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Assessing the neurotoxic effects of palytoxin and ouabain, both Na^+/K^+-ATPase inhibitors, on the myelinated sciatic nerve fibres of the mouse: An *ex vivo* electrophysiological study

Alexia Kagiava [a], Katerina Aligizaki [b], Panagiota Katikou [c], Georgios Nikolaidis [b,1], George Theophilidis [a,*]

[a] *Laboratory of Animal Physiology, Department of Zoology, School of Biology, Aristotle University of Thessaloniki, University Campus, Thessaloniki 54 124, Greece*
[b] *Department of Botany, School of Biology, Aristotle University of Thessaloniki, 54 124, Greece*
[c] *National Reference Laboratory on Marine Biotoxins, Ministry of Rural Development and Food, 3A Limnou St., 546 27 Thessaloniki, Greece*

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ABSTRACT

Palytoxin (PlTX) is a marine toxin originally isolated from the zoantharians of the genus *Palythoa*. It is considered to be one of the most lethal marine toxins that block the Na^+/K^+-ATPase. This study was designed to investigate the acute effects of PlTX and ouabain, also an Na^+/K^+-ATPase blocker, on the mammalian peripheral nervous system using an *ex vivo* electrophysiological preparation: the isolated mouse sciatic nerve. Amplitude of the evoked nerve compound action potential (nCAP) was used to measure the proper functioning of the sciatic nerve fibres. The half-vitality time of the nerve fibres (the time required to inhibit the nCAP to 50% of its initial value: IT_{50}) incubated in normal saline was 24.5 ± 0.40 h ($n = 5$). Nerves incubated continuously in 50.0, 10.0, 1.0, 0.5, 0.250 and 0.125 nM of PlTX had an IT_{50} of 0.06 ± 0.00, 0.51 ± 0.00, 2.1 ± 0.10, 8.9 ± 0.30, 15.1 ± 0.30 h, and 19.5 ± 0.20 h, respectively ($n = 5, 3, 4, 4, 10$). PlTX was extremely toxic to the sciatic nerve fibres, with a minimum effective concentration (mEC) of 0.125 nM ($n = 5$) and inhibitory concentration to 50% (IC_{50}) of 0.32 ± 0.08 nM (incubation time 24 h). Ouabain was far less toxic, with a mEC of 250.0 μM ($n = 5$) and IC_{50} of 370.0 ± 18.00 μM (incubation 24.5 h). Finally, when the two compounds were combined – e.g. pre-incubation of the nerve fibre in 250.0 μM ouabain for 1 h and then exposure to 1.0 nM PlTX – ouabain offered minor a neuroprotection of 9.1–17.6% against PlTX-induced neurotoxicity. Higher concentrations of ouabain (500.0 μM) offered no protection. The mouse sciatic nerve preparation is a simple and low-cost bioassay that can be used to assess and quantify the neurotoxic effects of standard PlTX or PlTX-like compounds, since it appears to have the same sensitivity as the haemolysis of erythrocytes assay – the standard *ex vivo* test for PlTX toxicity.

1. Introduction

Palytoxin (PlTX) is a marine toxin originally isolated from the zoantharians of the genus *Palythoa*. It is considered to be one of the most lethal marine toxins. It is a large, very complex molecule ($C_{129}H_{223}N_3O_{54}$) possessing the longest continuous chain of carbon atoms and the largest molecular weight (2680) of any known natural product

* Corresponding author. Tel.: +30 2310 998261, +30 2310 998275; fax: +30 2310 998269.
E-mail address: theophil@bio.auth.gr (G. Theophilidis).
[1] Diseased.

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(Cha et al., 1982; Moore and Bartolini, 1981; Wang, 2008). PlTX acts on the Na^+/K^+-ATPase in a unique way: converting the pump into an ion channel. Electrophysiological studies (Artigas and Gadsby, 2006) have revealed that this action results in K^+ efflux, Na^+ influx and, finally, membrane depolarisation. As a result, PlTX and PlTX-like compounds have a wide spectrum of secondary toxicological effects on lower and higher animals, including humans, because these toxins can enter the blood serum through the food chain (Deeds and Schwartz, 2010; Wu, 2009). Furthermore, PlTX-producing organisms have been expanding their range to temperate areas, including the Mediterranean Sea (Aligizaki et al., 2008; Ciminiello et al., 2006, 2008; Guerrini et al., 2010; Lenoir et al., 2004). This phenomenon has generated a great deal of concern about possible human health and economic impacts, on the part of both researchers, as indicated by the volume of recent publications (Aligizaki et al., 2011; Ares et al., 2005; Bellocci et al., 2008; Guerrini et al., 2010; Louzao et al., 2010; Ramos and Vasconcelos, 2010; Rossi et al., 2010; Sala et al., 2009), and the responsible European Food Safety Authority (EFSA, 2009). Thus, assessment of the toxicity of PlTX and PlTX-like compounds is an important avenue for future research.

In vivo tests on several animal species have shown that PlTX is a highly lethal substance for mammals (Wiles et al., 1974). For example, during gavage administration to mice, signs of toxicity and lethality that included scratching, jumping, respiratory distress, and paralysis were recorded for 24 h with a median lethal dose (LD_{50}) of 767 μg/kg (Sosa et al., 2009). In these toxicological studies, PlTX is diluted in the blood serum where red-blood cells are among the most probable PlTX targets. Indeed, one study showed that the haemolytic effects of PlTX had a minimum effective concentration (mEC) of 0.373 nM at 25 °C (exposure time 8.0 h); (Habermann et al., 1981). The haemolysis neutralisation red-blood cell assay (HNA), an *ex vivo* test, has therefore been used extensively to measure PlTX toxicity (Aligizaki et al., 2008; Riobó et al., 2008; Taniyama et al., 2003). The nervous system, however, is another probable PlTX target that could be evaluated through *ex vivo* toxicological studies. However, although the symptoms of PlTX-intoxication, respiratory failure and paralysis, suggest a nervous system malfunction, the toxic effects of PlTX have never been tested using *ex vivo* preparations based on either the central or peripheral nervous system of mammals. To date, *ex vivo* studies of toxic effects on nervous tissue have focused mainly on invertebrates and amphibians, using concentrations varying from 10.0 to 140.0 nM, or even higher (Castle and Strichartz, 1988; Dubois and Cohen, 1977; Kim et al., 1995; Kudo and Shibata, 1980; Lauffer et al., 1985; Pichon, 1982; Rakowski et al., 2007).

Cell-cultures based on cells derived from the mammalian nervous system have been used in an attempt to fill this gap. Neuroblastoma cells (Kim et al., 1995), BE(2)-M17 human neuroblastoma cells (Espiña et al., 2009), and the Neuro-2a neuroblastoma cell line (Ledreux et al., 2009) are all cell types that have been used for assessing the toxicity of PlTX. The latter two *in vitro* tests are considered to be the most sensitive for assessing PlTX toxicity, with a sensitivity ranging between 0.074 and 0.150 nM (exposure time 24 h). It is worth mentioning that there are a number of *in vitro* tests, based on cells from outside the nervous system, that appear to be more sensitive to PlTX (sensitivity in the range of 0.01–0.04 nM); (Bellocci et al., 2008; Bignami, 1993; Sala et al., 2009).

In almost all *ex vivo* and *in vitro* bioassays, the deleterious effects of PlTX have been confirmed using pre-incubation with ouabain (usually 1 h) prior to PlTX application (Aligizaki et al., 2008; Castle and Strichartz, 1988; Espiña et al., 2009; Ledreux et al., 2009). Ouabain also binds with the Na^+/K^+-ATPase and protects (100.0%) the exposed cells or tissue from the severe effects of PlTX. A number of studies have examined the effects of ouabain using a variety of *ex vivo* preparations (Castle and Strichartz, 1988; Habermann and Chhatwal, 1982; Riobó et al., 2008; Walz and Hertz, 1982), but none have used myelinated nerve fibres from the peripheral nervous system of mammals. In the optic nerve, in the central nervous system, ouabain (10.0 μM–1.0 mM) evoked nerve depolarisation in a dose-dependent manner, indicating a critical dependence of rat optic nerve axonal resting potential on continued pump (Na^+/K^+-ATPase) operation (Malek et al., 2005). Because the standard procedure for identifying the action of PlTX requires co-incubation of nerve tissue with both PlTX and ouabain, we also needed to determine the toxic effects of ouabain itself on the mouse nerve fibres.

The purpose of this study was to use an *ex vivo* test, based on the isolated sciatic nerve of the mouse, to assess the neurotoxic effects of standard PlTX, ouabain, and both combined, on the nerve fibres of mammals. This test of the toxic effects of synthetic PlTX will serve as a pilot study to assess the neurotoxic effects of the increasing number PlTX-like compounds found in *Ostreopsis* cells (Ciminiello et al., 2010; Rossi et al., 2010). A similar method, based on the isolated sciatic nerve of the rat, has been used extensively in a variety of neurotoxicological studies (Andreou et al., 2007; Kagiava et al., 2008; Moschou et al., 2008).

2. Materials and methods

2.1. The sciatic nerve preparation

This study used the sciatic nerve of 4–7 week old male mice (*Mus musculus*; Albino Swiss) weighing between 23.0 and 25.0 g. The intra-axonal recordings, only, were measured on the rat sciatic nerve (250.0–300.0 g). Animals were sacrificed using N_2 and cervical dislocation prior to nerve dissection. All experimental procedures were conducted in accordance with the animal care protocols outlined by Aristotle University of Thessaloniki, Greece and the Veterinary Authorities, and these protocols respect the recommended standard practices for Biological Investigations. Sciatic nerves were dissected from the spinal cord to the knee, immersed in oxygenated (O_2, 100%) saline solution, and desheathed to maximize drug access to nerve fibres (the sciatic nerve consists of approximately 2682 myelinated nerve fibres (Duchen and Scaravilli, 1977)). Nerves were immersed in a modified Krebs–Ringer solution containing (in mM): 136 NaCl, 4.7 KCl, 2.4 $CaCl_2$, 1.1 $MgCl_2$, 1 $NaHCO_3$, 11 glucose, and 10 HEPES; pH = 7.2. All experiments were performed at a constant temperature of 26.0 ± 1.0 °C.