Life Sciences 89 (2011) 931–938

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Characterization of a new muscarinic toxin from the venom of the Brazilian coral snake *Micrurus lemniscatus* in rat hippocampus

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ARTICLE INFO

Article history:
Received 23 March 2011
Accepted 28 September 2011
Available online 8 October 2011

Keywords:
Muscarinic acetylcholine receptor
Micrurus lemniscatus
Muscarinic toxin
Inositol phosphate
Elapidae
Three-finger toxin
Hippocampus
MT-Mlα

ABSTRACT

Aims: We have isolated a new muscarinic protein (MT-Mlα) from the venom of the Brazilian coral snake *Micrurus lemniscatus*.
Main methods: This small protein, which had a molecular mass of 7,048 Da, shared high sequence homology with three-finger proteins that act on cholinergic receptors. The first 12 amino acid residues of the N-terminal sequence were determined to be: Leu-Ile-Cys-Phe-Ile-Cys-Phe-Ser-Pro-Thr-Ala-His.
Key findings: The MT-Mlα was able to displace the [^{3}H]QNB binding in the hippocampus of rats. The binding curve in competition experiments with MT-Mlα was indicative of two types of [^{3}H]QNB-binding site with pK_i values of 9.08 ± 0.67 and 6.17 ± 0.19, $n = 4$, suggesting that various muscarinic acetylcholine receptor (mAChR) subtypes may be the target proteins of MT-Mlα. The MT-Mlα and the M_1 antagonist pirenzepine caused a dose-dependent block on total [^{3}H]inositol phosphate accumulation induced by carbachol. The IC_{50} values for MT-Mlα and pirenzepine were, respectively, 33.1 and 2.26 nM. Taken together, these studies indicate that the MT-Mlα has antagonist effect on mAChRs in rat hippocampus.
Significance: The results of the present study show, for the first time, that mAChRs function is drastically affected by MT-Mlα since it not only has affinity for mAChRs but also has the ability to inhibit mAChRs.

Introduction

Acetylcholine is a major neurotransmitter in the central and peripheral nervous systems (Caulfield, 1993; Wess et al., 1990). The nicotinic acetylcholine receptors (nAChRs) are activated by nicotine and belong to the ligand-gated ion channel superfamily, whereas muscarinic acetylcholine receptors (mAChRs) are activated by muscarine and belong to the family of G-protein-coupled receptors. The muscarinic actions of acetylcholine are mediated by five distinct mAChR subtypes (M_1 to M_5) (Caulfield, 1993; Caulfield and Birdsall, 1998; Wess, 1996). Different experimental approaches have shown that mAChRs are present in all organs, tissues or cell types (Caulfield, 1993; Levey, 1993; Vilaro et al., 1993; Wolfe and Yasuda, 1995). Central mAChRs are involved in regulating a large number of cognitive, behavioral, sensory, motor and autonomic functions (see Wess, 2004, for review). In addition, reduced or increased signaling through distinct mAChR subtypes has been implicated in the pathophysiology of several major diseases of the central nervous system, including Alzheimer's and Parkinson's disease, depression, schizophrenia and epilepsy (Eglen et al., 1999; Felder et al., 2000; Levine et al., 1999; Wess et al., 1990).

Muscarinic neurotoxins (MTs) from Elapidae venoms have been purified mainly from the venom of African green mamba (genus *Dendroaspis*) and characterized for their ability to inhibit the binding of selective muscarinic ligands such as [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) and [^{3}H]N-methylscopolamine to an enriched source of mAChRs (Adem et al., 1988; Bradley, 2000; Karlsson et al., 2000; Kornisiuk et al., 1995; Jolkkonen et al., 1994). Furthermore, MTs have been reported to possess high affinity for individual mAChR subtypes (Bradley, 2000; Bradley et al., 2003; Jerusalinsky and Harvey, 1994; Jerusalinsky et al., 2000).

Despite the fact that the MTs belong to a family of toxins containing three-finger type structures (Kini, 2002), they present functional differences and display distinct profiles as to their interactions with the various mAChR subtypes. MTs have a variety of functional characteristics, with competitive antagonist, allosteric modulator or even agonist effects (Bradley, 2000; Bradley et al., 2003; Servent and Fruchart-Gaillard, 2009). In addition to the classical MTs that were originally isolated from the genus *Dendroaspis* (Servent and Fruchart-Gaillard, 2009) other MT-like proteins endowed with PLA2 activity have been purified from *Naja naja sputatrix, Naja atra* (Chinese cobra) and *Crotalus atrox* venoms (Huang et al., 2008; Miyoshi and Tu,

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0024-3205
doi:10.1016/j.lfs.2011.09.024

1996; 1999; 2000). These molecules also display affinities for different mAChR subtypes.

Concerning the venoms of Brazilian snakes of the genus *Micrurus* (belong to Elapidae family), they are particularly rich in pre- and pos-synaptic neurotoxins that have the cholinergic system as their target of action (Dal Belo et al., 2005; Moreira et al., 2010; Silva et al., 1991; Vital-Brazil, 1987; Vital-Brazil and Fontana (1983/1984) However, few investigations have dealt with isolated toxins from venoms of these snakes. Some neurotoxins endowed of PLA2 activity (Oliveira et al., 2008) or neurotoxins that have high affinity to nAChRs (Olamendi-Portugal et al., 2008) have been isolated and characterized from genus *Micrurus*. In spite of the low incidence of Coral snake accidents and the fact that the signs and symptoms caused by *Micrurus sp.* envenomation are the result of a progressive blockade of neuromuscular transmission at the endplates, in severe cases, death may result from respiratory arrest (Olamendi-Portugal et al., 2008).

So, once the majority of the symptoms of the Coral snake envenomation are due to the involvement of the cholinergic nicotinic system, the effect of toxins acting on mAChRs has rarely been addressed by toxinologists. Moreover, MTs are minor components of the Coral snake venom which may impair the development of such studies. Although very few toxins targeting G protein-coupled receptors have been isolated and characterized from Elapidae venoms, MTs, different from the selective ligands described so far, are able to discriminate between diverse mAChR subtypes. Thus, their distinct pharmacological properties become valuable tools to the characterization of the structure and function of the peripheral and central nervous systems.

Taking this into consideration, we have undertaken a search for the components associated with mAChR in the Brazilian snake *Micrurus lemniscatus* venom. In the present work, we described for the first time the presence of a three-finger neurotoxin isolated from *M. lemniscatus* venom (MT-Mlα) with affinity for mAChRs. In the design of this work we performed the purification, molecular mass determination, partial amino acid sequencing of the MT-Mlα, and characterization of their effects on the inhibition of the binding of the selective muscarinic ligand [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) and inhibition of the [^{3}H]inositol phosphate accumulation in hippocampus from male rats.

Materials and Methods

Materials

The lyophilized *M. lemniscatus* crude venom was obtained from Laboratory of Venomous Animals, Federal University of Bahia, Brazil.

Animals

Male Wistar rats, 3 months old, were maintained on a 12 h light, 12 h dark schedule, at 22°C, with food and water *ad libitum*. The experimental procedures were conducted according to guidelines for the care and use of laboratory animals as approved by the Research Ethical Committee from Instituto Butantan (protocol nº.613/09).

RP-HPLC purification of M. lemniscatus venom

Crude *M. lemniscatus* venom (10 mg) was diluted in 5 ml of Milli-Q water. After filtration in a 0.45-μm filter (Millipore), 400-μL samples (800μg) were applied to a C8 reversed-phase column (Shim-Pack; 4.6 mm × 250 mm, 5μm particle) coupled to a HP 1100 series HPLC system. The elution was carried out under a flow rate of 1 mL.min^{-1}, and monitored at 214 nm. A linear gradient of trifluoroacetic acid (TFA) (solvent A) (0.1% TFA in water) and acetonitrile (solvent B) (90% acetonitrile + 10% A), from 10% to 35% of B over 50 min, was used to elute proteins. Nineteen peaks were manually collected according to the absorbance (Fig. 1). The peaks 1, 2, 3, 4, 5 and 6 were assayed for their ability to inhibit the binding of selective muscarinic ligands [^{3}H]quinuclidinyl benzilate. For this bioassay, only peak 6 was active and therefore was selected for further biochemical characterization and the [^{3}H]inositol phosphate measured.

Mass spectrometry

Previously lyophilized samples were dissolved into 50% Acetonitrile (ACN), containing 0.1% formic acid (FA) and deposited into the 384 well plate of the autosampler for ESI-MS analysis in a MSQ Surveyor mass system (Thermo Finnigan). Typically, 20 μL sample aliquots were infused in 50% ACN, 0.1% (FA), under a 50 μL.min-1 constant flow, in positive ionization. Instrument control, data acquisition and processing were performed by the XCalibur suite (Thermo Finnigan).

N-terminal sequence determination

For N-terminal sequence determination, MT-Mlα was re-purificated in a C18 RP–HPLC column (SUPELCOSIL-LC-18-DB 15 cm × 4.6 mm Cat. No. 58348) eluted with a 0 to 90% Acetonitrile (ACN JT Baker) gradient containing 0.1% of Trifluoracetic acid (TFA Sigma-Aldrich). Solvent A: 0.1% TFA (in Milli-Q water), solvent B 90%ACN (in 0.1% TFA). To determine the N-terminus sequence, the purified protein (500 pmol) was dissolved in ACN 37%, and submitted to the automated Edman degradation, using a PPSQ-21A Protein Sequencer, following the manufacturer instructions and protocols (Shimadzu, Kyoto, Japan). The N-terminal sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and the alignment performed with ClustalW (http://www.ebi.ac.uk/clustalw/).

[^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB) binding assay

Hippocampus membrane, obtained from 6 animals for each experiment, was prepared as described by Cardoso et al. (2004). Briefly, the hippocampi were isolated from rats, minced and homogenized in 25 mM Tris–HCl, pH 7.4 (containing 0.3 M sucrose, 5 mM $MgCl_2$, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride) with a Ultra-Turrax homogenizer (T-25, Ika Labortechnik, Staufen, Germany). The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was filtered through two layers of gauze and then centrifuged at 100,000 × *g* for 60 min. The final 100,000 × *g* pellet was re-suspended in 1 ml of 25 mM Tris–HCl, pH 7.4 (containing 5 mM $MgCl_2$, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride), using a Dounce homogenizer and stored at −70°C. All procedures were carried out at 4°C, and all solutions contained freshly added 1 mM phenylmethylsulfonyl fluoride to inhibit proteolysis. Protein concentration of membrane preparations was determined with a protein reagent assay (Bio Rad Laboratories Inc., Hercules, CA, USA).

Saturation and competition binding experiments were performed as described by Abdalla et al. (2000). Briefly, hippocampus membrane preparation (80 μg protein/ml) was incubated with 0.05 nM to 8.0 nM [^{3}H]QNB (specific activity 44 Ci/mmol; New England Nuclear, Boston, MA, USA) in the absence (total binding) and presence (nonspecific binding) of 1 μM atropine (Sigma Chemical Co., St Louis, MO, USA) for 1 h at 30°C. Specific binding was calculated as the difference between total and nonspecific binding. The nonspecific binding, near the K_D value, was about 10% of the [^{3}H]QNB total binding.

In competition binding experiments, hippocampus membrane preparation (80 μg protein/ml) was incubated with [^{3}H]QNB (concentration near the K_D values) for 1 h at 30°C in the absence and presence of increasing concentrations of MT-Mlα or atropine as a control of the assay.

Saturation and competition binding data were analyzed using a weighted nonlinear least-squares interactive curve-fitting program GraphPad Prism (GraphPad Prism Software Inc, San Diego, CA, USA).