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A FUNCTIONAL ASSAY FOR PARALYTIC SHELLFISH TOXINS THAT USES RECOMBINANT SODIUM CHANNELS

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A functional assay for paralytic shellfish toxins that uses recombinant sodium channels

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Abstract

Saxitoxin (STX) and its derivatives are highly toxic natural compounds produced by dinoflagellates commonly present in marine phytoplankton. During algal blooms ("red tides"), shellfish accumulate saxitoxins leading to paralytic shellfish poisoning (PSP) in human consumers. PSP is a consequence of the high-affinity block of voltage-dependent Na channels in neuronal and muscle cells. PSP poses a significant public health threat and an enormous economic challenge to the shellfish industry worldwide. The standard screening method for marine toxins is the mouse mortality bioassay that is ethically problematic, costly and time-consuming. We report here an alternative, functional assay based on electrical recordings in cultured cells stably expressing a PSP target molecule, the STX-sensitive skeletal muscle Na channel. STX-equivalent concentration in the extracts was calibrated by comparison with purified STX, yielding a highly significant correlation ($R = 0.95$; $N = 30$) between electrophysiological determinations and the values obtained by conventional methods. This simple, economical, and reproducible assay obviates the need to sacrifice millions of animals in mandatory paralytic shellfish toxin screening programs.

Keywords: Saxitoxins; Electrophysiological assay; Sodium channel

1. Introduction

Harmful algal blooms are natural phenomena triggered by complex environmental stresses including human pollution (Smayda, 1997a,b). Some 300 phytoplankton species produce "red tides" but only 60–70 species are actually harmful (Sournia, 1995). Dinoflagellates, in particular, produce potent non-peptide neurotoxins (Hall et al., 1990; Yasumoto and Murata, 1993). Among these, saxitoxins (Hall et al., 1990; Shimizu, 1996), brevetoxins (Baden, 1989) and ciguatoxins (Lewis, 1995) have the sodium channel protein as their sole molecular target and bind with high affinity to specific sites on the α subunit (Ritchie and Rogart, 1977; Barchi and Weigele, 1979). Fatal paralytic shellfish poisoning (PSP) intoxication represents the most serious threat of marine origin worldwide (Hallegraeff, 1995), with prominent public health and economic impact in Asia, Europe, North America (Anderson, 1989, 1997) and South America (Uribe et al., 1999). As a consequence, most seafood-exporting countries have established mandatory PSP toxin screening programs. The method most widely employed is the semi-quantitative mouse mortality bioassay (Horwitz, 1990). While reliable for regulatory purposes, this assay is costly and time-consuming. Its major limitation, however, is the controversial use of live animals. The assay measures the time to death after intraperitoneal injection of seafood extract, a procedure that has received such ethical criticism that it can no longer be carried out in some European countries (Cembella et al., 1995). Analytical HPLC methods require oxidation of STX analogues for fluorescent detection (Oshima et al., 1993) and the availability of

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scarce sets of analogues as internal standards. Acidic extracts from naturally contaminated PSP shellfish samples may contain over 20 different analogues of STX in variable proportions (Oshima et al., 1993; Lagos et al., 1996). Analytical methods provide the molar composition of toxic extracts, a quantity that has to be transformed into intraperitoneal toxicity values. This calculation relies on scales of relative toxicity obtained by the mouse bioassay carried out with pure STX analogues (Hall et al., 1990). Recently, the application of radioassays (Doucette et al., 1997) has been further hampered by rigid constraints on the international transfer of STX and radiolabelled derivatives owing to biological warfare conventions.[1] These considerations motivated our development of a molecularly based electrophysiological assay for the detection and quantification of PSP toxins. These data have been presented in preliminary form (Vélez et al., 1999; Suárez-Isla et al., 2000).

2. Materials and methods

2.1. Materials

Standard saxitoxin dihydrochloride was kindly provided by Dr Sherwood Hall (US Food and Drug Administration, Office of Seafood). Aliquots from batches No. 086-252B and 087-181A were used in this study. Saxitoxin diacetate was purchased from Sigma-Aldrich Chemical Corp. (St. Louis, MO) and all reagents used were of analytical grade.

2.2. Shellfish samples

A set of 41 acidic extracts of PSP toxic shellfish samples was used in displacement assays of tritiated saxitoxin. The extracts were obtained from mussels (*Mytilus edulis*), ribbed mussels (*Aulyacoma ater*) and clams (*Venus antiqua antiqua*). A second set of 30 acidic extracts of mussels and *locos* (a Chilean abalone-like gastropod; *Concholepas concholepas*) was used in the electrophysiological experiments. All toxic shellfish samples were obtained during official monitoring cruises from areas closed to commercial fishing. The extracts were kindly provided by the Servicio de Salud Magallanes, Punta Arenas, Chile.

2.3. Mouse bioassay

Mouse bioassays were performed at the Laboratory of Marine Toxins, University of Chile, with the sets of PSP toxic samples provided by the Servicio de Salud Magallanes. Bioassays were performed with CF-1 mice of 20 ± 2 g reared in a local facility from parents provided by the Instituto de Salud Pública, Chile, following the standard procedure of the Association of Official Analytical Chemists (Horwitz, 1990). A correction factor (relationship between STX concentration and time of death), was obtained by injecting aliquots of standard STX dihydrochloride (U.S.F.D.A. Batch No 086-252B, Office of Seafood) intraperitoneally into three groups of ten mice each over four different days. The final correction factor was 0.234.

2.4. Radioassay

Membranes were isolated by homogenization and differential centrifugation from chick cerebellum (Sierralta et al., 1996). Binding assays were run in duplicate with 100–200 μg protein per vial in Tris HCl buffer, pH 7.4, in the presence of choline chloride, calcium and KCl. Free [^{3}H]STX (Amersham, UK) was separated on GFC filters (Millipore, Bedford, MA). Scatchard analysis of reference [^{3}H]STX displacement curves gave: K_D (nM) = 0.80 ± 0.41 and B_{max} (pmol/mg protein) = 1.68 ± 0.01 ($n = 3$). The extrapolated K_D for STX obtained by the infinite dilution method was 0.47 nM. Overall apparent K_D obtained with chick cerebellar membranes was 0.87 ± 0.07 (mean ± S.E.M.) (±8.3% coefficient of variation) for 38 reference curves obtained in the range of 0.5–50 nM cold STX using eight different batches of [^{3}H]STX in a period of 22 months. Toxicity evaluations of natural PSP extracts were performed in triplicate on dilutions of untreated acid extracts (dilution range was 1:125–1:12500).

2.5. Electrophysiological recordings

HEK 293 cells stably expressing STX-sensitive rat skeletal muscle Na channels (μ1) (Yamagishi et al., 1997) were patch clamped in the whole-cell configuration. Na currents were recorded under control conditions and after perfusion with several dilutions of extracts of shellfish samples. The control external solution was (mM): 70 NaCl, 70 TEACl (or 70 TMACl), 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4. The patch pipette (1–2 Mohms) contained (nM) 140 CsF, 5 NaCl, 1 $MgCl_2$, 10 EGTA, 10 Hepes, pH 7.2. Peak sodium currents were elicited every 2 or 3 s by 10-ms depolarizing pulses from a holding voltage of −100 to −10 mV. A P/4 protocol was used to subtract linear capacitative and leak currents. To ensure appropriate voltage control, cells expressing no more than 4 nA of peak sodium current were used. The cells were continuously perfused at 1 ml/min at 21–22°C. Signals from an Axopatch 200-B patch clamp amplifier (Axon Instruments, Foster City, CA) were low-pass filtered at 10 kHz, acquired at 50 kHz and analyzed using pCLAMP software (Axon Instruments, Foster City, CA).

The half-blocking concentration (IC_{50}) for STX from the reference curves was determined by a least-squares fit (Levenberg–Marquardt algorithm) of the data to the

[1] STX was included by the Organization for the Prohibition of Chemical Weapons in the list of toxic compounds qualified as potential chemical weapons. As of August 1, 1997, STX cannot be re-exported even among treaty signing countries.

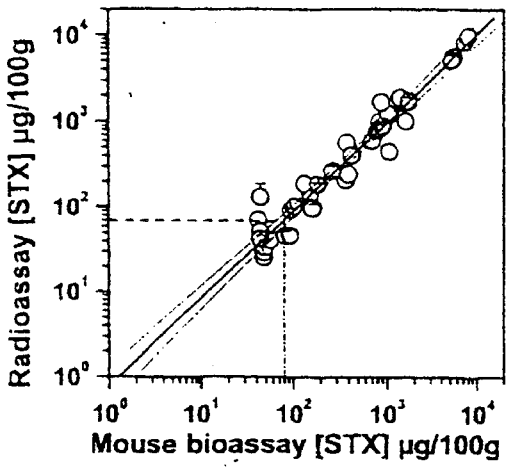

Fig. 1. Correlation between PSP toxicity levels determined by the mouse mortality assay and toxin binding assessed by the STX displacement binding radioassay. The displacement of [^{3}H]STX by natural PSP extracts was performed with toxic samples provided by the Servicio de Salud Magallanes (40–10 000 µg STX/100 g). A significant correlation ($R = 0.972$ for 41 samples) was found between the toxicity levels determined by mouse bioassay and displacement of labeled toxin. The dotted lines indicate the safety limit of 80 µg STX/100 g.

function $I/I_0 = I\{1 + ([STX]/IC_{50})^n\}$, where I and I_0 are, respectively, the currents in the presence and absence of STX and n is the Hill coefficient obtained from the fit. The fit was performed by a subroutine included in the ORIGIN 6.0 plotting and analysis program (OriginLab Corporation, Northampton, MA). Calibration curves were generated with increasing concentrations (0.01–100 nM) of STX-diacetate (Sigma Chemical Corp.) or STX-dihydrochloride (US Food and Drug Administration, Office of Seafood; batch 087-181A).

3. Results

3.1. [^{3}H]STX displacement assay

The samples of toxic extracts used in this study were collected from fjords of the Magallanes region in Chile (49–55°S). Naturally contaminated shellfish extracts from that region contain up to 10 STX analogues in variable molar ratios (HPLC analysis with fluorescent detection; Oshima et al., 1993) including a high proportion of the extremely potent gonyautoxins 1 to 4 and neo-STX (Lagos et al., 1996). To establish whether the intraperitoneal toxicity of natural PSP extracts from this region as measured by mouse bioassay was due to these saxitoxins, a [^{3}H]STX displacement assay (Doucette et al., 1997; Barchi and Weigele, 1979) was performed. Displacement assays provide a measure of the capacity of toxin mixtures to displace radiolabelled STX from the common binding site on the channel protein (Ritchie and Rogart, 1977; Barchi and Weigele, 1979; Backx et al., 1992; Lipkind and Fozzard, 1994). However, biological toxicity is the end result of a complex interaction of bioavailability factors and access of the saxitoxins to their target molecule. To determine the relationship between STX displacement and in vivo toxicity, we compared various shellfish extracts for their ability to displace [^{3}H]STX binding (Ritchie and Rogart, 1977; Barchi and Weigele, 1979) with lethal toxicity in mice. Fig. 1 shows a good correlation between STX binding and toxicity evaluated by these two conventional tests ($R = 0.97$; $N = 41$). These data suggest that the binding of mixed PSP toxins present in different ratios and amounts elicits biotoxicity proportional to the capacity of the toxic mixture to displace [^{3}H]STX from its binding site.

3.2. A functional assay using patch-clamp current recordings

Displacement of a radiolabeled ligand does not directly assay how saxitoxins impair Na channel function. To determine how the biotoxicity of PSP extracts reflects blockage of sodium channels, we established a functional assay using patch-clamp current recordings in HEK 293 cells stably expressing the STX-sensitive rat skeletal muscle Na channel (µ1) (Yamagishi et al., 1997). The cells displayed robust Na currents (Fig. 2a) and their small size (diameter 14 ± 5 µm) enabled us to avoid voltage-clamp inhomogeneities during runs that lasted on average more than 90 min. As shown in Fig. 2b, bath-applied STX blocked peak sodium currents (I_{Na}) in a concentration-dependent manner. After perfusing the cells with a solution containing STX, I_{Na} decreased with a time constant that depended on the frequency of stimulation (Conti et al., 1996) and reached a new steady-state level determined by the STX concentration. Steady-state values of I_{Na} were measured at least 2 min after solution exchange.

3.3. Concentration dependence of STX block of sodium current

Fig. 2b displays the concentration-dependent decrease of I_{Na} amplitude in the presence of 0.3, 1.0 and 3.0 nM standard STX. Fig. 3 shows full dose-response curves obtained with saxitoxin from two suppliers. Sigmoidal fits to the experimental data indicated an IC_{50} of 1.17 ± 0.05 nM (12 cells; Hill coefficient $n = 0.967$) for STX-diacetate (Sigma Chemical Co., St. Louis, MO) (filled circles) and 1.46 ± 0.05 nM (five cells; Hill coefficient $n = 0.825$) for standard STX-dihydrochloride provided by the US Food and Drug Administration (open circles). This slight difference underlies the need to generate calibration curves for each batch of standard.

3.4. Evaluation of PSP toxicity with the electrophysiological assay

We next used the electrophysiological assay to evaluate

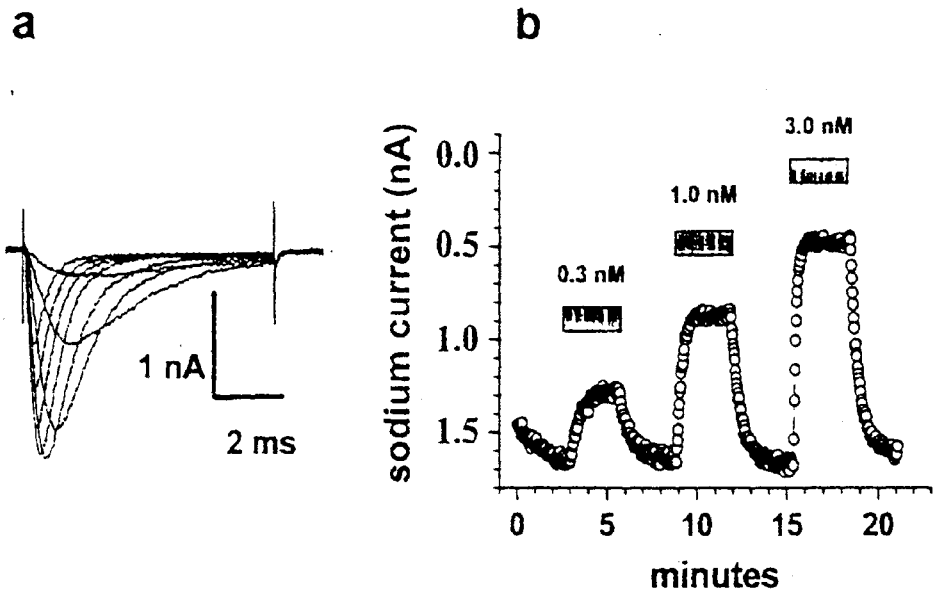

Fig. 2. (a) A family of currents recorded from HEK 293 cells stably expressing the skeletal muscle Na channel α subunit in response to voltage pulses from −40 to +30 mV from a holding potential of −100 mV. The peak I_{Na} was elicited at a test potential of −10 mV and pulses of 10 ms duration were applied every 2 or 3 s. (b) Dose-response of bath-applied STX on sodium currents in HEK 293 cells. Each point represents peak sodium current at −10 mV. The concentration-dependent decrease of sodium current amplitude was measured at 0.3, 1.0 and 3.0 nM standard STX, after a steady state level was reached.

PSP toxicity in naturally contaminated samples. Fig. 4 demonstrates the effect of application and washout of 1 nM STX after stabilization of the electrical recording. Diluted PSP samples were then applied sequentially, interspersed with purified STX (1 nM). The regulatory limit of 80 μg of STX equivalent per 100 g tissue is comparable to a solution of 1910 nM STX-diacetate or 2149 nM for STX-dihydrochloride. Thus, it was necessary to dilute toxic samples approximately 1000-fold (~1 nM final concentration) to fall within the dynamic range of I_{Na} blockage. The transformation factor of wet tissue weight to volume was 1.00 ± 0.02 (n = 36) as determined in tests with five different shellfish species. The dilution factor of three orders of magnitude reduced significantly variations in pH and divalent cation concentration, factors that are known to modify STX binding (Doyle et al., 1993). In addition, matrix effects that could interfere with the assay were minimized.

To compare toxicity evaluations by mouse bioassay and the electrophysiological assay, 30 samples of PSP extracts ranging from 35 to 800 μg STX eq./100 g were used. Fig. 5 demonstrates a robust correlation between the two assays (R = 0.946).

3.5. Practical detection limit

The practical detection limit was determined by the addition of decreasing STX concentrations. Exposure to 0.1 nM STX produced a small but reproducibly detectable current inhibition (Fig. 6). Average values of peak I_{Na} before and during exposure to STX were significantly different, and the observed deflection yielded a signal-to-noise ratio >3:1. Even though smaller toxin-induced current reduction could be detected, 0.1 nM STX represents a robust practical detection limit. This is equivalent to 0.042 μg STX/100 g, or 19100 times below the regulatory limit and 8550 times below the mouse test detection limit (based on STX-diacetate).

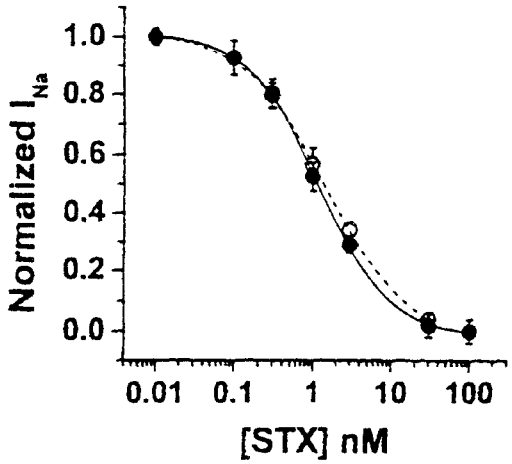

Fig. 3. Reference inhibition curves for STX. Open circles: STX-dihydrochloride (US Food and Drug Administration, Office of Seafood; batch 087-181A). IC_{50} = 1.46 ± 0.05 nM (mean ± S.D.; five experiments; N = 0.825), Hill coefficient). Filled circles: STX-diacetate (Sigma Chemical Corp.). IC_{50} = 1.17 ± 0.05 nM; 12 experiments; N = 0.967). The points represent the fractional inhibition of peak I_{Na} and the error bars indicate standard deviations. Solid and dotted curves represent least square-fits to the data obtained as described in Section 2.

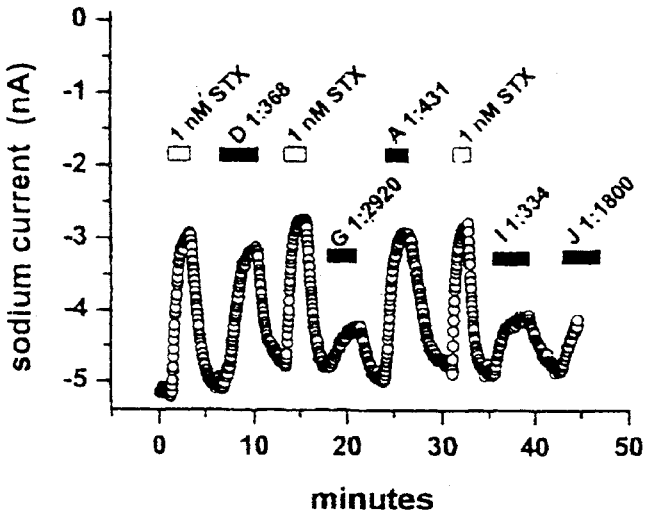

Fig. 4. Na channel inhibition by extracts from contaminated shellfish. Each point represents peak I_{Na} at −10 mV. The open bars represent bath application of standard STX; other bars indicate application of toxic PSP extracts. Extracts were first tested at 1000-fold dilution and then diluted to produce fractional inhibition of I_{Na} near 0.5. Dilution factors are shown in the figure. In this example, five samples were tested within 50 min.

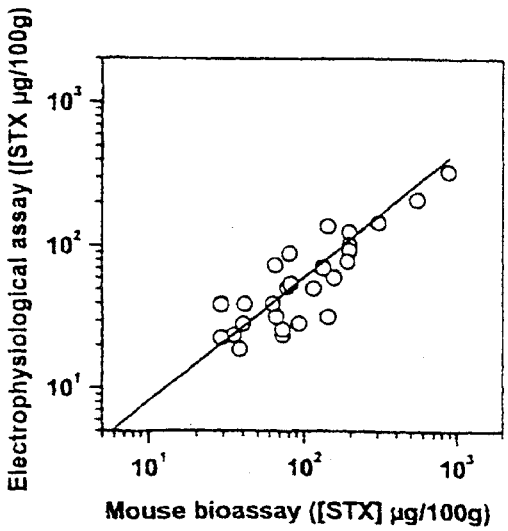

Fig. 5. Correlation between PSP toxicity levels determined by the mouse bioassay and inhibition of Na current electrophysiologically determined. Na channel blockade was determined in dilutions of shellfish extracts from 1:800 to 1:8000. A significant correlation ($R = 0.95$ for 30 samples) was found between the toxicity levels determined by mouse bioassay and inhibition of expressed Na current.

3.6. Reproducibility

The percent Na current inhibition within different experiments and cells with different maximal I_{Na} (0.8–4.0 nA) was very reproducible, consistent with the clonal origin of the expressed channels. The percent current inhibition was 36.40 ± 2.36 for 0.7 nM STX (mean ± SD; $n = 5$) and 52.28 ± 3.76 ($n = 12$) for 1.0 nM STX (diacetate salt, Sigma Chem. Corp.).

4. Discussion

Accumulation of phycotoxins by filter-feeding shellfish is a well-known global phenomenon and has become a continuous threat to public health worldwide (Hallegraef, 1993, 1995). These phycotoxins include diarrheic shellfish poisoning (DSP) toxins, amnesic shellfish poisoning (ASP) toxins, neurotoxic shellfish poisoning (NSP) toxins, and PSP toxins known as saxitoxins (Strichartz and Castle, 1990).

Saxitoxin-producing dinoflagellates cause great economical losses around the world owing to closure of shellfish harvesting grounds during algal blooms and the negative impact on seafood marketing resulting from such events (Hallegraef, 1993, 1995). Since harmful algal blooms are complex events and cannot be predicted, the only way to avoid the human health threat is to detect the toxin levels before shellfish reach human consumers. Thus, there is a need for efficient saxitoxin detection methods. Most of the available methods for toxin detection are expensive, require costly analytical standards, use large numbers of laboratory animals or are based on radiolabelled compounds that have been catalogued as chemical weapons by international treaties. Therefore, there are additional economical and social effects because of the need for expensive or ethically questionable monitoring programs to ensure product safety.

We tested here the feasibility of a simple, economical and ethically acceptable functional assay based on electrophysiological recordings in cultured cells stably expressing a PSP target molecule, the STX-sensitive skeletal Na channel (Yamagishi et al., 1997; Catterall, 1980, 1992). The

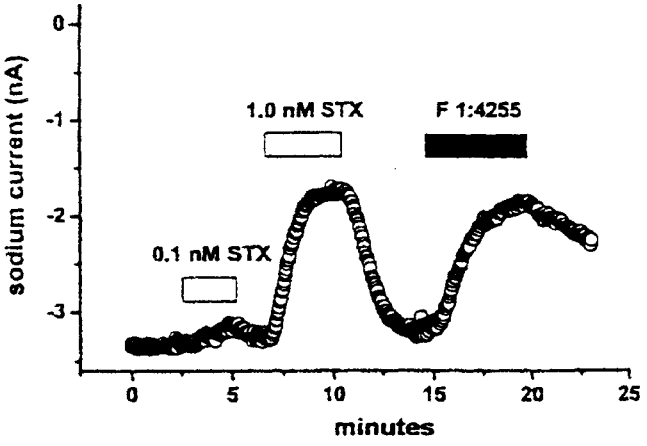

Fig. 6. Determination of the practical detection limit. In this experiment, two bath applications of 0.1 and 1.0 nM standard STX preceded exposure to a diluted toxic sample. Addition of 0.1-nM STX produced reproducibly detectable current inhibition. Average values of peak I_{Na} before and after STX were significantly different and the observed deflection is equivalent to a signal to noise ratio >3:1. Smaller inhibitions could still be detected, but 0.1 nM STX was selected as the practical detection limit.

assay is straightforward and, while it requires personnel trained in electrophysiology, it is actually less laborious than the empirical mortality bioassay. Standard culture methods are sufficient to maintain the cell line used to express the toxin receptor channel (HEK 293 cells). The small size and simple geometry of these cells avoid artifacts resulting from poor voltage control (Marty and Neher, 1995). Moreover, these cells are easy to patch and current recordings can last as long as 2 h allowing several samples to be tested in the same cell. A trained technician can analyze 8–10 samples per work shift (8 h). The reproducibility of our electrophysiological assay is demonstrated by the small standard errors (<4%) obtained with the same sample measured in different cells. The high correlation found between measurements of PSP toxicity levels by the inhibition of Na current in HEK cells compared to those obtained with the standard bioassay prove the reliability of the electrophysiological test. Furthermore, this assay is three orders of magnitude more sensitive than the mouse bioassay (40 vs. 0.04 μg STX/100 g, respectively). Thus, this functional electrophysiological assay is a promising tool to complement and replace eventually the conventional mouse bioassay in regulatory screening of shellfish samples for PSP contamination. Indeed, given the assay's high sensitivity, natural ecological variations in subtoxic PSP levels can be tracked, providing an early detection of harmful algal blooms. Given its circumvention of animal testing and compliance with biological warfare treaties, the electrophysiological assay has obvious ethical advantages avoiding the use of experimental animals and radioactive STX or STX analogues as internal standards. These results are of immediate utility and provide the basis for simplified biosensors based on recombinant Na channels that can be tailored to specific toxins of the Na channel, other than saxitoxins. In 1986, the Royal Society (London), defined a biosensor as: "a device that recognizes an analyte in an appropriate sample and interprets its concentration as an electrical signal via a suitable combination of a biological recognition system and an electrochemical transducer". In this sense, ion channels fulfill this definition without the need of ancillary proteins as they are molecular devices that combine a recognition site (i.e. the toxin binding site) and conductive moieties in the same macromolecule. Saxitoxin binding results in blockade of the conduction pore, interrupting the conduction of ions and the electrical current. Therefore, sodium channels appear as logical candidates to develop biosensors for natural toxins that target the macromolecule, a strategy that is currently being developed in other laboratories (Sackmann, 1995; Nikolelis et al., 1996; Kasianowicz et al., 1996; Cornell et al., 1997; Costello et al., 1998, 1999, 2000; Branton and Golovchenko, 1999; Bayley, 1999; Gu et al., 1999, 2000). In conclusion, we report a highly sensitive functional assay that directly monitors and quantifies the interaction of saxitoxins with their natural receptor by measuring the fractional Na current inhibition by PSP toxic extracts.

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The detection limit of the AOAC mouse bioassay for Paralytic Shellfish Toxins (PSP) as determined by an electrophysiological assay.

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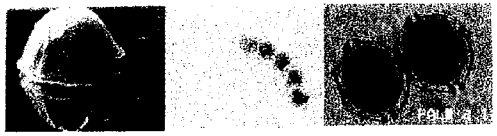
Poster N° 2202

ABSTRACT

The AOAC mouse bioassay for saxitoxins (STX) is the main regulatory tool applied worldwide to analyze shellfish and prevent PSP intoxications and deaths. The end point of the bioassay is the time of death (T_D) of 20 ± 2 g mice, i.p. injected with 1 mL of shellfish meat extracts diluted to give T_D between 5-7 min. Measurements are standardized with i.p. injections of known dilutions of standard STX. One of the critical limitations of the method (besides the occurrence of false positives and ethical concerns involved in the use of live animals), is that near 40 µg STX equivalent /100 grams of tissue, T_D shows an increasing variability, introducing a large degree of uncertainty in public health decisions, as this value is very near to the action level (80 µg STX eq./100 g). We have applied an electrophysiological assay for STXs (Vélez y cols., Toxicon 39:929, 2001) to determine toxicity and the limit of detection of the mouse bioassay. Naturally intoxicated shellfish samples obtained during a recent PSP outbreak in the island of Chiloé (January-May 2002) were utilized (N=157). All samples higher than 60 µg STX/100 g were detected by mouse bioassay. In contrast, samples between 40 and 60 µg STX eq/100 g had a chance of 80,0% of being detected, a percent that decreased to only a 52,8% for the range 30 to 40 ug/100 g. and to 17,2% for 20-30 µg/100 g. This suggests that the operational detection limit for the mouse bioassay should be set at 40 µg STX eq/100 g. For regulatory decisions and in view of the uncertainty, values lower than 40 µg/100 g as determined by mouse bioassay, should be reported as "below the detection limit".



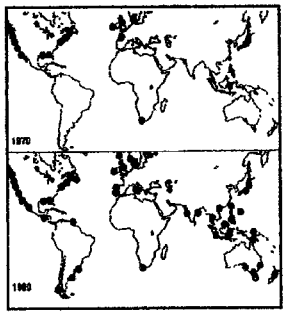
Alexandrium catenella, a saxitoxin producing dinoflagellate

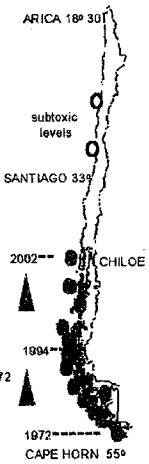
INTRODUCTION

Sodium channel toxins pose a significant and increasing public health threat and an enormous economic challenge to the shellfish industry worldwide. Several species of toxic dinoflagellates produce saxitoxins, brevetoxins and ciguatoxins that bind to specific sites on the α subunit (Cestele & Catterall, 2000). Saxitoxins produce Paralytic Shellfish Poisoning (PSP), a potentially lethal syndrom resulting from high-affinity block of voltage-dependent Na channels in neuronal and muscle cells.

Fatal PSP intoxications represent the most serious threat of marine origin worldwide, with prominent public health and economic impacts in Asia, Europe, North America and South America. As a consequence, most seafood-exporting countries have established mandatory PSP toxin screening programs. The method most widely employed is the semi-quantitative mouse mortality bioassay (Horwitz, 1990). While reliable for regulatory purposes, this assay is costly and time-consuming. Its major limitation, however, is the controversial use of live animals. The mouse bioassay is the main regulatory tool applied worldwide to analyze shellfish and prevent PSP intoxications and deaths. The end point of the bioassay is the time of death (T_D) of 20 ± 2 g mice, after i.p. injection of 1 mL of shellfish meat extracts diluted to give T_D between 5-7 min. Measurements are standardized with i.p. injections of known dilutions of standard STX. One of the critical limitations of the method (besides the occurrence of false positives and ethical concerns involved in the use of live animals), is that near 40 µg STX equivalent /100 grams of tissue, T_D show an increasing variability, introducing a large degree of uncertainty in public health decisions, as this value is very near to the action level (80 µg STX eq./100 g). We have applied a highly sensitive, electrophysiological assay for STXs (Vélez y cols., Toxicon 39:929, 2001) to determine toxicity and the limit of detection of the mouse bioassay.

Changes in worldwide distribution of PSP





Expansion of PSP in Chile since 1972

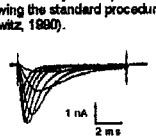
IMPACTS OF PSP IN CHILE

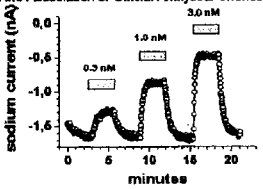
In the last 10 years high levels of PSP have been reported in southern Chile (41°- 55° SL). The main causative toxic dinoflagellate species has been identified as *Alexandrium catenella*. (Uribe et al., 1999; Vélez et al., 2001). The first reported PSP human intoxication was detected in 1972 in Bell Bay, Magallanes region (52° SL). Blooms of *Alexandrium catenella* and PSP outbreaks have taken place in the Magallanes region (50-55° LS) in 1972, 1981 and 1989. Since 1991 regular annual blooms have been detected and they have been associated with widespread and persistent toxic levels of PSP in shellfish. A similar situation developed since 1994 in the contiguous northern region of Aysen (44-55° LS). In these two largely unpopulated provinces a total of 387 cases of PSP human intoxications have been officially reported, including 26 fatalities. In year 2002 and for the first time, a severe PSP outbreak in the island of Chiloe (41-42° LS), an area of intensive salmon farming and shellfish aquaculture, caused 63 intoxications and 3 fatalities.

METHODS

HEK 293 cells stably expressing STX-sensitive rat skeletal muscle Na channels (µ1) (Yamagishi et al., 1997) were patch clamped in the whole-cell configuration. Na currents were recorded under control conditions and after perfusion with several dilutions of extracts of shellfish samples (Vélez et al. Toxicon 39 (2001) 929-935.

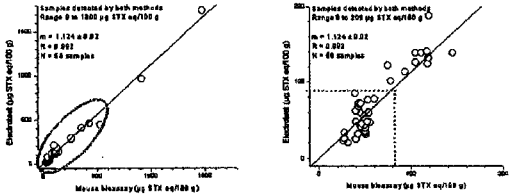
Mouse bioassays were performed with CF-1 mice of 20 ± 2 g reared in a local facility following the standard procedure of the Association of Official Analytical Chemists (Horwitz, 1990).





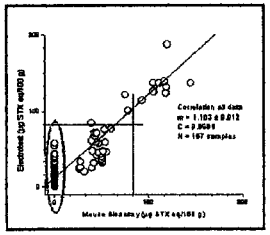
RESULTS

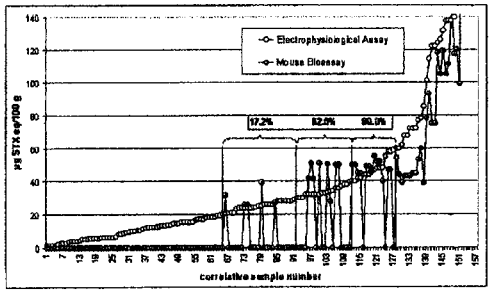
A set of 157 samples was analyzed by the electrophysiological assay and the AOAC mouse bioassay (MB). Levels of toxicity determined by the electrophysiological assay ranged from 1.1 to 1744 µg STX eq/100 g. 20 samples contained more than 80 µg STX/100 g and 29 were higher than 60 µg STX/100 g. All samples higher than 60 µg STX/100 g were detected by the mouse bioassay (MB) and a good correlation was found between the two methods. In contrast, only 20.3% of all samples under 60 µg STX/100 g (26 out of 128) could be found to contain PSP as measured by MB.

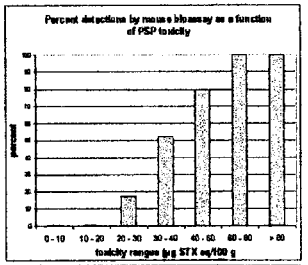


UNCERTAINTIES IN THE MOUSE BIOASSAY

Closer examination of lower toxicity ranges confirmed that management decisions taken solely on the basis of MB results are increasingly risky for samples below 60 µg STX/100 g.







TOXICITY RANGE (µg STX eq/100)	% CHANCE TO BE DETECTED
40 and 60	80.0
30 and 40	52.8
20 and 30	17.2

CONCLUSIONS

This is the first estimation of the operational detection limit of the AOAC mouse bioassay for PSP toxins obtained with an independent methodology that directly measures the funcional effect of saxitoxins.

Results confirm that the AOAC MB is a robust regulatory tool for samples that exceed 60 µg STX/100 g and suggest that an operational detection limit for the MB should be set at 40 µg STX eq/100 g.

Calculated values obtained from death times below 40 µg STX eq/100 g are meaningless and should be reported as a "below the detection limit" result.

These observations confirm the utility of the patch clamp technique in PSP toxicity measurements and demonstrate the need to modify current monitoring strategies with the aim to determine the intra-population variability of PSP toxicity in an aquaculture site or shellfish bed.

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