

High affinity binding of red tide neurotoxins to marine mammal brain

Vera L. Trainer ^{a,*}, Daniel G. Baden ^b

^a National Marine Fisheries Service, Northwest Fisheries Science Center, Environmental Conservation Division,
2725 Montlake Blvd. E., Seattle, WA 98112, USA

^b National Institute of Environmental Health Sciences Marine and Freshwater Biomedical Sciences Center, University of Miami,
RSMAS, 4600 Rickenbacker Cswy, Miami, FL 33149, USA

Received 7 July 1998; received in revised form 27 October 1998; accepted 4 November 1998

Abstract

During a period of several weeks in the spring of 1996, over 200 manatees (*Trichechus manatus latirostris*) were found dead or dying in coastal waters or on beaches of the Florida west coast. Concurrent with this event, high densities of *Gymnodinium breve*, the dinoflagellate which produces the potent neurotoxin called brevetoxin, were observed in the same coastal areas. Our study demonstrates that brevetoxin binds to isolated nerve preparations from manatee brain with similar affinity as that reported for a number of terrestrial mammals. Analysis of receptor binding of tritiated brevetoxin to manatee brain, illustrates saturable specific binding, competition of specific binding by a non-radioactive toxin of the same structure, and temperature dependence of binding. Complementary studies with the red tide neurotoxin, saxitoxin, which is responsible for the intoxication syndrome paralytic shellfish poisoning, show high affinity and specific binding of this toxin to isolated nerve preparations from several marine mammals, including manatee, gray whale (*Eschrichtius robustus*), humpback whale (*Megaptera novaeangliae*), and sea lion (*Zalophus californianus*). These results demonstrate the specific binding of brevetoxin and saxitoxin to excitable brain tissue of marine mammals and support the hypothesis that the exposure of manatees to brevetoxin in the spring of 1996 was a factor in their stranding and death. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Red tides; Marine mammal; Brevetoxin; Saxitoxin; Sodium channel; Receptor binding

1. Introduction

The primary neurotoxins responsible for the intoxication syndromes paralytic shellfish poisoning (PSP) and neurotoxic shellfish poisoning

* Corresponding author. Tel.: +1-206-8606788; fax: +1-206-8603335.

E-mail address: vera.l.trainer@noaa.gov (V.L. Trainer)

(NSP) exert their effects by binding to specific sites on the voltage-sensitive sodium channels of nerves. Sodium channels are essential for the initiation and propagation of action potential in excitable cells. The PSP toxins, consisting of saxitoxins, gonyautoxins, and a corresponding suite of sulfocarbamate derivatives, bind to site 1 at the mouth of the sodium channel pore, thereby blocking the flow of sodium ions into the channel (Ritchie and Rogart, 1977). The NSP toxins, a suite of at least nine toxic isoforms called brevetoxins, bind to sodium channel site 5 to shift activation to more negative potentials, resulting in subsequent membrane depolarization (Huang et al., 1984).

A recent indication of a severe environmental consequence of exposure to red tide toxins from *Gymnodinium breve*, which causes NSP, was a massive manatee kill in early 1996 (Landsberg and Steidinger, 1998), which was thought to be the result of the lipophilic neurotoxins exerting their effects on the lung and central nervous system of these aquatic species. Autopsy results were consistent with neurointoxication facilitated by oral and inhalation exposure (Bossart et al., 1998). Cell counts of *G. breve* were approximately $23.3 \text{ million cells l}^{-1}$ in Florida west coast waters and remained high between 1 March and 1 May 1996, coincident with the manatee epizootic, during which time 158 animals died (Landsberg and Steidinger, 1998). This intoxication episode is consistent with the direct, deleterious effect of algal toxins on marine mammals via ingestion or inhalation.

Saxitoxin, the causative agent of PSP and the parent compound of the more than 21 toxic derivatives that exist, can also affect marine mammals by specific interaction with sodium channels. The death of 14 humpback whales in the late 1980s off Cape Cod, MA, was believed to be due to saxitoxin transfer from the planktonic algae through the viscera of Atlantic mackerel (Geraci et al., 1989). Incompletely digested mackerel were found in the stomachs of several dead whales, illustrating the potential of food web transfer of toxins as a lethal route of exposure in marine mammals.

The sequence of events in toxic episodes during red tides begins with entry of the toxins into the animal. The route of exposure is an important

consideration in the ability of a toxin to actually reach its target, a specific site of action on nerve membranes. Specific, high affinity interaction of algal toxins with the nerves of terrestrial mammals has been extensively documented (Catterall et al., 1979; Tamkun and Catterall, 1981; Rogart et al., 1983; Poli et al., 1986; Querfurth et al., 1987). Although numerous investigators have described the symptoms of marine mammals exposed to red tides (for example, Geraci et al., 1989; Steidinger, 1989; Landsberg and Steidinger, 1998), the specific binding of toxins to excitable tissues of aquatic mammals has not yet been characterized.

The voltage-sensitive sodium channel is a highly conserved protein. It might, therefore, be expected that evolutionarily-related organisms such as aquatic and terrestrial mammals would have a high amino acid sequence similarity at toxin binding sites and therefore demonstrate similar affinity for toxins that bind to those specific receptor sites. However, this must not be assumed since it is known that even single amino acid changes can affect toxin binding to sodium channels (Noda et al. 1989). Employing a rapid filtration technique to separate bound from free toxin following binding in vitro, we illustrate that both brevetoxin and saxitoxin bind with high affinity to brain synaptosomes of manatee. Furthermore, saxitoxin binds with high affinity to excitable brain tissue of a number of marine mammals, demonstrating the potential for intoxication of these organisms upon exposure to a sufficient level of saxitoxin in vivo. This study clearly illustrates that excitable tissue of marine mammals has similar affinity to red tide neurotoxins as terrestrial mammals, such as the rat, in which a direct correlation between toxin exposure and deleterious health effects has been previously demonstrated.

2. Materials and methods

2.1. Materials

Tritiated saxitoxin was purchased from Amersham Life Science and unlabeled saxitoxin was obtained from ICN Pharmaceuticals. Both

[³H]PbTx-3 and unlabeled PbTx-3 were supplied courtesy of the National Institute of Environmental Health Sciences Marine and Freshwater Biomedical Sciences Center at the University of Miami. All other chemicals were of reagent grade or of the highest purity available.

2.2. Brain tissue collection

Brain tissue was collected as part of the National Marine Fisheries Service Marine Mammal Health and Stranding Response Program. In all cases, tissue was obtained immediately after death from live, stranded organisms (manatee, *Trichechus manatus latirostris*; California sea lion, *Zalophus californianus*; gray whale, *Eschrichtius robustus*) or soon after death from a humpback whale (*Megaptera novaeangliae*) and immediately placed in a plastic bag on ice before storing at -20°C .

2.3. Brain tissue preparation

Using 10 strokes of a motor-driven tissue homogenizer, approximately 4 g of tissue from each mammal was homogenized in 20 ml ice-cold sucrose buffer (5 mM sodium phosphate, 0.32 M sucrose, pH 7.4) to which 10 μM PMSF was added immediately prior to use. The homogenate was sedimented at $1000 \times g$ for 10 min at 4°C . The supernatant suspension was removed and the pellet resuspended in 20 ml sucrose solution, homogenized, and centrifuged as before. The combined supernatant suspensions were centrifuged at $17\,000 \times g$ for 60 min at 4°C . The resulting pellet was resuspended in 4 ml sucrose buffer, homogenized with five strokes of the tissue homogenizer and stored frozen at -80°C in 1 ml aliquots until use.

2.4. Binding assays

Samples from organisms which inhabit coastal locations where PSP is common were used in saxitoxin binding studies; likewise, only manatees, which are typically found in coastal areas where NSP is common were used in brevetoxin binding experiments. All binding experiments were per-

formed in a 1 ml total volume in binding medium containing 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg ml^{-1} bovine serum albumin (BSA), 1 mM EGTA, 370 mM sucrose, and 0.01% Emulphor EL-620 emulsifier (GAF Corporation). Aliquots (0.1 ml) of mammal brain synaptosomes (approximately 0.1 mg total protein) were suspended in binding medium minus BSA, and added to a reaction mixture containing tritiated polyether brevetoxin, [³H]PbTx-3, or tritiated saxitoxin, [³H]STX, and nonradioactive standard toxin in 0.9 ml binding medium in borosilicate glass tubes. For calculation of dissociation constant and binding maxima, tritiated brevetoxin concentrations ranged from 0 to 45 nM, and saxitoxin concentrations ranged from 0 to 8 nM. During competition experiments, unlabeled brevetoxin concentrations ranged from 0 to 1 μM with tritiated brevetoxin PbTx-3 held constant at 5 nM.

After incubation at 0°C for 1 h (for brevetoxin) and at 0°C for 30 min (for saxitoxin), the reaction was stopped by adding 5 ml ice-cold wash medium (5 mM HEPES, pH 7.4, 163 nM choline chloride, 1.8 mM calcium chloride, 0.8 mM magnesium sulfate, 370 mM sucrose, and 1 mg ml^{-1} BSA) to each tube. Contents of each tube were immediately poured over a Whatman GF/C filter and tubes were rinsed twice with 5 ml each of wash medium.

Filters were transferred to liquid scintillation vials containing 10 ml Ecolume and radioactivity was quantified by scintillation spectroscopy. Non-specific binding was measured in parallel tubes containing a saturating concentration (1 μM) of unlabeled toxin (either PbTx-3 or saxitoxin) and was subtracted from total binding to yield specific binding. Protein was estimated by bicinchonic acid assay (BCA, Pierce Chemical).

3. Results and discussion

The model system for characterization of toxin binding to sodium channels in vitro is derived from a preparation of axon terminals in rat brain called synaptosomes. Rat brain synaptosomes, as

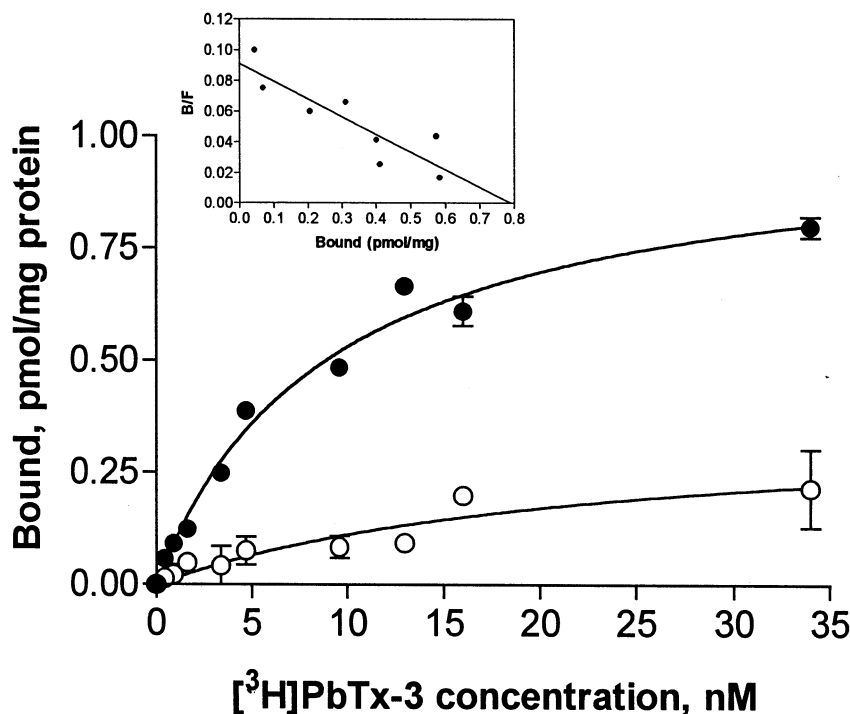


Fig. 1. Binding of $[^3\text{H}]\text{PbTx-3}$ to manatee brain synaptosomes. Synaptosomes were incubated with increasing concentrations of $[^3\text{H}]\text{PbTx-3}$ for 1 h at 0°C using the rapid membrane filtration assay as described in Section 2. Total binding ($\bullet$) and nonspecific binding ($\circ$) measured in the presence of $1\ \mu\text{M}$ PbTx-3 are plotted versus the measured free brevetoxin concentrations. Free brevetoxin concentrations were calculated by determining the radioactivity in an aliquot of each diluted standard. Values are means ($\pm$ S.D.) of three separate analyses. Blank values (no added synaptosomes) were subtracted in all cases. Inset: analysis of the specific binding of $[^3\text{H}]\text{PbTx-3}$ to manatee brain synaptosomes in a Scatchard plot. The regression line was obtained using GraphPad Prism (GraphPad Software, San Diego, CA) transformation and regression of mean specific binding values from saturation experiments. The B_{max} value is the x intercept.

well as excitable brain tissue prepared from other terrestrial mammals, have been demonstrated to bind with nanomolar affinity and high specificity to the neurotoxins, brevetoxin and saxitoxin (Catterall et al., 1979; Tamkun and Catterall, 1981; Rogart et al., 1983; Poli et al., 1986; Querfurth et al., 1987). This demonstration of a specific *in vitro* effect is correlated with the potential for intoxication due to *in vivo* exposure to toxins.

Several criteria of specific toxin–receptor interactions have been met in this study: saturability of specific binding, competition of specific binding by non-radioactive toxin of the same structure, linearity of binding with increasing tissue protein concentration, temperature dependence, and a binding maximum in the pmol mg^{-1} protein range. A complete analysis of both brevetoxin and

saxitoxin binding to manatee brain tissue is shown. Tritiated PbTx-3 binds with high affinity and specificity to manatee brain synaptosomes. Under the conditions used in this assay at brevetoxin concentrations near the K_D , the specifically bound counts are 80% of the total providing a good signal-to-noise ratio (Fig. 1). Analysis of specific binding in the Scatchard plot (Fig. 1, inset) suggests that a single class of saturable, non-interacting binding sites is present. A dissociation constant (K_D) of 7.5 ± 2.5 nM is determined for manatee synaptosomes and a binding maximum of 0.8 ± 0.1 pmol toxin bound per mg protein is calculated by linear regression. This number of saturable brevetoxin binding sites is less than that reported for other vertebrates at 4°C , i.e. 1.9 ± 0.9 pmol mg^{-1} (Edwards et al.,

1992) and 6.8 pmol mg^{-1} (Poli et al., 1986), which may be accounted for by the different incubation temperature used in this study (e.g. 0°C).

A temperature dependence of brevetoxin binding to manatee synaptosomes is observed (Fig. 2) as a progressive reduction in toxin–receptor affinity and lower measured percentages of specific binding as the temperature increases from 0 to 37°C . A similar temperature dependence of brevetoxin binding to rat synaptosomes has been shown previously (Poli et al., 1986).

Saxitoxin binding is linear with increasing tissue concentration up to $0.32 \text{ mg protein ml}^{-1}$ binding medium (Fig. 3). Manatee synaptosomes also bind saxitoxin with high affinity and specificity (Fig. 4). A K_D of $3.0 \pm 0.6 \text{ nM}$ is determined for manatee synaptosomes and a binding maximum of $1.0 \pm 0.1 \text{ pmol toxin per mg protein}$ is calculated by linear regression. At saxitoxin concentrations near the K_D and an incubation temperature of 0°C , the specifically bound counts are 98% of the total providing a very good signal-to-noise ratio. The percent specific binding is greater for saxitoxin than brevetoxin, likely due to the hydrophobicity of the latter toxin and its correspondingly higher affinity for lipid.

The number of saturable saxitoxin-binding sites in manatee is similar to that reported for other nerve preparations. The B_{max} for $[^3\text{H}]\text{STX}$ bind-

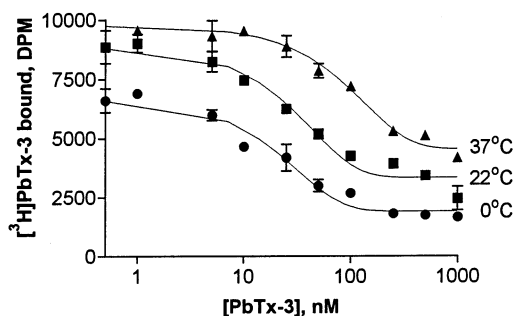


Fig. 2. Temperature dependence of $[^3\text{H}]\text{PbTx-3}$ specific binding to manatee brain synaptosomes. Samples were incubated with $5 \text{ nM } [^3\text{H}]\text{PbTx-3}$ at three different temperatures and competitive displacement was achieved with increasing concentrations of up to 1000 nM unlabeled PbTx-3. Specific binding was calculated as the difference between total and nonspecific binding. Values are means ($\pm$ S.D.) of three separate analyses.

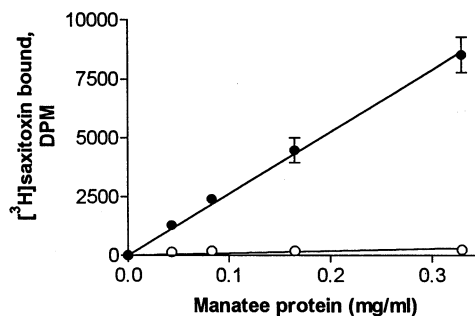


Fig. 3. Tissue linearity of $[^3\text{H}]\text{STX}$ binding to manatee brain synaptosomal protein. Increasing concentrations of manatee brain protein were incubated for 30 min at 0°C with $1 \text{ nM } [^3\text{H}]\text{STX}$. Nonspecific binding was determined in the presence of $1 \text{ }\mu\text{M}$ unlabeled saxitoxin. Values are means ($\pm$ S.D.) of three separate analyses. Protein was assayed using the BCA protocol (Pierce Chemical).

ing in manatee ($0.8 \pm 0.1 \text{ pmol per mg protein}$) is lower than the values calculated for rat brain preparations ($1.7 \text{ pmol per mg protein}$; Edwards et al., 1992 and $1.9 \text{ pmol per mg protein}$; Weigele and Barchi, 1978). This may simply be due to the difference in incubation temperatures or, alternatively, may reflect a loss of binding activity due to the collection of brain tissue several minutes after the death of the animal. However, all marine mammals tested in this study have similar B_{max} values.

We also compared the stoichiometry of $[^3\text{H}]\text{PbTx-3}$ and $[^3\text{H}]\text{STX}$ binding sites in manatee brain tissue obtained in parallel experiments (Figs. 1 and 4, insets; B_{max} is the x intercept). The observation of an approximately 1:1 stoichiometry suggests that there are equal numbers of brevetoxin and saxitoxin binding sites in manatee brain. A single, high affinity binding site likely exists for each toxin.

The number of saxitoxin binding sites in a number of marine mammals is shown in Fig. 5. Binding maxima are similar, indicating that all mammals tested have similar sodium channel density in their brain tissues. All marine mammal neuronal tissues tested demonstrate nanomolar affinity and specificity for saxitoxin, with humpback whale showing the lowest affinity at 4.9 nM (Table 1). The linearity of Scatchard plots indicates that each species contains a single class of

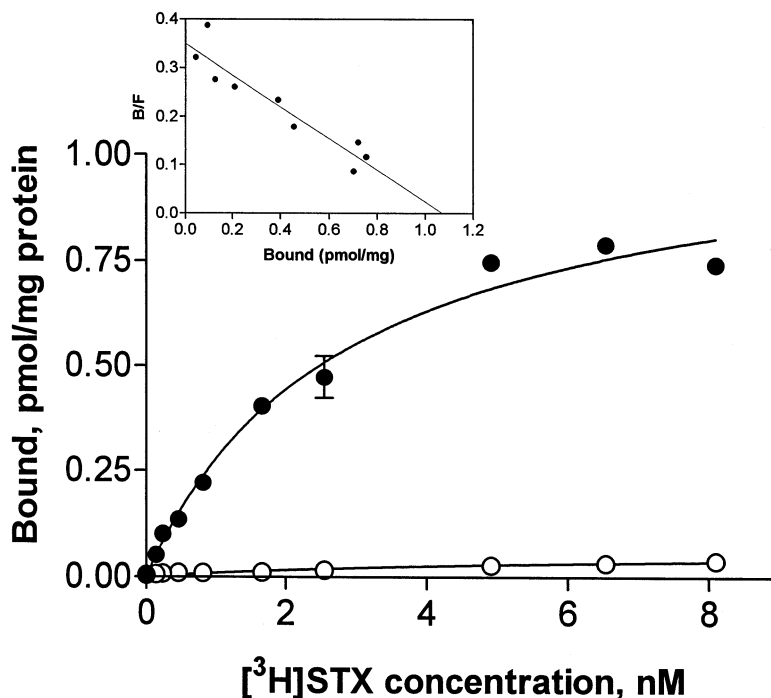


Fig. 4. Binding of [³H]STX to manatee brain synaptosomes. Synaptosomes were incubated with increasing concentrations of [³H]STX for 30 min at 0°C using the rapid membrane filtration assay as described in Section 2. Total binding (●) and nonspecific binding (○) measured in the presence of 1 μM unlabeled saxitoxin are plotted versus the measured free saxitoxin concentrations. Values are means (± S.D.) of three separate analyses. Free saxitoxin concentrations were calculated by determining the radioactivity in an aliquot of each diluted standard. Blank values (no added synaptosomes) were subtracted in all cases. Inset: analysis of the specific binding of [³H]STX to manatee brain synaptosomes in a Scatchard plot. The regression line was obtained as described in Fig. 1.

receptor sites for saxitoxin in brain tissue. These findings suggest that a high degree of homology exists at the sodium channel receptor sites in terrestrial and marine mammals. The sequencing of toxin binding sites on sodium channels of marine organisms in future experiments is needed to confirm this hypothesis.

The severity of the biological response to toxin exposure in marine mammals is directly correlated not only with the physiological factors including toxin affinity and number of binding sites in neuronal tissue described earlier, but is also dependent upon the route of toxin exposure. Dead fish have been known to appear when *G. breve* concentrations approach 5×10^5 cells l^{-1} (Steidinger, 1983). The route of brevetoxin exposure in fish is via absorption directly across the gill membrane (Abbot et al., 1975) which is simi-

lar to intravenous administration. Therefore, access to the specific toxin binding site is direct and not hampered by the biological barriers encountered by other routes of exposure, such as ingestion. In an analogous manner, brevetoxins aerosolized by breaking wave action can be inhaled by marine mammals (Bossart et al., 1998). Additionally, brevetoxins and saxitoxin can be ingested by marine mammals with food: this route of access to the specific binding site on neuronal tissue is indirect, and therefore may be hampered by biological barriers, and bioconversion can act to enhance or reduce potency and change the relative proportions of toxic isoforms ultimately reaching the site of binding.

Chronic and acute signs and symptoms of intoxicated manatees during the spring 1996 Florida event were consistent with neurointoxication. The

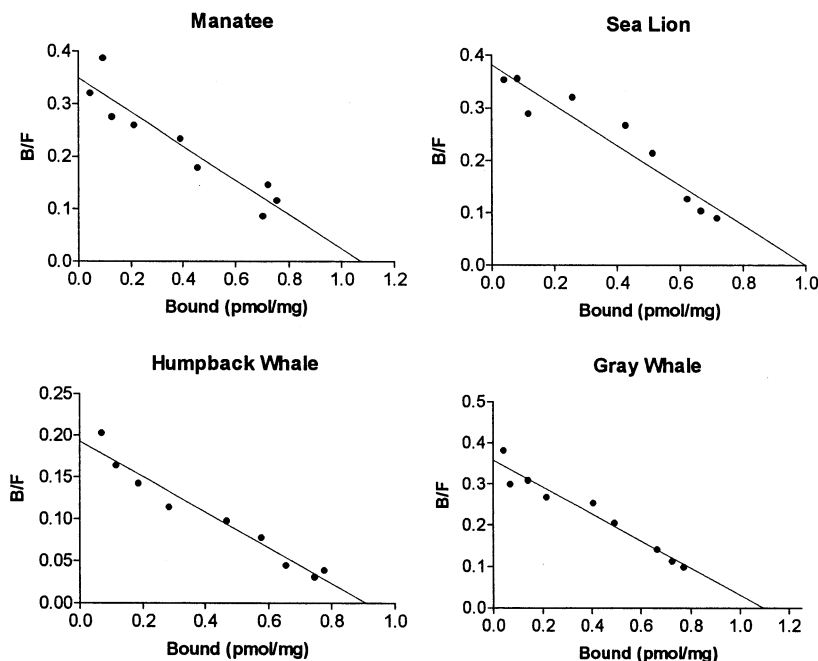


Fig. 5. Analysis of the specific binding of [3 H]STX to various marine mammal brain synaptosomes in a Scatchard plot. The regression lines were obtained as described in Fig. 1.

route of manatee exposure to brevetoxin was by both inhalation, as evidenced by lung damage, and ingestion, as evidenced by toxins detected in the gut using receptor binding analysis (Bossart et al. 1998). Necropsies of manatees demonstrated the presence of gross lesions which included thickened, erythematous nasal passages, trachea and bronchi. Lungs were frequently congested, hemorrhagic, and edematous. This is comparable to the effects due to human exposure to the aerosolized *G. breve*, e.g. conjunctival irritation, rhinorrhea, and cough accompanied by motor, bulbar, respiratory, or reflex changes (Hughes and Merson, 1976). In the manatee exposure event, cerebellar tissues were commonly affected with lymphocytic infiltrates and mild hemorrhage demonstrating a direct interaction of toxins with brain tissue. Given the affinity of manatee for brevetoxin at 7.5 nM and assuming a concentration of 11 picograms total toxin produced by *G. breve* on a per cell basis (Stuart and Baden, 1988), approximately 3×10^5 cells l^{-1} were required to yield a concentration of toxin at the level of the K_D . Indeed, the

cell concentrations at the time of manatee deaths were persistently greater than $100\,000$ cells l^{-1} for a period of several days during the spring of 1996, indicating that a sufficient number of cells were present for lethal exposure to eventually occur by inhalation.

Environmental factors also play an important role in determining the dose of toxin to which the marine organism is exposed. Dilution effects will be present in an aqueous system, thereby requiring a physical means of toxin concentration in the environment for lethal exposure to result. For example, at the times of the manatee deaths that occurred in conjunction with a *G. breve* bloom in the years 1963, 1982, and 1996, large numbers of manatees congregated in the warmer waters of southwest Florida where the upstream power plants or canals are poorly flushed (Landsberg and Steidinger, 1998). This created a scenario in which the bloom of *G. breve* in saline canals persisted for a number of days. Consequently, the sustained exposure of manatees to brevetoxin resulted in their slowed motor responses and inability to

escape the canals containing high numbers of toxic algae.

Other physiological factors that may increase the effect of a given dosage of toxin, especially in cetaceans, have been postulated. Upon toxin exposure, a whale may lose control over its vital peripheral heat-conserving mechanism (Ridgway et al., 1974) or become unable to return to the surface to breathe by the same processes that result in peripheral nerve impairment in poisoned humans (McFarren et al. 1960). During a

cetacean dive, blood is channeled to the heart and brain (Ridgway 1972), thereby concentrating the toxin in those organs while limiting access to the liver and kidney where metabolism and elimination occur. Water-soluble saxitoxin would tend to concentrate in metabolically active, physiologically sensitive tissues such as the heart and brain, whereas brevetoxin would more likely concentrate in lipid-rich tissues due to its hydrophobicity. Therefore, this mechanism of toxin concentration in cetaceans during dives would be specific to water-soluble toxins such as saxitoxin which was a likely factor in humpback whale mortalities off the New England coast in the late 1980s (Geraci et al., 1989).

Organisms which already have compromised health, such as a gram-negative bacterial infection which was prominent in some manatee specimens in the 1996 Florida incident (Bossart et al. 1998), may be particularly sensitive to further health stressors such as toxins from harmful algal blooms. For example, a *Vibrio* species was present at the same time that brevetoxins were identified in tissues of dolphin which had stranded off the US east coast between New Jersey and Florida during the late 1980s. Alternatively, the health of animals could initially be compromised by exposure to toxins, thereby making them more susceptible to subsequent opportunistic infections. With the recent reports of brevetoxins and their ability to competitively inhibit cathepsin enzymes (Sudarsanam et al. 1992), which are important in cellular immune function, and the demonstration of brevetoxins in lymphocytes (a cell type rich in cathepsins), it could be postulated that the latter compromised situation may actually prevail.

Marine mammal strandings have occurred over the past several decades without clear explanation. It has been suggested that marine mammal strandings are escalating (Gerber et al. 1993); likewise, the incidence of harmful algal blooms is believed to be increasing (Boesch et al. 1997). Often, strandings are associated with seizures, gastroenteritis, hepatic failure, and pneumonia-like symptoms of unknown etiology (Gerber et al. 1993). Although several explanations of these symptomologies are possible, they may be indicative of neurotoxin exposure, which is linked to

Table 1
Brevetoxin and saxitoxin binding affinity in excitable tissue

Organism	K_D^a	
	Brevetoxin	Saxitoxin
<i>Terrestrial</i>		
Cat		0.6 ^b
Rat	2.9 ^c	2.0 ^d
		0.2 ^e
Chick		0.3 ^f
Locust		0.5 ^g
Drosophila		1.9 ^h
<i>Aquatic</i>		
Squid		4.3 ⁱ
Eel		6.0 ^j
Tilapia	6.1 ^k	
Gambusia	10.0 ^l	
Trout		3.8 ^m
Manatee	7.5 ⁿ	3.0
Gray whale		2.7
Sea lion		1.9
Humpback whale		4.9

^a K_D values are expressed in nM. Amphibians are not included in this table.

^b Querfurth et al., 1987 (3°C).

^c Poli et al., 1986 (4°C).

^d Catterall et al., 1979 (36°C).

^e Tamkun and Catterall, 1981 (4°C).

^f Rogart et al., 1983.

^g Moskowitz et al., 1991 (0°C).

^h Gitschier et al., 1980 (4°C).

ⁱ Strichartz et al., 1979, using solvent exchange technique with giant axon preparations (4°C).

^j Reed and Raftery, 1976, using plasma membrane preparations and [³H]TTX binding (2–4°C).

^k Stuart and Baden, 1988 (23°C).

^l Lewis, 1992; Baden et al., 1988. Represents LD₅₀ value at 23°C.

^m Rubin and Soderlund, 1993 (7°C).

ⁿ All remaining organisms, this study (°C).

intoxication by the suite of toxins found in algal blooms. It is likely that, in conjunction with stressors such as bacterial infections that have deleterious effects on the health of a variety of marine organisms, marine toxins play an important ecological role in the health and survival of the global marine mammal population.

Acknowledgements

We thank Dr Scott Wright of the Florida Department of Environmental Protection Marine Research Institute, St. Petersburg, FL, for providing manatee brain tissue for this study. We acknowledge the following agencies/groups for their help with tissue collection: the Marine Mammal Center, Sausalito, CA (California sea lion), Robert L. Brownell, Southwest Fisheries Science Center, LaJolla, CA and S. A. Blokhin, Pacific Research Institute of Fisheries and Oceanography (TINRO), Vladivostok, Russia (gray whale), and the New England Aquarium, Boston, MA (humpback whale). The collection of all marine mammal brain tissue, not including manatee, was supported, in part, by the Marine Mammal Health and Stranding Response Program of the National Marine Fisheries Service. We thank Elena Boeva for her excellent technical support during this study.

References

- Abbot, B.C., Siger, A., Spiegelstein, M., 1975. Toxins from blooms of *Gymnodinium breve*. In: LoCicero, V.R. (Ed.), The First International Conference on Toxic Dinoflagellate Blooms. Massachusetts Science and Technology Foundation, Wakefield, MA, pp. 355–365.
- Baden, D.G., Mende, T.J., Szmant, A.M., Trainer, V.L., Edwards, R.A., Roszell, L.E., 1988. Brevetoxin binding: molecular pharmacology versus immunoassay. *Toxicon* 26, 97–103.
- Boesch, D.F., Anderson, D.M., Horner, R.A., Shumway, S.E., Tester, P.A., Whitledge, T.E., 1997. Harmful Algal Blooms in Coastal Waters: Options for Prevention, Control and Mitigation. In: NOAA Coastal Ocean Program Decision Analysis Series, vol. 10. NOAA Coastal Ocean Office, Silver Spring, MD, p. 46.
- Bossart, G.D., Baden, D.G., Ewing, R.Y., Roberts, B., Wright, S.D., 1998. Brevetoxicosis in manatees from the 1996 epizootic: gross, histologic and immunohistochemical features. *Toxicol. Pathol.* 26, 276–282.
- Catterall, W.A., Morrow, C.S., Hartshorne, R.P., 1979. Neurotoxin binding to receptor sites associated with voltage-sensitive sodium channels in intact, lysed and detergent-solubilized brain membranes. *J. Biol. Chem.* 254 (22), 11379–11387.
- Edwards, R.A., Trainer, V.L., Baden, D.G., 1992. Brevetoxins binding to multiple classes of sites in rat brain synaptosomes. *Mol. Brain Res.* 14, 64–70.
- Geraci, J.R., Anderson, D.M., Timperi, R.J., St. Aubin, D.J., Early, G.A., Prescott, J.H., Mayo, C.A., 1989. Humpback whales (*Megaptera novaeangliae*) fatally poisoned by dinoflagellate toxin. *Can. J. Fish. Aquat. Sci.* 46, 1895–1898.
- Gerber, J.A., Roletto, J., Morgan, L.E., Smith, D.M., Gage, L.J., 1993. Findings in pinnipeds stranded along the central and northern California coast 1984–1990. *J. Wildlife Dis.* 29 (3), 423–433.
- Gitschier, J., Strichartz, G.R., Hall, L.M., 1980. Saxitoxin binding to sodium channels in head extracts from wild-type and tetrodotoxin-sensitive strains of *Drosophila melanogaster*. *Biochim. Biophys. Acta.* 595, 291–303.
- Huang, J.M.C., Wu, C.H., Baden, D.G., 1984. Depolarizing action of a red tide dinoflagellate brevetoxin on axonal membranes. *J. Pharmacol. Exp. Ther.* 229, 615–621.
- Hughes, J.M., Merson, M.H., 1976. Current concepts fish and shellfish poisoning. *New Engl. J. Med.* 295 (20), 1117–1120.
- Landsberg, J.H., Steidinger, K., 1998. A historical review of red tide events caused by *Gymnodinium breve* as related to mass mortalities of the endangered manatee (*Trichechus manatus latirostris*) in Florida, USA. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Microalgae. IOC of UNESCO and Xunta de Galicia, Spain, pp. 97–100.
- Lewis, R.J., 1992. Ciguatoxins are potent ichthyotoxins. *Toxicol.* 30 (2), 207–211.
- McFarren, E.F., Schafer, M.L., Campbell, J.E., Lewis, K.H., Jensen, E.T., Schantz, E.J., 1960. Public health significance of paralytic shellfish poison. *Adv. Food Res.* 10, 135–179.
- Moskowitz, H., Zlotkin, E., Gordon, D., 1991. Solubilization and characterization of the insect neuronal sodium channel. *Neurosci. Lett.* 124 (2), 148–152.
- Noda, M., Suzuki, H., Numa, S., Stuhmer, W., 1989. A single point mutation confers tetrodotoxin and saxitoxin insensitivity on the sodium channel II. *FEBS Lett.* 259, 213–216.
- Poli, M.A., Mende, T.J., Gaden, D.G., 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels bind to specific sites in rat brain synaptosomes. *Mol. Pharmacol.* 30, 129–135.
- Querfurth, H.W., Armstrong, R., Herndon, R.M., 1987. Sodium channels in normal and regenerated feline ventral spinal roots. *J. Neurosci.* 7 (6), 1705–1716.
- Reed, J.K., Raftery, M.A., 1976. Properties of the tetrodotoxin binding component in plasma membranes isolated from *Electrophorus electricus*. *Biochemistry* 15 (5), 944–953.

- Ridgway, S.H., 1972. Homeostasis in the aquatic environment. In: Ridgway, S.H. (Ed.), *Mammals of the Sea: Biology and Medicine*. Thomas, Springfield, IL, pp. 590–603.
- Ridgway, S.H., McCormick, J.G., Wever, E.G., 1974. Surgical approach to the dolphin's ear. *J. Exp. Zool.* 188 (3), 265–276.
- Ritchie, J.M., Rogart, R.B., 1977. The binding of saxitoxin and tetrodotoxin to excitable tissue. *Rev. Physiol. Biochem. Pharmacol.* 79, 1–49.
- Rogart, R.B., Regan, L.J., Dziekan, L.C., Galper, J.B., 1983. Identification of two sodium channel subtypes in chick heart and brain. *Proc. Natl. Acad. Sci. U.S.A.* 80, 1106–1110.
- Rubin, J.G., Soderlund, D.M., 1993. Binding of [^3H]batrachotoxinin a-20- α -benzoate and [^3H]saxitoxin to receptor sites associated with sodium channels in trout brain synaptoneuroosomes. *Comp. Biochem. Physiol.* 105C (2), 231–238.
- Steidinger, K.A., 1983. A re-evaluation of toxic dinoflagellate biology and ecology. In: Round, F.E., Chapman, D.J. (Eds.), *Progress in Phycological Research*. Elsevier, New York, pp. 147–188.
- Steidinger, K.A., 1989. Implications of 1986–1987 *Ptychodiscus brevis* red tide and 1987–88 mass bottlenose dolphin mortalities. In: *Toxic Dinoflagellates and Marine Mammal Mortalities*. Tech. Rep. Woods Hole Oceaogr. Inst, Woods Hole, MA, pp. 56–65.
- Strichartz, G.R., Rogart, R.B., Ritchie, J.M., 1979. Binding of radioactively labeled saxitoxin to the squid giant axon. *J. Membr. Biol.* 48 (4), 357–364.
- Stuart, A.M., Baden, D.G., 1988. Florida red tide brevetoxins and binding in fish brain synaptosomes. *Aquat. Toxicol.* 13, 271–280.
- Sudarsanam, S., Virca, G.D., March, C.J., Srinivasan, S., 1992. An approach to computer-aided inhibitor design: application to cathepsin L. *J. Comput. Aided Mol. Des.* 6 (3), 223–233.
- Tamkun, M.M., Catterall, W.A., 1981. Ion flux studies of voltage-sensitive sodium channels in synaptic nerve-ending particles. *Mol. Pharmacol.* 19, 78–86.
- Weigele, J.B., Barchi, R.L., 1978. Analysis of saxitoxin binding in isolated rat synaptosomes using a rapid filtration assay. *FEBS Lett.* 91, 310–314.