Evaluation of in vivo and in vitro recovery rate of anatoxin-a through the microdialysis probe

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A B S T R A C T

In vivo microdialysis is a versatile sampling technique commonly employed to observe changes in neurotransmitters levels that occur in response to different treatments, being these treatments administered through a microdialysis probe implanted into a specific brain region in living animals. In previous works we have used this technique to study the effects of the drug anatoxin-a, a nicotinic acetylcholine receptor agonist, on dopamine release in striatum. The aim of the present study was to assess the recovery of anatoxin-a through the microdialysis probe. This information allows knowing the exact amount of the drug crossing the microdialysis membrane, acting on extracellular tissue. High Performance Liquid Chromatography (HPLC) with Fluorescence Detection (FLD) has been used for the analysis of anatoxin-a. We observed that the recovery of anatoxin-a was about 0.5%. Under our experimental conditions, the results suggest that anatoxin-a can be used as an important tool in the study of neuronal nicotinic receptors by in vivo microdialysis technique and also show a reliable estimation of the anatoxin-a recovery through the microdialysis probe under both in vivo and in vitro conditions.

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1. Introduction

In vivo microdialysis is a versatile sampling technique commonly employed to observe changes in neurotransmitters levels that occur in response to different treatments, being these treatments administered through a microdialysis probe implanted into a specific brain region in living animals (Chaurasia, 1999; Elmquist and Sawchuk, 1997; Hansen et al., 1999).

The microdialysis probe has a small dialysis membrane at its tip which allows the movement of extracellular compounds, as neurotransmitters, into the probe and the diffusion of different treatments, as drugs or toxins, from the probe to the extracellular tissue, depending on the concentration gradient. Treatments are administered through the inlet tube of the probe and subsequently the neurotransmitters are collected from the outlet tube of the probe (Fig. 1).

Since the movement of substances occurs under non-equilibrium conditions and because the microdialysis membrane acts as a semipermeable barrier excluding the transport of larger molecules, only a fraction of the treatment administered will be able to cross the microdialysis membrane to the extracellular tissue, and only a fraction of the extracellular neurotransmitters will be found in the microdialysates samples (Sasongko et al., 2000).

The amount of treatment which crosses the microdialysis membrane in relation to the concentration...
concentration of the drug diffused outside and concentration of the drug perfused.

...where \( R \) is the recovery in percentage, \( C_{\text{out}} \) is the concentration of the drug diffused outside and \( C_{\per} \) is the concentration of the drug perfused.

This method is limited since in vivo physiological factors which act on membrane probe, such as extracellular tortuosity, are not taken into account. By this, assessment of probe recovery performance in vivo is required to ensure the validity of the recovery data obtained using in vitro procedures (Chaurasia, 1999; Zhou and Gallo, 2005).

In previous works, we have been using the toxin, anatoxin-a, to study the involvement of neuronal nicotinic receptors on striatal dopamine release by means of the microdialysis technique (Campos et al., 2006a,b, 2007).

Anatoxin-a is a low molecular weight alkaloid (165 Da) neurotoxin produced by some toxigenic strains of cyanobacteria, principally Anabaena and Oscillatoria genera. It was responsible for the death of livestock, pets and wildlife and it has also been implicated in cases of human illness (Carmichael et al., 1975). This toxin acts as a potent agonist on nicotinic acetylcholine receptors in the central and peripheral nervous system as well as in the neuromuscular junction (Spivak et al., 1980). In fact, anatoxin-a has been observed to be a more potent agonist than the typical nicotinic agonist, nicotine, and the endogenous agonist, acetylcholine (Swanson et al., 1986; Amar et al., 1993; Thomas et al., 1993). Moreover, this toxin is a secondary amine and not an ester, being resistant to enzymatic cholinesterase hydrolysis. These properties make it a useful natural tool in the study of neuronal nicotinic receptors (Aronstam and Witkop, 1981; Macallan et al., 1988).

In line with this, in our previous works about the effects of anatoxin-a, in vitro studies carried out to assess the drug recovery through microdialysis probe, we have estimated a recovery value around 0.5% (Gago-Martinez et al., 2003), however this data has not been confirmed yet with in vivo experiments in living animals.

The aim of the present manuscript was to demonstrate the use of this toxin as an important tool in the study of neuronal nicotinic receptors by in vivo microdialysis technique and to assess a reliable estimation of the in vitro and in vivo recoveries of anatoxin-a through the microdialysis probe. High Performance Liquid Chromatography with Fluorescence detection (HPLC/FLD) has been used to evaluate the concentration of anatoxin-a.

2. Materials and methods

2.1. Animals

Female Sprague-Dawley rats (250–300 g) were used for all experiments. Rats were housed in plastic cages under controlled temperature conditions (22 ± 2 °C) and light/dark cycles (14 h/10 h) with free access to food and water. The experiments were performed according to the Guidelines of the European Union Council (86/609/EU) on the use of laboratory animals.

2.2. Chemicals

Anatoxin-a fumarate was purchased from Tocris (Bristol, UK), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was purchased from Sigma–Aldrich (Gillingham, UK). All other solvents used were HPLC grade and were supplied from Panreac (Barcelona, Spain). Deionized water was prepared...
by passing glass-distilled water through a Milli-Q water purification system. Stock standard solution (1 mg/ml) of anatoxin-a fumarate was prepared by using Milli-Q water and stored in an amber glass vial at −20 °C. Working solutions were prepared daily and stored in dark at 4 °C.

2.3. Protocol of anatoxin-a administration

In previous microdialysis studies (Campos et al., 2006a,b, 2007) concentration of 3.5 mM anatoxin-a was chosen as a control dose to study its effects on striatal dopamine release. In the present study, all experiments were performed using the same protocol administration and the same microdialysis materials. Briefly, anatoxin-a was dissolved in the perfusion medium (Ringer’s medium: 147 mM NaCl, 4 mM KCl, 3.4 mM CaCl2; pH 7.4), and perfused through microdialysis probe into the Eppendorf vial or striatum nucleus at rate flow of 1.5 μL/min over 20 min by means of a microdialysis infusion pump (CMA/Microdialysis, Sweden). Subsequently, the microdialysis samples were collected using a CMA/142 microsampler (CMA/Microdialysis, Sweden). The microdialysis probes used (CMA/Microdialysis, Sweden) had a polycarbonate–polyether copolymeric membrane with a molecular cut-off of 20,000 Da and a length of 3 mm (cut-off reflects the highest weight of a substance which can cross the membrane probe).

2.4. In vitro microdialysis recovery

Following the same procedure mentioned in Section 1, the in vitro recovery of the probe was evaluated. With this aim, at the beginning of every experiment, the inlet tube of the microdialysis probe was connected to the infusion pump and the outlet tubing was connected to the microsampler. After the elimination of any dead volume in the system, the probe was placed in an Eppendorf vial containing 1 ml of Ringer’s solution and subsequently 3.5 mM anatoxin-a was perfused at rate flow of 1.5 μL/min over 20 min. At the end of the experiment, the Eppendorf vials were used to assay the amount of anatoxin-a crossing the membrane probe.

2.5. In vivo microdialysis recovery

In order to check the in vivo recovery of 3.5 mM anatoxin-a, the toxin was administered in the striatum of living animals through the microdialysis probe. After that, the striatum where the probe was located was taken out and the concentration of anatoxin-a diffused to the neuronal tissue was determined.

Surgical insertion of microdialysis probe was performed according to our previous studies (Faro et al., 2002; Alfonso et al., 2003). In brief, female rats (250–350 g) were anaesthetized (i.p.) with chloral hydrate (400 mg/kg) and placed in a stereotaxic apparatus (Narishige SR-6) for the implantation of a guide-cannula. The skull was exposed and a hole was drilled through the skull in the area overlying the right striatum, using the following coordinates with respect to Bregma: A/P +1 mm; M/L +3 mm, D/V +6 mm according to Paxinos and Watson (Paxinos and Watson, 1986). A guide-cannula was lowered into the brain and fixed to the skull with miniature screws and acrylic dental cement and the incision was closed with sutures. Surgery was performed using sterile instruments and aseptic conditions. Right location of the probe was determined on serial coronal sections (Fig. 2) and only data obtained from rats with correctly implanted probes were included in the results. All experiments were made with awake, conscious and freely moving rats.

Twenty-four hours after surgery, the inlet tube of the probe was connected to the infusion pump and the outlet tube was connected to the microsampler. After the removal of the dead volume in the system, the microdialysis was slowly inserted through the guide-cannula into the striatum and perfused for 1 h with Ringer’s medium (following the same protocol used in our previous works) and subsequently 3.5 mM anatoxin-a was administered for 20 min at a flow rate of 1.5 μL/min. After anatoxin-a administration, the probe was taken out and the animal sacrificed. The entire right striatum was taken out by means of free hand dissection and subsequently the amount of anatoxin-a in it was determined.

2.6. Extraction of anatoxin-a from striatum

The conditions previously used for the analysis of anatoxin-a in tissue samples by HPLC/FLD (Rellan et al., 2007), were applied in this work (with some modifications) for the analysis of anatoxin-a from striatal samples. Striatal samples (50 mg approximately) were homogenized for 1 min by sonication using an ultrasonic processor (Vibra cell™, Sonics & Materials inc, Danbury, CT., USA) in 1 ml of perchloric acid 0.1 M in freeze. The solution was ultracentrifuged at 3000 g for 15 min. The supernatant was collected and make up to a final volume of 5 ml with ultrapure water.

The sample extracts were adjusted to pH 7 with NaOH 0.1 mM before solid-phase extraction (SPE) clean-up using a weak cation-exchange material, Supelclean LC-WCX 3 ml tubes (Supelco, Bellefonte, PA, USA). The SPE cartridge was conditioned with methanol (6 ml) and water (6 ml). The extracts were transferred to the cartridge and washed with 3 ml of methanol 100%. After air drying the cartridges for 30 min, the anatoxin-a was eluted using 10 ml methanol containing 0.2% trifluoroacetic acid and then the solvent was evaporated under nitrogen stream at 40 °C. Samples obtained were re-dissolved in 1 ml of methanol and kept at −22 °C in amber vials until HPLC/FLD analysis.

2.7. Analysis of anatoxin-a

HPLC/FLD analysis of anatoxin-a was performed after derivatisation with 4-fluoro-7-nitro-2,1,3-benozox-adizole (NBD-F) (James et al., 1998). Samples of anatoxin-a from in vitro and in vivo recovery experiments and standards solutions were evaporated and then reconstituted with 100 μL of sodium borate (0.1 M) in a 2 ml amber vial. 50 μL of NBD-F in acetonitrile (1 mg/ml) were added and the mixture was allowed to stand (10 min) in the dark at room temperature. 100 μL of hydrochloric acid (1 M) were added to terminate the reaction. HPLC–FLD was performed.
directly on the products (20 μl injection) using a LC reversed phase, Luna C18 column (5 μm, 250 × 4.6 mm i.d., Phenomenex) at room temperature. The mobile phase was acetonitrile–water (50:50) with a flow rate of 0.8 ml/min. The excitation and emission wavelengths for fluorimetric detection were set at 470 and 530 nm respectively. The HPLC system consisted of a quaternary pump, Agilent 1100 Series QuatPump a degasser Agilent 1100 Series and a Fluorescence detector Agilent 1100 FLD (Agilent Technologies, Waldbronn, Germany). Under these experimental conditions the detection and the quantification limit were 8 and 29 ng/g of anatoxin-a, respectively (Rellan et al., 2007).

2.8. Statistical analysis

In vivo and in vitro recovery data are presented as the mean (%) ± S.E (n = 9). The comparisons of results were made using a two-tailed Student’s paired t-test.

3. Results

3.1. Analysis of anatoxin-a standards

Ten microlitre of anatoxin-a standard solution (20 μg/ml) was derivatized with NBD-F following the procedure above described and 20 μl of derivate was then injected into the HPLC system. Fig. 3 shows the results for the HPLC/FLD analysis of 20 ng a standard solution of anatoxin-a.

3.2. Analysis of anatoxin-a from in vitro recovery experiments

To assess the in vitro recovery of anatoxin-a, the concentration of the toxin diffused to the Eppendorf vials where the probe was located, was compared with the concentration of the standard solution perfused. Fig. 4 shows the results corresponding to the standard solution of anatoxin-a and a sample solution from Eppendorf vials. After considering the dilution factors, the ratio between the levels of anatoxin-a in samples and standard solutions was 0.45 ± 0.12%. These results suggest that when the in vitro perfusion of 3.5 mM is performed for 20 min at a flow of 1.5 μl/min (17.04 μg of anatoxin-a/striatum), only 76.7 ng of anatoxin-a/striatum ± 20.5 would be able to cross the membrane probe.
3.3. Recovery of the extraction of anatoxin-a from the neuronal tissue

Due to the loses of anatoxin-a in the extraction procedure from neuronal tissue, the assessment of the extraction of anatoxin-a from the neuronal tissue was necessary. With this aim, neuronal samples (50 mg) were spiked with a known amount of anatoxin-a (5 ng). After the analysis, the amount of anatoxin-a present in the tissue samples was 4.25 ng/striatum ± 0.25 (Fig. 5). The recovery found for this extraction was 85 ± 5% (n = 3).

3.4. Analysis of anatoxin-a from in vivo recovery experiments

The analysis of anatoxin-a present in neuronal samples was carried out after the extraction and purification in the optimized conditions previously described. The quantification of the anatoxin-a present in these samples was carried out taking into account the dilution factors as well as the recovery percentage previously found (Fig. 6). The recovery percentage found for this in vivo experiments was 0.5 ± 0.15% being these results significantly similar than the ones observed in previous in vitro experiments (Fig. 7). Therefore, under our experimental conditions, when we perfused anatoxin-a 3.5 mM for 20 min at a flow of 1.5 µl/min (17.04 µg of anatoxin-a/striatum), 86.6 ng of anatoxin-a/striatum ± 25 were administered in the striatum.

4. Discussion

Anatoxin-a has been used extensively in in vitro experiments, mainly in slices and synaptosomes, in order to study the nicotinic receptors subtypes involved in the release of different neurotransmitters such as glutamate, noradrenaline, acetylcholine or dopamine in different brain regions (Clarke and Reuben, 1996; Wilkie et al., 1996; Sershen et al., 1997; Marchi et al., 1999; Anderson et al., 2000; Cao et al., 2005). However, in contrast with other nicotinic agonists, this drug has never been employed in in vivo microdialysis studies.

With the aim of comparing the effects of anatoxin-a on striatal dopamine release with those observed in in vitro works, for the first time, we checked the effects of this drug by in vivo microdialysis technique. In agreement with in vitro works, we found that the in vivo effects of anatoxin-a were inhibited by neuronal nicotinic receptors antagonists and being this mediated by an exocytotic mechanism (Campos et al., 2006a, 2007).

Studies about the drugs effect by in vivo microdialysis technique made necessary to check that those are able to cross the membrane probe to the external neuronal tissue. In our case, we observed that the effect of anatoxin-a on dopamine release was inhibited by nicotinic antagonists, such as, mecemylanine, methyllycaconitine or α-bungarotoxin (Campos et al., 2006a). Being anatoxin-a a nicotinic agonist, these observations, show that this drug is able to cross the membrane probe acting on neuronal tissue.

However, the common way to check if the drug used is able to cross the membrane probe and to know the exact amount of drug acting on neuronal tissue is to estimate the recovery of the drug through the probe.

As above mentioned, the recovery of a drug through the microdialysis probe is defined as the rate between the amount of the drug which crosses the membrane and the known drug concentration perfused. This date can be estimated by in vitro or in vivo experiments, analyzing the levels of the drug which crosses the membrane of the probe.

In both cases, in vitro and in vivo recovery, it is necessary to employ a specific analytical method to quantify the drug levels in the samples. In the case of in vivo recoveries, it is necessary a previous step, the extraction and purification of...
the drug from the tissue for subsequent analyses (see Section 3).

Owing to this, when a drug or other chemical agent is employed to study its effects by in vivo microdialysis technique, it becomes very complicated to assess its recovery because there are not specific analytical methods to do so, or because the method is very complicated. This problem is also bigger in the case of in vivo recovery, due to the purification step before analysis. Because of that, the most practical way is to assume that in vitro recovery is a reliable estimation of in vivo recovery (Sasonget al., 2000). Nevertheless, this procedure is not exact because factors such as tortuosity of interstitial space, volume of the interstitial compartment, pressure of the tissue on the membrane probe and also certain physiological barriers (e.g., blood–brain barrier) are not taken into account (Chaurasia, 1999). Indeed, some authors suggest that in vivo methods to assess the recovery in microdialysis experiments are more reliable and allow a better estimation than those from the in vitro methods (Zhou and Gallo, 2005).

In previous studies, using an HPLC/UV method for the analysis of anatoxin-a, we estimated that the in vitro recovery of this toxin was about 0.5% (Gago-Martinez et al., 2003). The sensitivity of this method was not enough to be applied to the analysis of the striatum from rats treated with anatoxin-a to assess the in vivo recovery, in addition the UV method was not selective enough due to a number of interferences of endogenous compounds.

The lack of information about the in vivo recovery represents an important limitation to compare the effects of anatoxin-a on in vivo dopamine release with other nicotinic agonists, because it is necessary to ensure the exact amount of the toxin acting on neuronal tissue in living animals.

To overcome the limitations of the sensitivity for the determination of the recoveries of anatoxin-a, an HPLC method with Fluorescence detection was used which conditions had been optimised in a previous work (Rellan et al., 2007). Under our experimental conditions, we observed that the in vitro and in vivo of recoveries of anatoxin-a were about 0.5%, being these results significantly similar than the ones observed with the HPLC/UV method. These data

Fig. 6. Chromatogram corresponding to the analysis of anatoxin-a from in vivo recovery experiments. Chromatogram a shows the analysis of a neural tissue sample from striatum of animals previously treated with anatoxin-a (1.7 ng). Little chromatograms shows (in different scale) the analysis of a standard solution of anatoxin-a (20 ng). After considering the dilution factors as well as the recovery extraction of anatoxin-a from striatal neural tissue, the recovery percentage was 0.5 ± 0.15%.

Fig. 7. Representation of in vitro and in vivo data. Recovery data are presented the mean (%) ± S.E. (n = 9). The comparisons of in vivo and in vitro results were made using a two-tailed Student’s paired t-test.
suggest that if we perfused a dose of 3.5 mM over 20 min (17.04 µg of anatoxin-a), only 86.6 ng of anatoxin-a would be able to cross the membrane of the probe to the neuronal tissue, reflecting why it is necessary to use high doses of anatoxin-a (3.5 mM) to study its effects by in vivo microdialysis technique. Obviously, the recovery of this toxin will be different if change the experimental conditions: perfusion flux, time recovery sample or kind of probe. In our case, the same recovery observed in vitro and in vivo, suggests that the assessment of in vivo recovery it is not affected by tissual factors, contrary to what we at first thought.

Among the different agonists used in the study of nicotinic receptors, nicotine is one of the most widely used. In a previous study about this agonist on striatal dopamine release, nicotine is one of the most widely used. In the in vivo case, the same recovery observed in vitro and in vivo, suggests that the assessment of in vivo recovery it is not affected by tissual factors, contrary to what we at first thought.

In conclusion, under our experimental conditions, our results show that anatoxin-a can be used as an important tool in the study of neuronal nicotinic receptors by in vivo microdialysis technique and also show a reliable estimation of the anatoxin-a recovery through the microdialysis probe under both in vivo and in vitro conditions.

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Conflict of interest

No conflict of interest.

References


