to increase as the oxygen concentration in the mobile phase increased. Because oxygen is necessary for the ChO catalyzed reaction in the IMER, it cannot be simply eliminated from the system to reduce the background current. In the present study, a vacuum degassing unit was used to reduce the oxygen concentration of the mobile phase and thus the background current. A piece of microbore Teflon tubing (I.D. = 0.2 mm) was used to connect the analytical column and the IMER in order to regain some oxygen and maintain the activity of the IMER.

This report describes a sensitive method for the determination of ACh. With this system, which utilized a novel enzyme-modified electrode and a microbore separation column, a practical detection limit of 10 fmol was obtained for ACh. The presented data suggest that the basal ACh concentration in rat brain microdialysates can be determined even in a very small sample (5 μl) without the use of an esterase inhibitor.

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