Toxicon xxx (2013) 1–7

Contents lists available at ScienceDirect

Toxicon

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

Neuromuscular blocking activity of pinnatoxins E, F and G

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ARTICLE INFO

Article history:
Received 29 July 2013
Received in revised form 18 September 2013
Accepted 2 October 2013
Available online xxxx

Keywords:
Pinnatoxin
Neuromuscular block
Harmful algal toxins
Electrophysiology

ABSTRACT

Pinnatoxins are produced by dinoflagellates and belong to the cyclic imine family of toxins. They are fast-acting and highly toxic when administered *in vivo* in rodent bioassays, causing death by respiratory depression within minutes. Studies have revealed that some cyclic imine toxins cause their toxicity by antagonizing both muscle type and heteromeric and homomeric neuronal nicotinic acetylcholine receptors (nAChRs). Pinnatoxins E, F and G all display potent toxicity in *in vivo* bioassays, with symptoms of toxicity similar to other cyclic imine toxins. However, very little work has been done on the mechanism of action of these pinnatoxin isomers. Thus the aim of the current study was to investigate the rank order of potency and mechanism of action of pinnatoxins E, F and G. The effects of pinnatoxin E, F and G on *in vitro* rat hemidiaphragm preparations were investigated using twitch tension and electrophysiological techniques to determine the effects of these toxins on cholinergic transmission at the neuromuscular junction. Pinnatoxins E, F and G all produced concentration-dependent reductions in the nerve evoked twitch response of the rat hemidiaphragm, with IC_{50} values ranging from 11 to 53 nM and a rank order of potency of F > G > E. Only complete washout of pinnatoxin E was evident, with pinnatoxins F and G displaying slow and incomplete washout profiles. Pinnatoxins F and G also reduced the amplitudes of spontaneous miniature endplate potentials and evoked endplate potentials at the neuromuscular junction, without affecting miniature endplate potential frequency or the resting membrane potential of the muscle fibres. These results show that pinnatoxins E, F and G are all potent neuromuscular blocking agents and cause toxicity by acting as antagonists at muscle type nicotinic acetylcholine receptors.

1. Introduction

Pinnatoxins are members of the cyclic imine family of toxins, which include spirolides, gymnodimine, pteriatoxins, prorocentrolides and spiroprorocentromine. These fast-acting toxins are produced by a variety of dinoflagellates, including *Karenia*, *Alexandrium* and *Prorocentrum* species (Molgó et al., 2007). They are highly toxic when administered *in vivo* in rodent bioassays, causing an "all or nothing" response and death by respiratory depression within minutes at toxic doses (Munday, 2008). The cyclic imine moiety common to all of the members of this toxin family is thought to be the main structural determinant for toxicity. Binding and functional studies have shown that cyclic imines cause their toxicity by antagonizing both muscle type and heteromeric and homomeric neuronal nicotinic acetylcholine receptors (nAChRs) (Bourne et al., 2010; Kharrat et al., 2008). Recent studies also suggest that antagonism of muscarinic ACh receptors may also play a role in cyclic imine toxicity (Wandscheer et al., 2010).

Pinnatoxin A, the first of the pinnatoxin isomers to be isolated, was discovered in extracts of the bivalve

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0041-0101/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.toxicon.2013.10.009

mollusc *Pinna muricata* in Okinawa, Japan (Uemura et al., 1995; Zheng et al., 1990). Pinnatoxins B, C and D were subsequently isolated from the same species (Chou, 1996; Takada et al., 2001). Routine biotoxin monitoring of Pacific oysters (*Crassostrea gigas*) from Rangaunu Harbour in New Zealand's sub tropical north revealed the presence of a fast acting toxin, which was later found to be a mixture of novel pinnatoxins E and F (Selwood et al., 2010). Pinnatoxin G was also isolated from *C. gigas*, collected in South Australia. Analysis of sediment samples from Rangaunu Harbour and the French Mediterranean coast revealed the dinoflagellate species responsible for pinnatoxin production, *Vulcanodinium rugosum*, strains of which are also found in South Australia, China, Spain, Hawaii and Japan (Nézan and Chomérat, 2011; Rhodes et al., 2011, 2010; Satta et al., 2013; Smith et al., 2011; Zeng et al., 2012).

Initial reports claimed that pinnatoxins were activators of calcium channels (Zheng et al., 1990). However, recent work has shown that, like the spirolides and gymnodimine, pinnatoxin A also binds to and antagonizes nAChRs of both the muscle type and heteromeric/homomeric neuronal subtypes (Araoz et al., 2011). The lack of nAChR antagonism by the pinnatoxin A ketone, which lacks the cyclic imine moiety, highlights the importance of this structural feature in the toxicity of pinnatoxin A. Recent studies have shown that pinnatoxins E and F cause an inhibition of the nerve-evoked twitch response in an *in vitro* hemidiaphragm preparation, with no effect on direct muscle stimulation (Hellyer et al., 2011). This is suggestive of antagonism of muscle type nAChRs at the neuromuscular junction. Pinnatoxin G has also recently been found to bind to nAChRs from the electric eel *Torpedo* species, which are similar in structure to mammalian muscle type nAChRs (Hess et al., 2013).

In the current study the rank order of potency and the mechanism of action of pinnatoxins E, F and G were investigated in rat hemidiaphragm using *in vitro* twitch tension and electrophysiological techniques. The present study shows potent neuromuscular blocking actions of all three pinnatoxin isomers, with effects on endplate potentials indicative of post-synaptic nAChR antagonism.

2. Materials and methods

2.1. Chemicals

Neostigmine was obtained from AstraZeneca Ltd (Auckland, New Zealand). Pinnatoxins E, F and G were isolated and purified from algae according to the methods of Selwood et al. (2010), with the resulting lyophilised toxin dissolved in 0.1% acetic acid (Pinnatoxin F), isopropanol (Pinnatoxin G) or deionised water (Pinnatoxin E), and frozen at −20 °C before use.

2.2. Animals and tissue preparation

All animal procedures were conducted with approval of the University of Otago Animal Ethics Committee, in accordance with guidelines. Young (6–8 weeks) male Sprague–Dawley rats were anaesthetised with CO_2 and sacrificed by rapid decapitation. Hemidiaphragms with attached phrenic nerves were dissected into Krebs–Hensleit buffer, consisting of (in mM) 120 NaCl, 2.5 $CaCl_2$, 4.7 KCl, 2.1 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11 glucose, which had been saturated with 95% O_2/5% CO_2 prior to use. For experiments involving intracellular recording, the concentration of $CaCl_2$ was lowered to 0.3 mM to prevent muscle contraction (Knight et al., 2003). Hemidiaphragms were then cleaned of extraneous tissue and immediately transferred to a holding chamber containing Krebs–Hensleit buffer maintained at room temperature and saturated with 95% O_2/5% CO_2.

2.3. Twitch tension experiments

Isolated phrenic nerve-hemidiaphragm preparations were mounted in organ baths (40 mL volume) and superfused with oxygenated Krebs buffer. Organ bath temperature was maintained at 34–37 °C. One end of each preparation was attached to a force transducer in order to record muscle tension. Resting tension was adjusted to 2 g for each preparation investigated. The phrenic nerve of each preparation was drawn through the loops of a bipolar electrode connected to a Grass SD9 stimulator (Grass Instruments, West Warwick, RI, USA). Each preparation was then left for up to 1 h to equilibrate. Following equilibration, motor nerve-evoked muscle twitches were recorded in response to square wave pulses of 0.1 ms duration and supramaximal voltage (~10–20 V) delivered at 0.2 Hz. All waveforms were acquired digitally at 10 kHz, amplified and displayed on an eMac computer using a PowerLab 2/25 analogue digital converter (AD Instruments, Sydney). Recordings were monitored and stored using Chart™ analytical software (Version 6–7, AD Instruments, Sydney) for offline analysis.

After maintenance of stable baseline twitch responses (~30 min), toxins were added directly to the organ bath at various concentrations. Twitch tension was then monitored for up to 2 h, or until complete abolition of the response was observed. This was followed by a washout period of up to 2 h, in which fresh buffer was applied every 20–30 min. In some preparations, neostigmine (15 μM) was also applied at the commencement of this washout period. For KCl response studies, muscle response to the administration of KCl (40 mM) for 30 s was determined both before and after toxin administration.

2.4. Intracellular recording

For miniature endplate potential (mEPP) recordings, hemidiaphragms were placed in a tissue recording chamber (Kerr Scientific Instruments, Christchurch, NZ) constantly perfused at a rate of 1 ml/min with oxygenated, low calcium (0.3 mM) Kreb's buffer maintained at 34 ± 2 °C. For endplate potential (EPP) recordings, the cut-muscle preparation was used (Barstad, 1962). Briefly, 1–2 mm of tissue was removed from each end of the diaphragm and the muscle subsequently bathed in low potassium (2.5 mM) solution for the remainder of the experiment. The phrenic nerve was drawn through the loops of a bipolar electrode connected to a Grass S48 stimulator. EPPs were evoked via supramaximal square wave stimulation of 0.1 ms duration, delivered at 0.2 Hz.

Glass microelectrodes (10–20 MΩ) filled with 3 M KCl were used to record mEPPs and EPPs and resting membrane potentials (RMPs). Signals were acquired using a

Please cite this article in press as: Hellyer, S.D., et al., Neuromuscular blocking activity of pinnatoxins E, F and G, Toxicon (2013), http://dx.doi.org/10.1016/j.toxicon.2013.10.009