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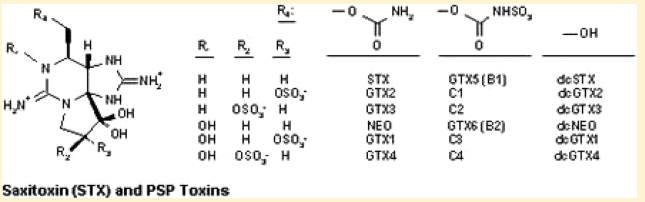
Determination of Toxicity Equivalent Factors for Paralytic Shellfish Toxins by Electrophysiological Measurements in Cultured Neurons

Sheila Perez,[†] Carmen Vale,[†] Ana M. Botana,[‡] Eva Alonso,[†] Mercedes R. Vieytes,[§] and Luis M. Botana[*,‡]

[†]Departamento de Farmacología, Facultad de Veterinaria, USC, Lugo, Spain

[‡]Departamento de Química Analítica and [§]Departamento de Fisiología, Facultad de Veterinaria, USC, Lugo, Spain

ABSTRACT: The establishment of toxicity equivalent factors to develop alternative methods to animal bioassays for marine-toxin detection is an urgent need in the field of phycotoxin research. Paralytic shellfish poisoning (PSP) is one of the most severe forms of food poisoning. The toxins responsible for this type of poisoning are highly toxic natural compounds produced by dinoflagellates, which bind to voltage-gated Na^+ channels causing the blockade of action potential propagation. In spite of the fact that several standards of PSP toxins are currently commercially available, there is scarcity of data on the biological activity of these toxins, a fact that limits the calculation of their toxicity equivalent factors. We have evaluated the potency of the commercial PSP toxin standards for their ability to inhibit voltage-dependent sodium currents in cultured neuronal cells by electrophysiological measurements. The *in vitro* potencies of the PSP toxin standards as indicated by their IC_{50} values were in the order Neosaxitoxin (NeoSTX) > decarbamoylsaxitoxin (dcSTX) > saxitoxin (STX) > gonyautoxin 1,4 (GTX1,4) > decarbamoylneosaxitoxin (dcNeoSTX) > gonyautoxin 2,3 (GTX2,3) > decarbamoylgonyautoxin 2,3 (dcGTX2,3) > gonyautoxin 5 (GTX5) > *N*-sulfocarbamoyl-gonyautoxin-2 and -3 (C1,2). The data obtained in this *in vitro* analysis correlated well with their previously reported toxicity values.



Saxitoxin (STX) and PSP Toxins

INTRODUCTION

Paralytic shellfish poisoning (PSP) is one of the most severe forms of food poisoning. The toxins responsible for this type of poisoning are highly toxic natural compounds found in different phyla of both prokaryotic and eukaryotic organisms.[1] PSP toxins comprise a series of heterocyclic guanidines with more than 57 known analogues of the parent compound saxitoxin.[2,3] The prevalent PSP toxins include saxitoxin and its related compounds neosaxitoxin, decarbamoylsaxitoxin, decarbamoylneosaxitoxin, gonyautoxins 1 to 6 (GTX) and their decarbamoyl analogues and four C toxins.[4] Recently, several analogues of saxitoxin possessing a hydroxybenzoate moiety have been identified.[5,6] Paralytic shellfish poisoning is a public health concern worldwide,[7] and it constitutes a serious illness in which neurological symptoms predominate. PSP toxins are neurotoxic, and PSP poisoning exhibits symptoms including tingling sensation of the lips, numbness of extremities, gastrointestinal problems, and difficulty breathing.[8] The mechanism of action of PSP toxins is the blockade of sodium channels in excitable membranes that causes the arrest of action potential propagation.[9,10]

The mouse bioassay (MBA) as standardized by the Association of Official Analytical Chemists (AOAC)[11] is commonly used to quantitatively determine the PSP toxicities of shellfish in many countries.[7] In this reference method,[11] shellfish extracts are administered by intraperitoneal injection, and the assessment is based on symptomatology and time to death. However, the bioassay has been shown to have high variability and low sensitivity. In recent years, considerable progress has been made in developing alternatives to the MBA method for almost all of the common PSP analogues. Alternative testing possibilities include antibody-based quantitation,[12] assay of cytotoxicity blockade in neuroblastoma cells in culture,[13,14] binding assays,[15] and assays using voltage-sensitive fluorescent dyes.[16−19] Highly sensitive analytical methods such as HPLC,[20] liquid chromatography—mass spectrometry (LCMS), or capillary electrophoresis [21,22] have also been developed. Of these, a postcolumn liquid chromatographic method described by Oshima[4] is extensively used in research applications, while an alternative procedure based on precolumn oxidation[23,24] constitutes the only validated and approved alternative method to MBA for the detection paralytic shellfish toxins.[20] However, the toxicological database for PSP toxins is limited and comprises mostly studies on their acute toxicity following intraperitoneal administration. Since PSP analogues display different toxicities depending on their functional groups, and toxicity equivalent factors (TEFs) may differ depending on the system used, for monitoring purposes using high performance liquid chromatography (HPLC) techniques, TEFs have been applied to express the detected analogues as STX equivalents. Currently, the Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) proposed the TEFs for PSP toxins based on acute i.p. toxicity in mice.[25,26] The European Commision considers the TEFs provided by EFSA opinions as the reference values to apply for all calculations.[27] By using a fluorescent method, we have previously shown that PSP blockade of the changes in membrane potential in cerebellar

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granule cells provides an estimation of PSP potency that strongly correlates with the *in vivo* toxicity of paralytic toxins[19] for the vast majority of toxin standards. Therefore, the aim of this work was to obtain the toxicity equivalent factors of commercial PSP toxins by a functional method using electrophysiological recordings in primary cultured neurons. With this approach, the unanbiguous confirmation of TEF with different technologies would provide a solid ground to use them in any future screening program.

EXPERIMENTAL PROCEDURES

Chemicals and Solutions. Seven-day-old Swiss Mice were obtained from the animal care facilities of the University of Santiago de Compostela. Animals were used according to current European legal ethical regulations. Plastic tissue-culture dishes were purchased from Falcon (Madrid, Spain). Fetal calf serum was obtained from Gibco (Glasgow, UK), and Dulbecco's modified Eagle's medium (DMEM) was from Biochrom (Berlin, Germany). The certified PSP standards were STX-dihydrochloride, dc-STX, GTX 2&3-b, GTX1&4b, NEO-b, GTX5-b, dc-GTX2&3-b, and C1&2; all were obtained from the Institute for Marine Biosciences, National Research Council of Canada. All other chemicals were of reagent grade and purchased from Sigma.

Cell Cultures. Primary cultures of CGC were obtained from cerebella of 7-day-old mice following previously described methods.[28,29] In brief, cells were dissociated by mild trypsinization with trypsin from bovine pancreas (0.002% w/v) at 37 °C, followed by trituration in a DNase solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in DMEM containing 25 mM KCl, 31 mM Glucose, and 0.2 mM glutamine supplemented with *p*-amino benzoate (0.1% w/v), insulin (0.04% w/v), penicillin (0.03% w/v), and 10% fetal calf serum. The cell suspension was seeded in glass coverslips precoated with poly-L-lysine and incubated in 6-multiwell plates for 7−11 days in a humidified 5% CO_2/95% air atmosphere at 37 °C. Cytosine arabinoside (20 μM) was added before 48 h in culture to prevent glial proliferation.

Electrophysiology. Membrane currents from single cells were studied at room temperature (22−25 °C) by electrophysiological recordings in voltage-clamp mode[30,31] using a computer-controlled current and voltage clamp amplifier (Multiclamp 700B, Molecular Devices). Signals were recorded and analyzed using a Pentium computer equipped with a Digidata 1440 data acquisition system and pClamp10 software (Molecular Devices, Sunnyvale, CA). pClamp10 was also used to generate current and voltage-clamp commands and to record the resulting data. Signals were prefiltered at 10 kHz and digitized at 20 μs intervals.

Recording electrodes were fabricated from borosilicate glass microcapillaries (outer diameter, 1.5 mm), and the tip resistance was 5−10 MΩ. The internal pipet solution contained (in mM) 108 Cs gluconate, 1.7 NaCl, 0.9 EGTA, 9.0 HEPES, 1.8 $MgCl_2$, 4.0 Na_2ATP, and 0.3 NaGTP at pH 7.2.[32] The extracellular medium contained (in mM) 154 NaCl, 5.6 KCl, 3.6 $NaHCO_3$, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 5.0 glucose, and 10 HEPES (pH 7.4). Moreover, 20 mM TEA and 1.0 mM 4-AP were added in the extracellular recording solution in order to block voltage-dependent potassium currents. Voltage-dependent sodium currents were elicited in CGCs by applying a series of 25 ms depolarizing pulses (voltage steps), in 5 mV increments, from a holding potential of −100 mV.[33,34] Dose−response curves for the inhibition of voltage-gated sodium currents were obtained by plotting the percent inhibition of the peak sodium current amplitude at each toxin concentration.

Statistical Analysis. The percent inhibition of peak sodium current amplitude of all cells was averaged. All the experiments were carried out at least three times. Results were analyzed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the means ± SEM.

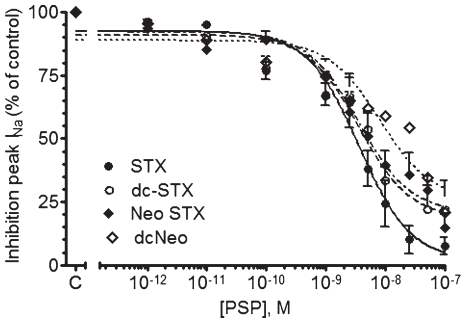

Figure 1. Concentration−response curves for the inhibitory effect of the PSP standards for STX, dcSTX, NeoSTX, and dcNeoSTX on voltage-dependent sodium currents in primary cultures of mouse cerebellar neurons. Values are the means ± SEM of 3 to 13 independent experiments.

RESULTS

Our objective was to determine the toxicity equivalent factor of commercial PSP standards by electrophysiological measurement; to do so, we evaluated their ability to inhibit voltage-dependent sodium currents in cultured cerebellar neurons. This cellular preparation was chosen because PSP toxins are known to block neuronal transmission through the inhibition of voltage-gated Na^+ channels.[9,10] In addition, this same neuronal system has been previously used to provide data on the relative potency of PSP toxins to inhibit veratridine-induced depolarization and show a good correlation with the data obtained by a standard mouse bioassay.[19]

Therefore, the ability of the different PSP standards to inhibit voltage-dependent sodium currents was evaluated directly. Figure 1 shows the effect of different saxitoxin-related compounds on the amplitude of voltage-dependent sodium currents. As shown in this figure, the parent compound saxitoxin inhibited VGSCs with an IC_{50} (95% confidence intervals) of 3.59 nM (1.8 to 7.2 nM), whereas dcSTX gave an IC_{50} of 3.57 nM (95% confidence intervals: 1.5 to 8.2 nM). For neosaxitoxin, the IC_{50} to inhibit the peak amplitude of VGSCs was 3.53 nM (95% confidence intervals: 1.9 to 6.5 nM), and dcNeoSTX yielded an IC_{50} of 8.06 nM (95% confidence intervals from 2.2 to 20.9 nM). Thus, the *in vitro* assay for the saxitoxin standards yielded the following order of potency: NeoSTX > STX ~ dcSTX > dcNeoSTX.

In the next series of experiments, the ability of the gonyautoxin standards to block the amplitude of voltage-dependent sodium currents was evaluated. As shown in Figure 2, the GTX 2,3 standard inhibited voltage-dependent sodium currents with an IC_{50} of 12.79 nM (95% confidence intervals from 5.8 to 28.1 nM), whereas decarbamoyl GTX2,3 (dcGTX2,3) yielded an IC_{50} of 27.0 nM (95% confidence intervals from 13.7 to 53.4 nM). The IC_{50} value for the GTX1,4 standard was 7.14 nM (95% confidence intervals from 2.1 to 23.7 nM) and for the GTX5 standard was 39.6 nM (95% confidence intervals from 16.0 to 97.8 nM). Therefore, the potency of the GTX standards to inhibit voltage-gated sodium channels was in the order GTX1,4 > GTX2,3 > dcGTX2,3 > GTX5.

In addition, we also evaluated the effect of the C1&2 PSP standards on voltage-gated sodium channels. As shown in Figure 3, this toxin inhibited the sodium channel amplitude with an IC_{50} of 151.3 nM (95% confidence intervals from 74.5 to 307.1 nM).

Therefore, the results presented here indicate that the toxicity of the GTX standards as evaluated with electrophysiological

recordings strongly correlated with the *in vitro* and *in vivo* potencies of the GTX standards previously reported by our laboratory[19] as shown in Table 1 and with the recently reported EFSA equivalent toxicity factors as shown in Figure 4.

DISCUSSION

Paralytic shellfish poisoning testing methods alternative to the mouse bioassay are currently being pursued. In this sense, the knowledge of toxicity equivalent factors for each toxin or toxin combination is very important as this will allow national monitoring systems to use analytical methods for toxin detection. To date only an HPLC method for paralytic toxins has been interlaboratory validated,[20,24] although single laboratory validation of additional methods is currently being pursued.[35] However, the toxicological database for PSP toxins is limited and comprises mostly studies on their acute toxicity after intraperitoneal administration.[26] For monitoring purposes using HPLC techniques, the European Food Safety Authority has applied TEF factors based on the acute intraperitoneal toxicity of these toxins in mice.[36] In this work, we examined the potency of commercial PSPs standards to inhibit voltage-gated sodium channels by electrophysiological recordings in cerebellar granule cells. This cellular model was chosen because we have previously shown that PSP blockade of the changes in membrane potential in cerebellar granule cells provides an estimation of PSP potency that strongly correlates with *in vivo* toxicity of paralytic toxins.[19]

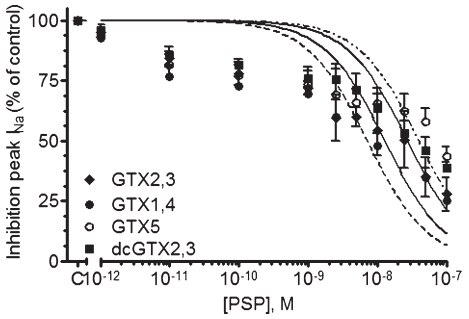

Figure 2. Concentration−response curves for the inhibitory effect of the PSP standards for GTX2,3, dcGTX2,3, GTX1,4, and GTX5 on voltage-dependent sodium currents in primary cultures of mouse cerebellar neurons. Values are the means ± SEM of 3 to 11 independent experiments.

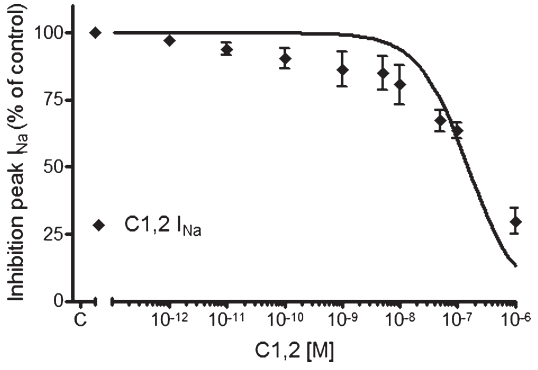

Figure 3. Concentration−response curves for the inhibitory effect of the PSP standards for C1,2 on voltage-dependent sodium currents in primary cultures of mouse cerebellar neurons. Values are the means ± SEM of three to five independent experiments.

Assays detecting the direct effect of PSP toxins on voltage-gated sodium channels are attractive because they more closely follow the underlying mechanism of *in vivo* toxicity. Although electrophysiological measurements applying patch-clamp techniques to single nerve cells have been used for screening shellfish,[37] the relative potency of PSP standards has not yet been evaluated by this approach. The results presented here indicate that electrophysiological recordings in neuronal cells may provide a valuable tool to develop functional assays for PSP toxin screening. In this regard, it is noteworthy to indicate that the IC_{50} for STX inhibition of neuronal sodium channels was very close to that reported previously using recombinant sodium channels yielding IC_{50} values of 3.6 nM in this work and 1.17 nM in recombinant sodium channels.[37] This point is important in order to develop automatic patch clamp functional assays for the screening of PSP toxins. Since granule cells in the cerebellum predominantly express Nav1.2 sodium channels in the axon, and Nav1.6, Naβ1, and Naβ2 sodium channels in the soma,[38] the development of automated patchclamp techniques using this channel should be considered for PSP screening. In this sense, it is interesting that besides providing a lower IC_{50} value for PSP standards than our previous *in vitro* method in cultured cerebellar neurons,[19] a fact probably due to the determination of isolated sodium currents in this work, the saxitoxin and gonyautoxins standards employed in this work also differed in their ability to inhibit voltage-dependent sodium currents at the highest

Table 1. Comparative Results of the Relative Potency and IC_{50} of PSP Standards to Inhibit Nav Current Amplitude in Electrophysiological Measurements and to Inhibit the Veratridine (VTD)-Induced Depolarization in Cerebellar Neurons (from ref 19)[a]

	relative potency Nav currents	IC_{50} (μM) Nav currents	relative potency[19] VTD depolarization	IC_{50} (μM)[19] VTD depolarization
STX	1.00	0.0035	1.00	0.0051
dcSTX	1.00	0.0035	0.84	0.0063
NeoSTX	1.02	0.0035	0.82	0.0062
dcNeoSTX	0.44	0.0080	0.48	0.0105
GTX1,4	0.50	0.0071	0.53	0.0095
GTX2,3	0.28	0.0128	0.37	0.0136
GTX5	0.09	0.0396	0.09	0.0520
dcGTX2,3	0.13	0.0270	0.22	0.0228
C1,2	0.02	0.1510	nd	nd

[a] nd: not determined.

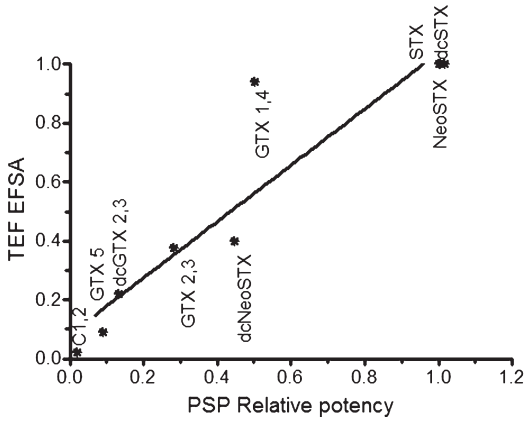

Figure 4. Linear relationship between the *in vitro* potency of PSP toxins to inhibit the peak sodium current amplitude in cerebellar neurons and the PSP toxicity equivalent factors reported by EFSA.

concentrations evaluated, a fact that may be useful to identify the PSP profile in a toxic sample.

The relative potencies of PSP toxin standards as indicated by their IC_{50} values to inhibit voltage-gated sodium currents were in the order NeoSTX > STX ≈ dcSTX > GTX1,4 > dcNeoSTX > GTX2,3 > dcGTX2,3 > GTX5 > C1,2, which closely correlates with their inhibitory potencies on the veratridine-induced depolarization and mouse bioassay.[19] In general, the IC_{50} values for PSP standards reported in this work are consistently lower than those previously reported in the same neuronal model by veratrine-induced depolarization; most likely, this observed effect is due to our measurements using isolated sodium currents in this work, thus eliminating potassium current interference. Noteworthy, the results reported here corroborate the proposed toxicity equivalent factors for PSP toxins reported by EFSA.[36] This fact is especially important in the case of GTX1,4 reference materials since the toxicity of this standard could have been overestimated previously (GTX1,4: 0.9[36] and 0.5 in this work).

Currently, the mouse bioassay and the Association of Official Chemists HPLC method (Lawrence method) are official methods for PSP detection in the European Union. Although not yet formally validated, other methods that have potential to determine STX-group toxins are receptor-based assays, antibody-based methods, and liquid-cromatography−tandem mass spectrometry (LC-MS/MS). However, most of these analytical methods do not provide information on the toxicity of the sample and require the use of toxicity equivalent factors. Here, we evaluated the potency of commercial PSP toxins by a simple, economical, and ethically acceptable functional assay and demonstrated that electrophysiological recordings in cultured neurons provide a sufficient tool to detect and quantify PSP toxins. While this method is also useful for ciguatoxins,[34] the method may discriminate between both types of toxins because PSP toxins do not change sodium channel activation. Therefore, this method could be suitable for high throughput screening of PSP toxins by automated patch-clamp in commercially available ready-to use cell lines expressing voltage-gated sodium channels.

AUTHOR INFORMATION

Corresponding Author

*Departamento de Farmacología, Facultad de Veterinaria, Universidad de Santiago de Compostela, Campus Universitario s/n, 27002, Lugo, Spain. Tel: 34-982 252 242. Fax: 34-982 252 242. E-mail: Luis.Botana@lugo.usc.es.

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ABBREVIATIONS

PSP, paralytic shellfish poisoning; TEF, toxicity equivalent factor; CGC, cerebellar granule cells; DMEM, Dulbecco's modified Eagle's medium; STX, saxitoxin; dcSTX, decarbamoylsaxitoxin; NeoSTX, Neosaxitoxin; dcNeoSTX, decarbamoylneosaxitoxin; GTX, gonyautoxin; dcGTX, decarbamoylgonyautoxin; C1,2, *N*-sulfocarbamoyl-gonyautoxin-2 and -3; MBA, mouse bioassay; MU, mouse unit; AOAC, Association of Official Analytical Chemists; VTD, veratridine.

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