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ORIGINAL PAPER

In vivo Effects of the Anatoxin-a on Striatal Dopamine Release

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Abstract Anatoxin-a is an important neurotoxin that acts a potent nicotinic acetylcholine receptor agonist. This characteristic makes anatoxin-a an important tool for the study of nicotinic receptors. Anatoxin-a has been used extensively in vitro experiments, however anatoxin-a has never been studied by in vivo microdialysis studies. This study test the effect of anatoxin-a on striatal in vivo dopamine release by microdialysis.The results of this work show that anatoxin-a evoked dopamine release in a concentration-dependent way. Atropine had not any effect on dopamine release evoked by 3.5 mM anatoxin-a. However, perfusion of nicotinic antagonists mecamylamine and α-bungarotoxin induced a total inhibition of the striatal dopamine release. Perfusion of α7*-receptors antagonists, metillycaconitine or α-bungarotoxin, partially inhibits the release of dopamine stimulated by anatoxin-a. These results show that anatoxin-a can be used as an important nicotinic agonist in the study of nicotinic receptor by in vivo microdialysis technique and also support further in vivo evidences that α7*nicotinic AChRs are implicated in the regulation of striatal dopamine release.

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Introduction

Anatoxin-a is a low molecular weight alkaloid (165 Da) neurotoxin produced by some toxigenic strains of cyanobacteria, principally *Anabaena* and *Oscillatoria* genera. This toxin is responsible for the death of livestock, pets and wildlife and it has also been implicated in cases of human illness [1]. Typical symptoms in animals include muscle fasciculation, gasping and convulsion, with death due to respiratory arrest within minutes after drinking contaminated water. This toxin is perhaps one of the most toxic of the cyanobacterial toxins [2, 3].

Anatoxin-a acts as a potent agonist on nicotinic acetylcholine receptors in the central and peripheral nervous system and in the neuromuscular junction [4, 5]. In fact, anatoxin-a has been observed to be more potent agonist than the typical nicotinic agonist, nicotine, and the endogenous agonist, acetylcholine [5–7]. Moreover, this toxin is a secondary amine, not an ester, being resistant to enzymatic hydrolysis. These properties make it an useful natural tool in the study of neuronal nicotinic receptors [8, 9].

Functional nicotinic receptors exist as heteromeric complexes, comprised of α (α2–α6) and β (β2–β4) subunits, or homomeric complexes, comprised of α (α7–α9) subunits [10]. However, a few subtypes of nicotinic receptors predominate, notably α4β2* and α7* (* denote the potential presence of additional subunits) subtypes are widespread in the vertebrate central nervous system [11–13].

A large body of evidence indicates that neuronal nicotinic receptors are present at presynaptic locations of the central nervous system, where they modulate the neurotransmitter release [14–16].

Anatoxin-a has been used extensively in vitro experiments (slices and synaptosomes) in order to identify the nicotinic receptors subtypes involved in neurotransmitters release: noradrenaline, acetylcholine or dopamine from hippocampus [17–20], acetylcholine from neocortex [21], glutamate from striatum [22] or noradrenaline from thalamus or frontal cortex [23].

However, anatoxin-a has been characterized, mainly, with respect to dopamine release from striatum, [18, 24–29], where there are a dense dopaminergic and cholinergic innervations [30–32].

In slices and synaptosomes from striatum, anatoxin-a induced [^{3}H]dopamine release in a concentration-dependent way, and this effect was blocked by mecamylamine (MEC), showing that anatoxin-a elicit the [^{3}H]dopamine release by interaction with nicotinic receptors [25–27].

Slices retain local anatomical integrity, enabling to observe neuronal interactions. However, synaptosomes represents isolated nerve terminals and provide a model for studying presynaptic receptors. With the aim to compare nicotinic receptors-evoked [^{3}H]dopamine release from rat striatal synaptosomes and slice preparations, the effect of anatoxin-a was observed in both kinds of neuronal preparations. It was observed that in slices, but not in synaptosomes, the [^{3}H]dopamine release induced by anatoxin-a was partially blocked by α7*-selective nicotinic receptors antagonists, α-bungarotoxin (α-bgt) or methyllycaconitine (MLA), suggesting that α7* nicotinic receptors are not present on dopaminergic terminals but they induce an indirect modulation of striatal [^{3}H]dopamine release.

These results show that the anatomical integrity of the preparations (slices and synaptosomes) play an important role in the evaluation of α7*receptors-mediated dopamine release.

However, to date, there are not studies using in vivo microdialysis technique about the striatal dopamine release and the involvement of α7* nicotinic receptor using anatoxin-a as nicotinic agonist. This technique let to see the effects of all neuronal interactions and therefore to achieve more physiological results rather in vitro preparations.

The purpose of the current study was to explore using in vivo microdialysis technique the effects of anatoxin-a on the dopaminergic neuronal system in the striatum of conscious and freely moving rats and try to test the involvement of α7* nicotinic receptors observed in slices experiments.

Methods

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.

Chemicals

Anatoxin-a and MLA were purchased from Tocris (USA); MEC, atropine and α-bgt were acquired from Sigma, St Louis (USA). All other chemicals and reagents were of analytical grade.

Microdialysis procedure

Surgical and perfusion procedures were performed according to previous studies [33]. Briefly, rats were anaesthetized (i.p.) with chloral hydrate (400 mg kg^{-1}) and placed in a stereotaxic apparatus Narishige SR-6) for the implantation of a guide-cannula. The coordinates for guide-cannula implantation above the striatum were 1 mm A/P, +3 mm L/M and +6 mm D/V, according to the rat brain atlas of Paxinos and Watson [34]. The skull was exposed, and a small hole was drilled in the skull over the left striatum. A guide-cannula was lowered into the brain and fixed to the cranium with miniature screws and acrylic dental cement and the incision was closed with sutures. Surgery was performed using sterile instruments and aseptic conditions.

The experiments were carried out 24 h after implantation of the guide-cannula. A microdialysis probe (CMA/Microdialysis, Sweden) with a 3 mm membrane length was inserted through the guide-cannula into the striatum. Continuous perfusion was performed with a Ringer's solution (147 mM NaCl, 4 mM KCl, 3.4 mM $CaCl_2$; pH 7.4) using a CMA/102 infusion pump (CMA/Microdialysis, Sweden).

Microdialysis samples were collected every 20 min by means of a CMA/142 microsampler (CMA/Microdialysis, Sweden) in order to quantify dopamine, dihydroxyphenilacetic acid (DOPAC) and homovanillic acid (HVA).

All experiments were made with awake, conscious and freely-moving rats.

At the end of experiments, animals were killed with an overdose of anaesthetic and brains were removed and fixed for subsequent sectioning in order to determine the location

of the microdialysis probe (Fig. 1). Placements were evaluated according to the atlas of Paxinos and Watson [34]. Only data from animals with correct probe placements in striatum were used.

Drug treatment

Initial studies were conducted to determinate the optimum concentration for the antagonist for these studies. Concentrations of antagonists selected (MEC, 1.75 mM; α-bgt, 40 μM; atropine, 3.5 mM; or MLA, 3.5 mM) did not affect the basal dopamine release from striatum (see Results). Higher concentrations could not be used because *per se* changed the basal levels of dopamine (data not shown). The concentration of antagonists selected was also higher than the concentrations used in other microdialysis studies [35–43].

All drugs used were dissolved in the perfusion fluid and administered locally into the striatum through the microdialysis probe. Dialysates were collected every 20 min and after four basal samples (80 min) the drugs were administered. Anatoxin-a was infused at different concentrations (1, 2, 3.5 and 7 mM) during 20 min at a flow rate of 1.5 μl min^{-1}. After this, the perfusate was switched back to the unmodified perfusion medium and the infusion continued in the absence of anatoxin-a for 150 min.

In other experimental groups, 1.75 mM MEC, 3.5 mM MLA and 3.5 mM atropine, were administered for 40 min, (20 min before anatoxin-a administration and 20 min together with 3.5 mM anatoxin-a, in the same microdialysis solution) at a flow rate of 1.5 μl min^{-1}.

Because the relatively high molecular weight of α-bgt limits its diffusion through the microdialysis membrane, it was directly injected into the striatum. Before anatoxin-a administration, 4 μl of α-bgt 40 μM was injected into the striatum (0.4 μl min^{-1} for 10 min). Higher flows were not tested in order to prevent neuronal damage by direct administration. Similar administration was used in previous microdialysis studies [35].

In another experiment, MEC and α-bgt were infused with anatoxin-a. MEC was administered for 40 min (20 min before anatoxin-a administration and 20 min with anatoxin-a 3.5 mM) at a flow rate of 1.5 μl min^{-1}, and α-bgt 40 μM (0.4 μl min^{-1} for 10 min) was directly injected into the striatum before of anatoxin-a and MEC co-administration.

Assay of dopamine and metabolites

The samples obtained from the microdialysis procedure (30 μl) were used to quantify the levels of dopamine and metabolites using High-Performance Liquid Chromatography (HPLC) with electrochemical detection [44]. The mobile phase contained 70 mM KH_2PO_4, 1 mM octanesulfonic acid, 1 mM EDTA, and 10% methanol. Dialysates were injected into a Hewlett-Packard Series 1050 Liquid Chromatograph, using a Rheodyne 7125 injection valve. Compounds were separated on a Phenomenex reverse-phase column type nucleosil 5 μ C18. Elution was carried out at a flow rate of 1.8 ml min^{-1}. The dopamine and metabolites detection was achieved using an ESA Coulochem 5100 electrochemical detector at a potential of

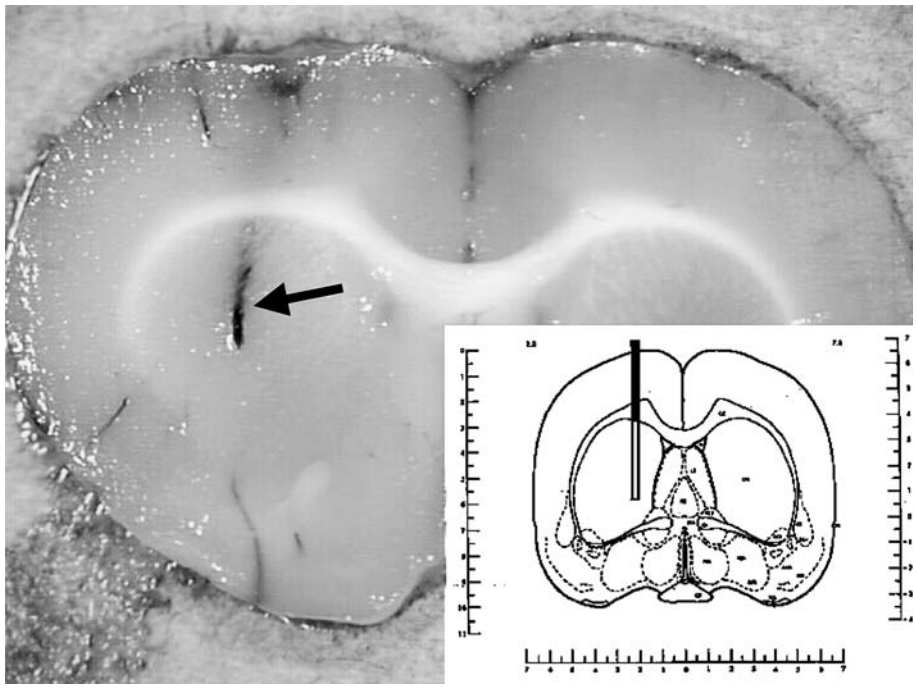

Fig. 1 Histological presentation of the microdialysis probe location in the rat striatum, following coordinates with respect to bregma, A/P +1 mm; M/L +3 mm, D/V +6 mm, according to Paxinos and Watson (1986). The arrow mark the probe tract

+400 mV. The retention times of dopamine and its metabolites were as follows: dopamine 8 min, DOPAC 12 min and HVA 15 min.

In vitro recoveries of dopamine, metabolites and anatoxin-a

The determination of the diffusion rate of dopamine, DOPAC and HVA across the dialysis membrane was carried out in vitro. So, the dialysis probe was placed in an standard solution of dopamine and metabolites (50 pg μl^{-1}) and flushed with Ringer at the same flow as in vivo conditions (1.5 $\mu l\ min^{-1}$ for 20 min). Levels of dopamine and metabolites in the dialysates were determined by HPLC. Recoveries were calculated from the concentration of substance in the perfusion fluid divided by its concentration in the standard solution. The recoveries of dopamine, DOPAC and HVA were over $16 \pm 0.18\%$, $19 \pm 0.24\%$ and $21 \pm 0.15\%$, respectively. All values of extracellular dopamine and metabolites were corrected using these recoveries.

The diffusion rate of anatoxin-a through the microdialysis probe was also estimated in vitro. In this case, the anatoxin-a was dissolved in Ringer solution and it was perfused through the dialysis probe. It was placed in an Eppendorf tube containing 1 ml of Ringer solution. The flow rate was the same as used in the experiments with freely-moving rats (1.5 $\mu l\ min^{-1}$). Under these conditions, the diffusion rate of anatoxin-a across the microdialysis membrane was 0.5 ± 0.08 % for 20 min. In this case, samples of anatoxin-a were measured by HPLC using fluorescence detection [45]. Therefore, perfusion of 3.5 mM anatoxin-a during 20 min, suggest that 5.15 ± 0.3 nmoles of anatoxin-a are administered in the striatum.

Expression of results and statistics

The results are shown as the mean ± S.E.M. respect to the basal levels, for 5–6 animals per group, except in the 3.5 mM anatoxin-a experimental group formed by 20 animals. The average concentrations of three stable samples before drugs administration were considered as the basal levels. These basal levels were taken as 100% in order to compare the different response of dopamine and metabolites after drug administration.

Statistical evaluation of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test, considering the following significant differences: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, respect to the basal and a $P < 0.01$ and b $P < 0.05$ with respect to anatoxin-a administration.

Results

The basal striatal dopamine, DOPAC and HVA contents were 0.15 ± 0.4, 13 ± 1.1 and 4 ± 0.87 ng 20 μl^{-1} of sample, respectively.

Effect of different doses of anatoxin-a on basal striatal dopamine release

Anatoxin-a (1 mM) had not significant effects on striatal dopamine levels. Perfusion of 2 mM anatoxin-a during 20 min evoked an increase in striatal dopamine levels of $225 \pm 15\%$. The maximum increase of dopamine was obtained 20 min after anatoxin-a administration and dopamine returned to the basal levels at 60 min. The perfusion of 7 and 3.5 mM doses of anatoxin-a for 20 min increased the dopamine levels to $1440 \pm 45\%$ and $632 \pm 51\%$, respectively. The maximum increase was obtained 40 min after anatoxin-a administration and dopamine returned to the basal at 80 min.

Intraestriatal infusion of anatoxin-a through the dialysis probe evoked increases in extracellular dopamine levels in a concentration-dependent way (Fig. 2).

No notable behavioural activation was observed during or after anatoxin-a perfusion.

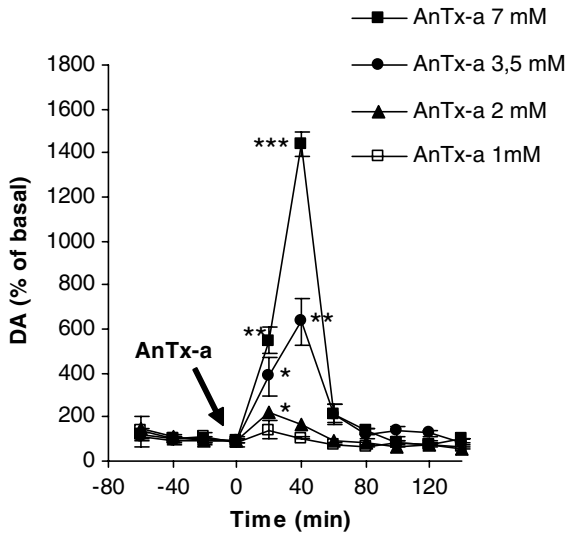

Fig. 2 Effects of intraestrial perfusion of different concentrations of anatoxin-a (AnTx-a) (1, 2, 3.5 and 7 mM) on dopamine (DA) extracellular levels from rat striatum. Arrow denotes the infusion of anatoxin-a during 20 min. The results are shown as means ± S.E.M. of 5–6 experiments with the doses of 1, 2, and 7 mM; and 20 animals per group for dose of 3.5 mM expressed as a percentage of the basal levels (100%). Basal levels were considered as the mean of dopamine concentration in the three samples collected before toxin administration. Significant differences: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ in respect to the basal levels. Statistical evaluation of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test

The perfusion of the four doses of anatoxin-a had no effect on dopamine metabolites, DOPAC and HVA. These are low at the beginning of every experiment, and then increase until stabilization, but there are no changes due to the anatoxin-a. These effects were similar to that induced by perfusion of Ringer medium alone (Fig. 3a and 3b).

Because that higher doses of anatoxin-a (7, and 3.5) mM induced significant increased of dopamine release, we select the dose of 3.5 mM as a control group (n=20) to observe the effects of the different treatments on dopamine release induced by the toxin (Figs. 2, 4–8).

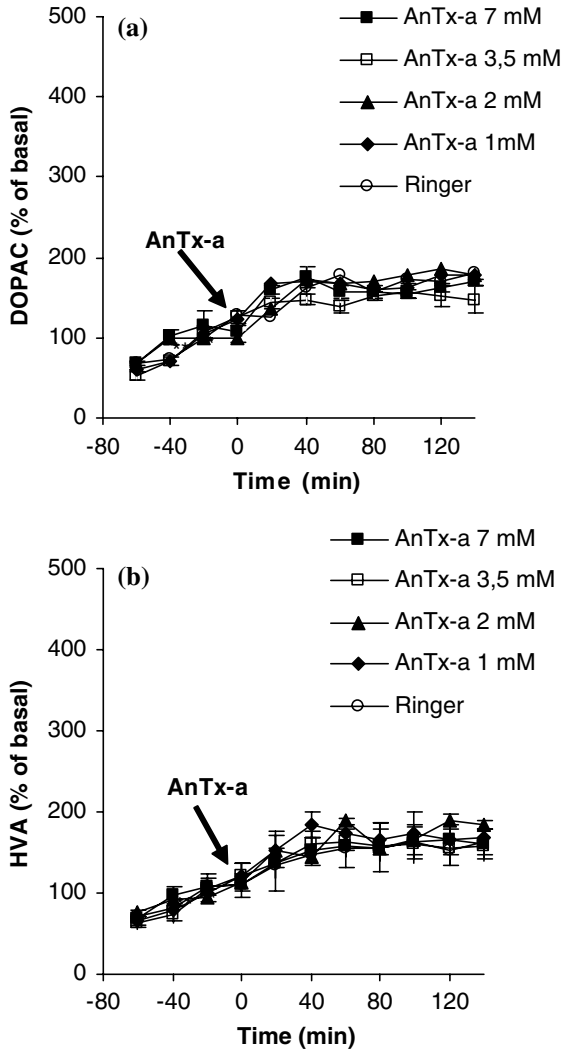

Fig. 3 Effects of intraestrial perfusion of different concentrations of anatoxin-a (AnTx-a) (1, 2, 3.5 and 7 mM) on (a) DOPAC and (b) HVA extracellular levels from rats striatum. Arrow denotes the infusion of anatoxin-a during 20 min. The results are presented as means ± S.E.M., expressed as a percentage of the basal levels (100%). Basal levels were considered as the mean of substance concentration in the three samples collected before anatoxin-a administration. Statistical evaluation of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test

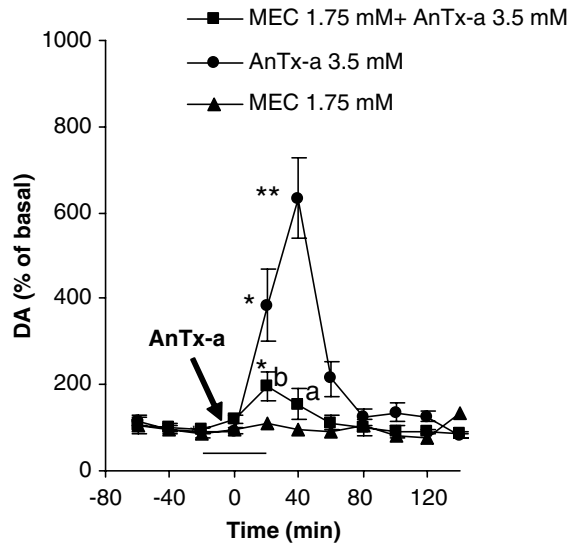

Fig. 4 Effects of MEC on anatoxin-a (AnTx-a) induced dialysate dopamine (DA) content in rat striatum. Anatoxin-a infusion started at the time indicated by the arrows over 20 min, and infusion of MEC was indicated by the black bar (40 min). The results are presented as means ± S.E.M., expressed as a percentage of the basal levels (100%). Basal levels were considered as the mean of dopamine concentrations in the three samples collected before anatoxin-a administration. Significant differences: $^{*}P<0.05$, $^{**}P<0.01$, respect to the basal levels and ^{a} $P<0.01$, ^{b} $P<0.05$ respect of the anatoxin-a alone administration. Statistical evaluation of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test

Effect of MEC on dopamine release stimulated by anatoxin-a

In order to test if striatal dopamine release induced by anatoxin-a is mediated by interaction with nicotinic receptors, the effect of anatoxin-a was studied in presence of MEC, a non-competitive nicotinic receptor antagonist [46]. About 1.75 mM MEC was administered as a control for 40 min, and no modification of dopamine basal levels was observed. The perfusion of anatoxin-a and MEC together decreased the dopamine release stimulated by anatoxin-a. In this case, the dopamine release was 196 ± 34%, being this increase 69% less than that observed with anatoxin-a alone (Fig. 4). These results show that anatoxin-a acts through nicotinic receptors evoking dopamine release.

Effect of atropine on dopamine release evoked by anatoxin-a

To investigate the possible interaction of muscarinic receptor on dopamine release evoked by anatoxin-a, the effect of anatoxin-a was studied in the presence of atropine, a muscarinic receptor antagonist. Infusion of 3.5 mM atropine for 40 min produced not significant effect on dopamine levels in striatum. Coinfusion of anatoxin-a and

atropine increased extracellular dopamine levels, with a maximum value reaching 512 ± 57%, 40 min after the treatment, and dopamine returned to the basal levels in 60 min. This effect was significantly similar to that obtained with anatoxin-a alone (Fig. 5).

Effect of MEC and α-bgt on dopamine release stimulated by anatoxin-a

In this experiment we observed the effect of anatoxin-a in presence of MEC and a selective α7*-receptor antagonist, α-bgt [47]. Perfusion of 1.75 mM MEC and 40 μM α-bgt not induce increase of striatal dopamine release. When 3.5 mM anatoxin-a was administrated in presence of MEC and α-bgt no significant effects respect to the basal levels were observed (Fig. 6).

The total inhibition of dopamine release by MEC and α-bgt show that effect of anatoxin-a is only mediated through interaction with nicotinic receptors.

Effect of MLA on dopamine release stimulated by anatoxin-a

To investigate if the α7* receptors could be implicated in the dopamine release induced by anatoxin-a, we infused MLA, a selective nicotinic α7*-receptor antagonist [12], through the microdialysis probe. Intrastrial infusion of

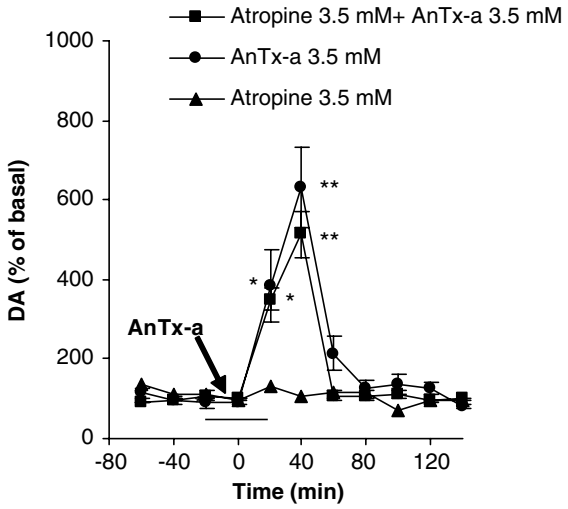

Fig. 5 Effect of atropine on anatoxin-a (AnTx-a) induced dopamine (DA) content in rat striatum. Anatoxin-a infusion started at the time indicated by the arrows over 20 min, and infusion of atropine is indicated by the black bar (40 min). The results are presented as means ± S.E.M., expressed as a percentage of the basal levels (100%).Basal levels were considered as the mean of dopamine concentrations in the three samples collected before anatoxin-a administration. Significant differences: $*P<0.05$, $**P<0.01$ respect to the basal levels. Statistical evaluation of the results were performed by means of ANOVA and Student–Newman–Kelps multiple range test

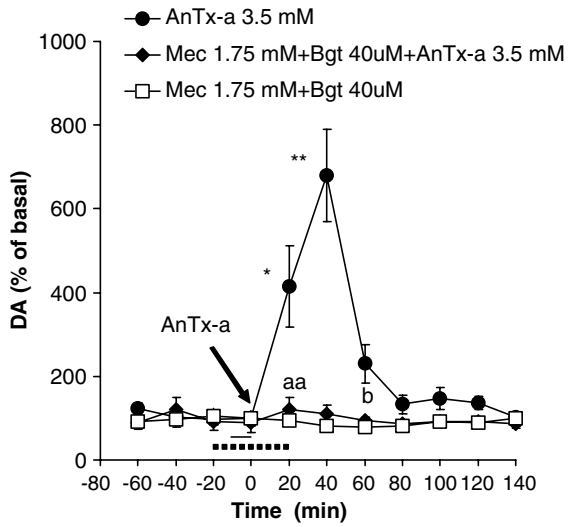

Fig. 6 Effects of MEC and α-Bgt on anatoxin-a (AnTx-a) induced dialysate dopamine (DA) content in rat striatum. Anatoxin-a infusion started at the time indicated by the arrows over 20 min, infusion of MEC is indicated by the discontinuous black bar (40 min), and α-Bgt is indicated by the black bar (10 min) The results were presented as means ± S.E.M., expressed as a percentage of the basal levels (100%). Basal levels were considered as the mean of dopamine concentrations in the three samples collected before anatoxin-a administration. Significant differences: $*P<0.05$, $**P<0.01$, respect to the basal levels and $^{a}P<0.01$, $^{b}P<0.05$ respect of the Anatoxin-a alone administration. Statistical evaluation of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test

3.5 mM MLA for 40 min had not significant effects on striatal dopamine levels. When anatoxin-a and MLA were administered, the dopamine release was 260 ± 49%, respect to the basal levels (Fig. 7). This increase in extracellular dopamine levels was 58% smaller than that observed with anatoxin-a alone, showing the participation of α7* receptors on the effects of anatoxin-a on striatal dopamine release.

Effect of α-bgt on dopamine release evoked by anatoxin-a

To confirm the involvement of α7*-selective nicotinic receptors on dopamine release induced by anatoxin-a, α-bgt was infused together with anatoxin-a. Injection 40 μM α-bgt into the striatum had no significant effects on dopamine basal levels. Infusion of anatoxin-a in α-bgt pretreated animals increased dopamine levels to 311 ± 35% over the basal (Fig. 8). In this case, the increase of dopamine levels was 49% smaller than that observed with anatoxin-a alone.

Metabolites of dopamine

The effect of the different treatments (MEC, MLA, α-bgt and atropine) on the dopamine metabolite levels, DOPAC

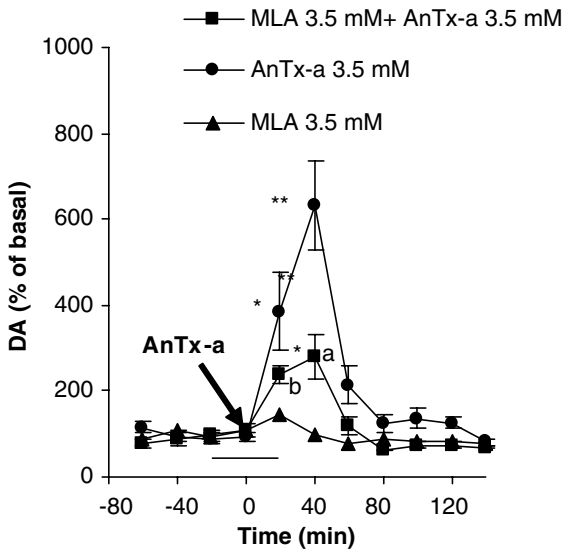

Fig. 7 Effect of MLA on anatoxin-a (AnTx-a) induced dialysate dopamine (DA) content in rat striatum. Anatoxin-a infusion started at the time indicated by the arrows over 20 min, and infusion of MLA was indicated by the black bar (40 min). The results are presented as means ± S.E.M., expressed as a percentage of the basal levels (100%).Basal levels were considered as the mean of anatoxin-a concentrations in the three samples collected before anatoxin-a administration. Significant differences: $*P<0.05$, $**P<0.01$ respect to the basal levels and ^{b} $P<0.05$ respect of the anatoxin-a alone administration. Statistical evaluation of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test

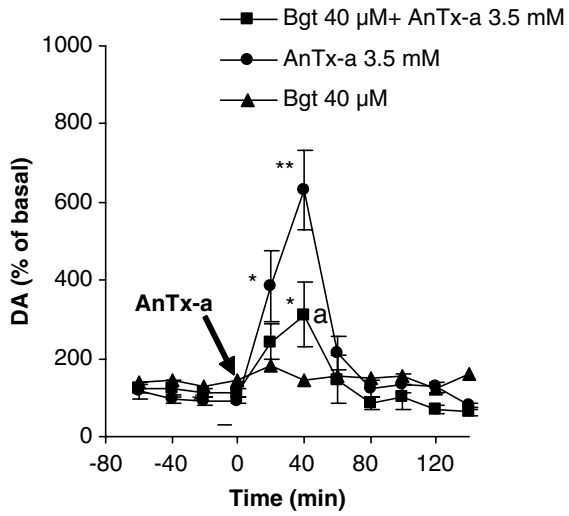

Fig. 8 Effect of α-Bgt on dopamine (DA) extracellular levels from rat striatum induced by anatoxin-a (AnTx-a). Anatoxin-a infusion started at the time indicated by the arrows over 20 min, and injection of α-Bgt was indicated by the black bar (10 min). The results are presented as means ± S.E.M., expressed as a percentage of the basal levels (100%).Basal levels were considered as the mean of dopamine concentration s in the three samples collected before anatoxin-a administration. Significant differences: $*P<0.05$, $**P<0.01$ respect to the basal levels and ^{b} $P<0.05$ respect to the anatoxin-a alone administration. Statistical evaluation of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test

and HVA, were similar to that observed with the different doses of anatoxin-a alone (results not shown).

Discussion

The presence of nicotinic acetylcholine receptors in presynaptic terminals in the central nervous system is well established and it is believed that modulatory actions by stimulating transmitter release or by enhancing synaptic efficacy are considered to constitute a major role of neuronal nicotinic acetylcholine receptors [16]. Nicotinic receptors are implicated in the presynaptic modulation of neurotransmitters release, including glutamate, GABA, norepinephrine, dopamine and acetylcholine in different brain areas [16, 48]. However, the most studied example of presynaptic nicotine modulation is the release of dopamine from striatum [49–53].

Striatum has a dense local innervations from cholinergic interneurones that closely interacts with dopaminergic projections [32], principally from the substantia nigra (nigrostriatal pathway). Distinct subtypes of nicotinic receptors modulate the release dopamine in the striatum, and the identification of nicotinic receptors subtypes involved in this process has been extensively studied [54].

Different nicotinic agonist have been used widely in the study of function of nicotinic receptors subtypes involved in the striatal dopamine release, such as nicotine, lobeline, cytisine or epibadidine, as studied in vitro using striatal slices [55–58] or synaptosomes [59–62] and in vivo microdialysis [57, 63–66]. Anatoxin-a, in the same way than those nicotinic agonists, has been used extensively in the study of nicotinic receptors-involvement on dopamine release from striatum using in vitro studies (slices and synaptosomes). However, to date, anatoxin-a, has never been used by in vivo microdialysis in the study of nicotinic receptors.

In the present study, we have investigated using in vivo microdialysis technique the effects of anatoxin-a on striatal dopamine release and we tried to compare these in vivo results with the effects of anatoxin-a observed in some in vitro experiments.

Under our experimental conditions, when anatoxin-a was administered through the microdialysis probe, it evoked a concentration-dependent increase in dopamine output in striatum. That anatoxin-a elicited extracellular dopamine release by interacting with nicotinic receptors was established by the ability of MEC to partially block the response of the toxin.

These results are consistent with previous in vitro experiments about the effect of anatoxin-a on [3H]dopa-

mine release. Using striatal slices and synaptosomes loaded with [3H]dopamine, anatoxin-a evoked [3H]dopamine release in a concentration-dependent and mecamylamine-sensitive way [25, 26, 29]. Also, similar findings using nicotine, lobeline or epibatidine were observed by in vivo microdialysis studies from striatum [37, 65, 67].

Our in vivo results, concentration-dependent and mecamylamine-sensitive, demonstrate that anatoxin-a evoke the dopamine release in the terminal fields of the nigroestriatal pathway, being consistent with the presynaptic nicotinic modulation of dopamine, established preparations in vitro [16, 25, 26, 29].

In all experiments, extracellular levels of metabolites DOPAC and HVA were not changed after treatment with anatoxin-a or nicotinic antagonists. The lack of effect of anatoxin-a on metabolites levels could indicate that anatoxin-a does not interfere in the processes of reuptake and/or metabolism of dopamine. These results agree with previous in vivo studies made with nicotine [37].

To support that dopamine release induced by anatoxin-a was of neuronal origin, in previous studies (under submission), we observed that this effect was blocked by tetrodotoxin and free-calcium medium, being consistent with the exocytotic dopamine release observed in synaptosomes studies [25, 27].

The dopamine release induced by anatoxin-a was not completely blocked by MEC. This observation may indicate that anatoxin-a could also act on non-nicotinic receptors such as muscarinic receptors, inducing dopamine release or, that MEC dose used, was not enough to block all nicotinic receptors. It was observed that α7* nicotinic receptors are somewhat less sensitive to MEC than α/β receptors, requiring higher concentrations for full blockade of them [68].

Because muscarinic receptors are implicated in striatal dopamine release observed in some microdialysis experiments [69, 70], we decided to investigate weather the muscarinic receptors had any mediation, directly or indirectly, on anatoxin-a actions. Perfusion of atropine, a general antagonist of muscarinic receptors, had not any effect on dopamine release evoked by anatoxin-a, suggesting that muscarinic receptors are not involved in this response.

Perfusion of MEC and the selective α7* receptor antagonist α-bgt induced a total inhibition. These results confirm that the effect of anatoxin-a is only mediated through activation of nicotinic receptors and also show the participation of α/β and α7*-receptors.

As mentioned in the in the introduction, it was observed that [3H]dopamine release induced by anatoxin-a was blocked by α7*-receptors antagonist (MLA or α-bgt) in slices but not in synaptosomes, showing that evaluation of α7*-receptors on striatal dopamine release is only possible in preparations which preserve some of the anatomical integrity, such as slices preparations [25]. In vivo microdialysis represent more anatomical integrity than in slices, and we can observe the effect of anatoxin-a on all neuronal striatum system.

In similar way that in slices and synaptosomes experiments, we tested the effect of two α7*-selective nicotinic antagonists, MLA and α-bgt, on dopamine release induced by anatoxin-a. The results showed that MLA attenuated 59% of striatal dopamine release induced by anatoxin-a. Therefore, this inhibition show the mediation of α7*-nicotinic receptors in the effect of anatoxin-a on striatal dopamine release.

Investigations about the selective antagonist MLA on α7* nicotinic receptors suggest that this toxin could interact on dopamine neurons with other nicotinic subunits different to α7 [71]. To confirm the mediation of α7* nicotinic receptors in the effect of anatoxin-a on in vivo striatal dopamine release, we used α-bgt as a more α7-selective antagonist. The dopamine release induced by anatoxin-a with α-bgt was 49% smaller than that induced by anatoxin-a alone. In this case, the inhibition elicited by α-bgt was smaller than that elicited by MLA, although we cannot compare the inhibition percentage of the two antagonists because their administration in the striatum was different (see Methods).

The ability of both α7*-receptors antagonists to partially inhibit the in vivo dopamine release stimulated by 3.5 mM anatoxin-a confirm the involvement of α7* nicotinic receptors, as it have been observed in slices from striatum [25, 29]. Although the concentration of α7*-antagonist used in these experiments would be enough to block the receptors, we cannot to sure total blocked. However, to demonstrate the participation of α7* nicotinic receptors on dopamine release was the aim of this experiment, but not to test the effects of total blockade of them.

Kaiser and Wonnacott [29] suggested a model about the relationship between nicotinic receptors and presynaptic boutons in the rat striatum. The model implies that non-α7* nicotinic receptors on dopamine terminals stimulate dopamine release; however, α7* receptors on striatal glutamatergic nerve terminals elicit release of glutamate which, in turn, acts on ionotropic glutamate receptors on dopamine terminals, enhancing the dopamine release. This explain why only in more integral preparations such as slices or in our in vivo studies, it is possible to check the indirect modulation of α7* receptors on striatal dopamine, but not in synaptosomes.

In vivo studies in progress in our lab are investigating the involvement of glutamatergic receptors on dopamine release induced by anatoxin-a.

In a recent study, in slices with nicotine, it was observed that α7* receptors did not appear to play a significant role

in dopamine release from striatum [72]. A possible explanation for this finding, could be that α7-subtype is not very sensitive to nicotine, and the dose of nicotine used in this study 10 μM, not enough to active α7* receptors [73, 74]. In synaptosomes studies, using anatoxin-a, it was also demonstrated, that glutamatergic axon terminals posses nicotinic receptor of the α7 subtype, able to mediate enhancement of striatal glutamate release [22].

In the same work [22], it was observed that anatoxin-a was much more potent at the α7* nicotinic receptor regulating glutamate release (EC_{50}=2.1 nM) than the non-α7* nicotinic receptor mediating enhancement of dopamine (EC_{50}=110 nM) [26], showing that anatoxin-a could represent an important tool in the studio of α7* nicotinic receptors.

In conclusion, our results show that anatoxin-a can be used as an important nicotinic agonist in the study of nicotinic receptors by in vivo microdialysis technique. These results also support further in vivo evidences that α/β and α7*nicotinic AChRs are implicated in the striatal dopamine release induced by anatoxin-a.

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