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Toxicon 50 (2007) 251–255

TOXICON

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A cellular target for the lipophilic toxins from *Karenia brevisulcata*

Penelope Truman*

Institute of Environmental Science & Research, Kenepuru Science Centre, P.O. Box 50348, Porirua, New Zealand

Received 25 January 2007; received in revised form 23 March 2007; accepted 23 March 2007
Available online 28 March 2007

Abstract

Crude lipophilic toxin from *Karenia brevisulcata* has been shown to be toxic to mammalian neuroblastoma (Neuro2A) cells in culture. This toxicity is partially antagonised by the addition of saxitoxin. The dose–response curves of saxitoxin acting to antagonise the action of *K. brevisulcata* toxin and of brevetoxin were examined and they displayed similar EC50 values. These results suggest that at least some of the effect on Neuro2A cells of the lipophilic toxicity found in *K. brevisulcata* results from an interaction with the mammalian voltage-dependent sodium channel.

Keywords: Marine biotoxin; *Karenia brevisulcata*; Saxitoxin; Brevetoxin; Voltage-dependent sodium channel

1. Introduction

Karenia brevisulcata is a dinoflagellate species first isolated from New Zealand waters in 1998, after it caused mass mortalities of marine life in Wellington Harbour (Chang et al., 2001; Wear and Gardner, 2001). People living and working close to affected coastlines also reported adverse effects (Chang, 1999). The toxicity displayed by this dinoflagellate was unusual in that it killed a very wide range of marine creatures, from fish, through a wide variety of larval and adult invertebrates to unicellular organisms (Chang et al., 1998; Wear and Gardner, 2001). Toxicity was shown to reside in the lipid fraction of algal extracts and could be assayed by a variation of the neuroblastoma assay for brevetoxin (Keyzers, 2003; Truman et al., 2005). The toxin is highly potent, although accurate potency determinations will not be possible until pure toxin is available. This paper describes results of experiments to determine whether or not the toxin acts via the mammalian voltage-dependent sodium channel (VDSC) in its action on neuroblastoma cells.

Earlier experimental results had suggested that *K. brevisulcata* toxin (KBT) might act to allow ions, possibly sodium ions, to enter cells in an uncontrolled manner (Truman et al., 2005). A wide variety of mechanisms for this are possible. Recent proteomic profiling experiments (unpublished) had given further, though inconclusive, evidence that the VDSC itself might be involved. We therefore decided to ask whether saxitoxin (a known VDSC blocker) could counteract the toxicity of KBT towards Neuro2A cells. The effect of saxitoxin on KBT toxicity is demonstrated here, together with its previously established effect in countering the effect of brevetoxin on VDSC function.

*Tel.: +64 4 914 0761; fax: +64 4 914 0770.
E-mail address: penelope.truman@esr.cri.nz.

0041-0101/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2007.03.016

2. Materials and methods

Cell culture and bioassay conditions have been described previously (Truman et al., 2005). Briefly, N2A cells (ATCC CCL-131, Neuro2A) at $1–2 \times 10^5$ cells/mL, were dispensed into 96 well plates and allowed to re-attach overnight. The following day medium was replaced with 200 μL/well of fresh culture medium (RPMI 1640, supplemented with glutamine, sodium pyruvate, 10% foetal calf serum and antibiotics; Truman and Lake, 1996). For brevetoxin/saxitoxin dose–response curves, the medium in the central wells of each plate included veratridine at 0.017 mM and ouabain at 0.083 mM (Truman et al., 2002). For KBT/saxitoxin dose–response curves and for the outer wells of the brevetoxin plates, no additions to the base medium were made.

Preliminary experiments indicated that responses to saxitoxin were most clearly seen with KBT at 10–14 toxicity units (TU) per well (data not shown). One toxicity unit of KBT is defined as the smallest amount of KBT detectable in the N2A assay in the presence of ouabain and veratridine at the above concentrations. Approximately equivalent effects on cell viability were seen with brevetoxin at 0.1 ng/well (0.58 nM) and these concentrations were used in all the experiments reported here.

Stock solutions of saxitoxin were obtained from Dr. Sherwood Hall, USFDA, and were 100 μg/mL in 20% ethanol, 1 mM HCl, stored at 4 °C. Brevetoxin PbTx-1 was a gift from Professor Hong Nong Chou, made up at 0.1 μg/mL in 80% ethanol and stored at −30 °C. KBT was a crude algal extract, supplied by Cawthron Institute and prepared by R. Keyzers to provide a "gold standard" for KBT analysis (Keyzers, 2003). An aliquot of this sample was adjusted to 25,000 TU/mL in 80% ethanol and stored at −30 °C. No change in biological activity of this solution had been detected over a 3-year period.

A series of saxitoxin standards was made. The most concentrated standard was a 1/10 dilution of the USFDA saxitoxin standard in cell culture medium (10 μg/mL). Further dilutions were made using medium diluted with 1/10 volume of 20% ethanol 1 mM in HCl. Saxitoxin standards ranged from 10 μg/mL to 0.1 ng/mL. Replicate additions of the standards were added to each 96 well plate to give saxitoxin at 0.1 μg/well to 1 pg/well (1.67 μM–16.7 pM).

KBT or brevetoxin PbTx-1 were then added to an appropriate 96 well plate (without or with ouabain or veratridine as required) so that each plate contained a saxitoxin dilution series plus either KBT or PbTx-1 at a constant concentration. Controls containing none of the toxins, saxitoxin only, and either KBT or PbTx-1 only were included. All standards and controls were in at least quadruplicate. In these experiments, the outer wells of the plate were not used except to check that the amount of ouabain and veratridine added to the central wells in the PbTx-1 plates caused a 5–20% reduction in cell viability. After an incubation period of 18–21 h (37 °C, 5% CO_2), viability was determined using the MTT viability assay (Manger et al., 1993; Truman and Lake, 1996).

Individual EC50s were determined for each experiment from raw absorbance figures by fitting a sigmoidal dose–response curve using Graphpad Prism software. Individual EC50 values for saxitoxin antagonising either KBT or brevetoxin were compared using Student's *t*-test (unpaired).

For ease of presentation and for inter-assay comparison, results of three clear dose–response curves obtained for each toxin were further processed by normalisation against the viability attained when both toxins were present and saxitoxin was at maximal concentration, as the 100% viability condition. Normalised values were used in deriving consensus dose–response curves (Figs. 1 and 2). The consensus EC50 values for each toxin were derived by fitting a curve to the combined normalised data.

Mean absorbance values for cells exposed to KBT alone or for cells exposed to KBT and at least 0.17 μM saxitoxin were compared using a paired

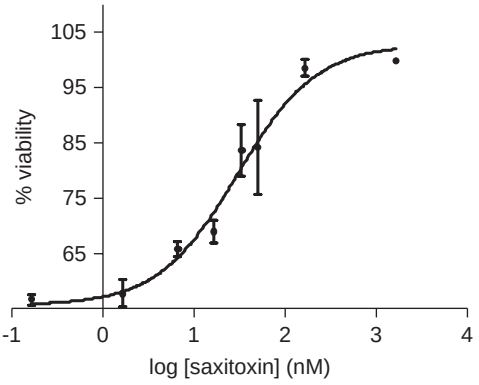

Fig. 1. Effect of saxitoxin in antagonising the effect of brevetoxin PbTx-1 in the neuroblastoma assay. Values are means ± S.E.M. of three independent dose–response experiments each normalised against the absorbance where cells were exposed to saxitoxin at maximal concentration as well as brevetoxin PbTx-1 (0.58 nM) as the 100% viability condition.