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The Molecular Biology of Marine Toxins from Dinoflagellates and their Effects on Causing Human Disease

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Abstract:

Marine toxins, produced by dinoflagellates, have been identified throughout the world as an increasing environmental problem, having caused significant diseases in both human and marine organisms. Dinoflagellates belong to one of the most species-rich groups of protists called, the Alveolates. Marine toxins from the dinoflagellates are naturally occurring chemicals that quickly move through the food web via trophic transfer and are bio-magnified. Marine invertebrates are the reservoirs for these toxins and are responsible for diseases in higher order animals and humans. Diseases in humans result mainly through the direct ingestion of contaminated fish or shellfish although certain diseases are caused by aerosol toxins which are inhaled into the lungs or through skin contact. In humans, the effects of marine toxins range from acute neurological to chronic diseases. This paper focuses on the molecular biology of two dinoflagellates marine toxins, saxitoxin and brevetoxin. The mode of action of these toxins is via their high affinity for the voltage-dependent sodium channels which interferes with normal neuronal activity and result in neurological diseases. The high affinity is achieved by structure functional groups that bind tightly to the sodium channels, changing the sodium channels conformation leaving them unable to close (saxitoxin) or remain open (brevetoxin). Studying these marine toxins has useful biomedical applications, such as gaining a better understanding of sodium channels, better development of anesthetics and cancer research underscore the value of these chemicals.

Background:

Dinoflagellates are single-celled, algae-like biflagellated protists dating back to about 800 million years ago with both prokaryotic and eukaryotic attributes (Freeman 2002). As protists, dinoflagellates are eukaryotes and occur in a variety of habitats; from pelagic to benthic, from temperate to tropical seas, and from estuaries to freshwater (Garrrison 2006). Remarkably, numerous species can survive in or on other marine organisms (as symbionts). Many species can also produce resting cysts that can survive in sediments for a long period of time and then germinate to initiate a bloom. Some are free swimming with a forward spiraling motion propelled by two dimorphic flagella. They have a large nucleus with condensed chromosomes and organelles such as the chloroplasts, mitochondria and Golgi bodies. Dinoflagellates are diverse in many aspects from their sexual reproduction process, their cell shapes and sizes to modes of obtaining food. Generally, dinoflagellates reproduce asexually through binary fission, but some species reproduce sexually and form cysts. Their nutrition varies from autotrophy to heterotrophy to mixotrophy (Garrison 2006). Similar to plants, the photosynthetic dinoflagellates have chlorophylls "a" and "c", and the light harvesting pigments peridinin, fucoxanthin and xanthophylls (Garrison 2006). It is believed that the broad life histories have made dinoflagellates among the most dominant eukaryote in marine environments.

Another key feature of dinoflagellates is that they are the primary producers of the sea, functioning like grass and plants on land. Thus, dinoflagellates convert sunlight into carbohydrate and serve as a rich food base for all other marine organisms. In favorable conditions, dinoflagellates proliferate to form dense concentrations of cells in a phenomenon referred to as an algal bloom. In many cases these blooms are harmless with toxic species

normally present in low concentration with no environmental or human health impacts. Large algal blooms can cause actual discoloration of the water to milky white or red (Tibbetts 1998). While the overwhelming majority of dinoflagellates are not harmful, some produce toxins as they redden the sea. These "red tides" are strongly associated with harmful algal blooms (HAB) which have great impact on a wide range of marine organisms and human health. HAB can cause substantial economic losses to coastal communities and commercial fisheries, and mass mortalities among fish, birds, and mammals (Tibbetts 1998). More worrisome has been the discovery of dinoflagellates that produce potent toxins but do not discolor the sea hence giving no advance warning to their effects (Tibbetts1998).

Two decades ago, about 25 toxic species of dinoflagellates were known. However, the number of toxic species has been increasing (Tibbetts 1998). Of several thousand marine algal species, about 60-80 are known to be toxic, and an estimated of 75 percent are dinoflagellates (Smayda 1990). Globally, about 60,000 known cases of toxic seafood poisoning are reported annually, with a 1.5 percent mortality rate (this is not including the unreported cases or the misdiagnosed cases) (Van Dolah 2002). Approximately 20 percent of all foodborne diseases outbreaks in the United States are due to consumption of seafood with 50 percent of those resulting from naturally occurring algal toxins such from dinoflagellates (Ahmed 1991). The growth and spread of harmful algal bloom in the United States coastal waters have been a major problem (Tibbetts 1998). Indeed, world-wide, there has been an increase of incidence of dinoflagellates toxins reported since the 1970's. This increase is in part due to increased monitoring for toxins but also a geographic expansion in the occurrence of toxic outbreaks worldwide over the past three decades. For instance, historically, the occurrence of Neurotoxic shellfish poisoning (NSP), which is caused by *Gymnodinium brev*, has be limited to Florida's

west coast, however, in 1987 blooms were detected in North Carolina with over 48 cases of shellfish poisoning and massive fish kills (Van Dolah 2000).

Similarly, Paralytic Shellfish Poisoning (PSP), caused by Gonyaulux toxins was endemic to the North America, Europe and Japan prior to the 1970s. Since then, PSP outbreaks have been documented in South America, Australia, India and Southeast Asia (Van Dolah 2000). Newly developed monitoring programs have increased awareness of the HABS. For instance, New Zealand, which had no reported algal toxins in the 1970's experienced a surprising toxic outbreak in 1992 which led to a wide-ranging monitoring program that has since detected HABS severe enough to cause closures of shellfish harvest (Van Dolah 2000).

Figure 1: North America increase in harmful algal blooms

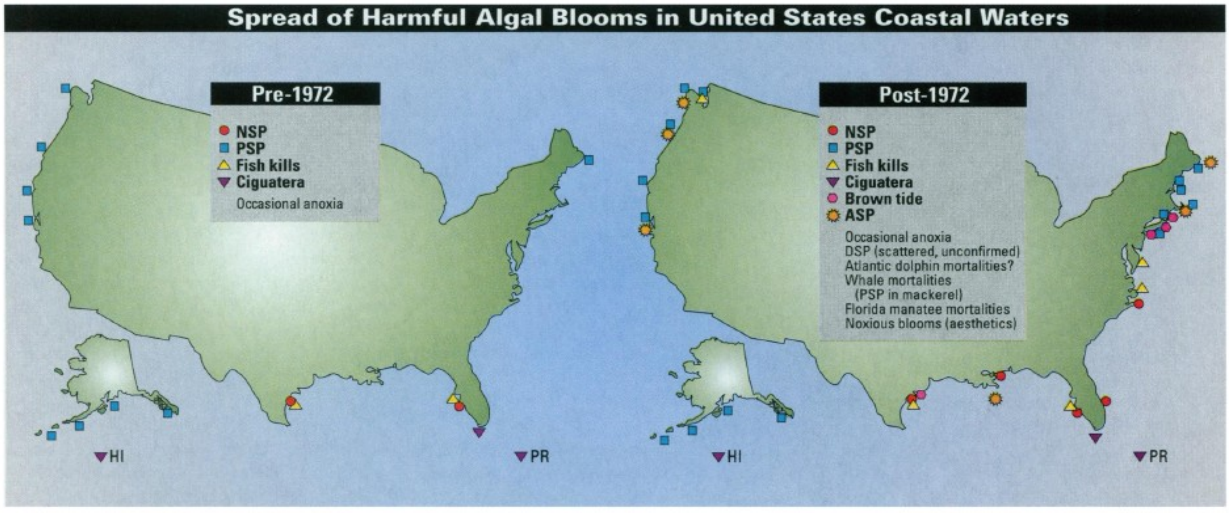
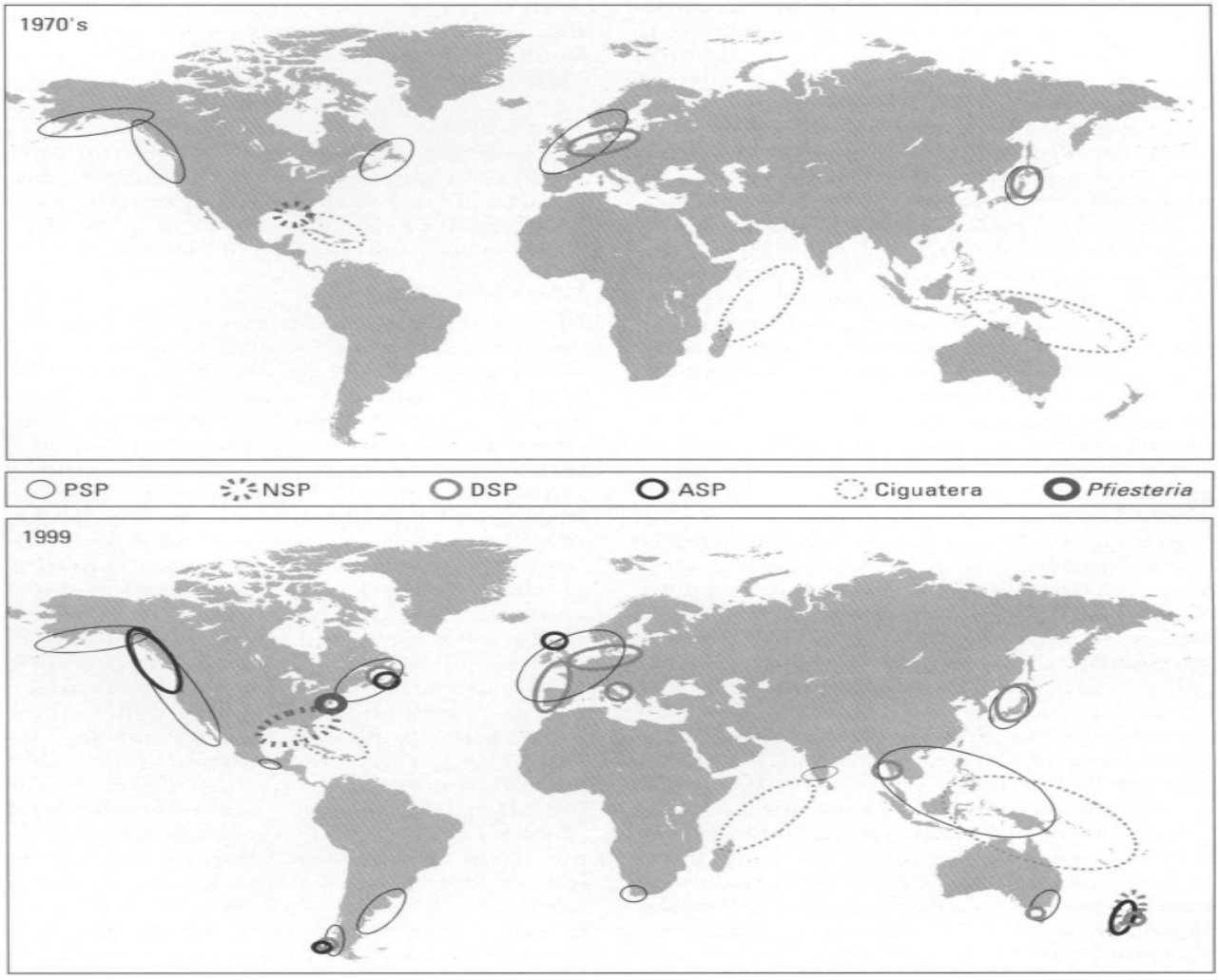


Figure 2: Global increase in reported incidence of algal toxins

(Van Dolah 2000)



Once produced and excreted into the environment, the toxins produced by dinoflagellates are quickly biomagnified through trophic level transfer. Clams, mussels, scallops, oysters, and other shellfish filter toxin from the water without great effect on the shellfish health. This is due to these shellfish lacking a complex nervous system which is present in higher order animals such as fish, marine animals and humans. Hence, these filter-feeding shellfish serve as vectors that carry the toxins to humans when consumed.

Human consumption of seafood contaminated with toxins can cause gastrointestinal disorders, respiratory diseases, memory loss and even death. One report suggests that a single clam or mussel can contain enough toxin concentration to cause serious illness and even kill a human (Tibbettes 1998). The danger of these dinoflagellates toxins is that they are odorless, tasteless and colorless, and even cooking seafood does not neutralize the toxins. Some toxins, such as brevetoxins can be aerosolized, and thus easily transmitted to humans by simply breathing the toxins into the lungs (Abraham 2005).

Dinoflagellate toxins can result in four seafood poisoning syndromes: Paralytic Shellfish Poisoning (PSP), Neurotoxic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), and Ciguatera Fish Poisoning (CFP). Most of the toxins are neurotoxins, targeting and interfering with normal neuronal activity in humans, marine mammals, and fish. The following Table 1 summarizes the four toxic syndromes associated with dinoflagellate toxins.

Table1: Toxic syndromes associated with dinoflagellate toxins

Syndrome	**Causative Agent**	**Toxin**	**Primary vector**	**Molecular Mechanism target**
Neurotoxic Shellfish Poisoning	*Gymodinium breve*	Brevetoxins: two forms (polyethers; PbTx-1 and PbTx-2)	Shellfish	Voltage-gated sodium channel site5
Paralytic Shellfish Poisoning	*Alexdanrium spp.* *Gymodinium spp.* *Pyrodinium spp.*	Saxitoxins: (sulfocarbamyl toxins)	Shellfish	Voltage-gated sodium channel site1
Ciguatera Finfish Poisoning	*Gambierdiscus Toxicus*	Ciguatoxins	Reef fish	Voltage-gated sodium channel site5
Diarrhetic Shellfish Poisoning	*Dinophysis spp.* *Prorocentrum spp.*	Dinophysistoxins Okadaic acid	Shellfish	Ser/thr protein phosphatases

Toxins and Diseases:

For PSP, globally, an estimated 2,000 cases of human poisonings are reported annually with a 15 percent mortality rate. CFP is estimated to affect 50,000 people annually and is no longer a disease limited to the tropics (Hallegraeff 1993). The epibenthic dinoflagellate *Gambierdiscus toxicu*s is primary cause of CFP, where the ladder-like polyether toxins target both the ion sodium and calcium channels. Red tide dinoflagellates *Dinophysis spp.* and *Prorocentrum spp* cause DSP through a class of acidic polyether toxins targeting the serine/threonine protein phosphatases and inhibiting the protein normal function. Both NSP and PSP are caused by toxins targeting the sodium voltage channel where binding of toxins result in activating or blocking the channel, respectively (Burkholder 1998).

Brevetoxin and Saxitoxin:

NSP and PSP diseases are caused by two different dinoflagellate toxins and yet the molecular target mechanism is the voltage gated sodium channel. This similarity at the molecular level of the two toxins results in a common disruption of neuronal activity. Brevetoxins causing NSP interact with the voltage gated sodium channel and persistently activates the ion channel and thus, prolongs the opening conformation of the channel. This interferes with the normal signaling pathway in neuron cells leading to symptoms of NSP which include loss of motor control, numbness of perioral area, severe muscular ache, seizures, and unconsciousness. Conversely, saxitoxins causing PSP blocks the sodium gated channel, preventing channel conductance. This also interferes with normal signaling pathways in neuron cell leading to symptoms of PSP, which include numbness of perioral areas and extremities, loss of motor control, respiratory paralysis, and even death (Van Dolah 2000).

The Cell and Signal Transport

The human nervous system has two major divisions, the central nervous system (CNS) which includes the brain and spinal cord and the peripheral nervous system (PNS) which consists of nerves. The two systems are connected and work together. One type of nervous tissues is neurons. Neurons are cells that transmit nerve impulses and sigals between parts of the nervous system. Neurons are important cells and consist of three types, sensory neurons, interneurons and motor neurons. Sensory neurons take nerve messages from sensory receptors to the CNS, where sensory receptors are specialized cells that detect changes in the environment. An interneuron located within the CNS and serves as a receiver, receiving input from sensory neurons and other

interneurons in order to communicate with motor neurons. Motor neurons take nerve impulses away from the CNS to an effector that carries out responses to changes in the environment (Mader 2006). The appearance and shape of neurons vary however all consists of three basic parts, the cell body, dendrites, and an axon. The cell body stores the nucleus and other organelles. The dendrites are short extensions that receive signals from sensory receptors and other neurons. An axon has a long structure and it is an important part of neuron cells which conducts nerve impulses. Axons are responsible for carrying messages to the CNS and away from the cell body (Albert 2002).

These axons are membrane bound and the voltage gated sodium channels are embedded in the plasma membrane of the axon. They serve as a gate keeper, trafficking sodium ions across the axon membrane. The different concentrations of sodium ions between the inside and outside of an axon are maintained by these channels. The stimulus that is known to open the sodium ion channel is a change in the voltage across the membrane. A phenomenon known as an action potential in nerve and skeletal muscle cells is the traveling wave of electrical excitation that can carry a message without attenuation from one end of a neuron to the other at great speed (over 100 meters per second). This action potential or nerve impulse carrying the message is triggered by a depolarization of the plasma membrane, a rapid change in polarity across an axomembrane. This depolarization causes sodium channels to open and small amounts of sodium ions enter the cell. The influx of positive sodium charge depolarizes the membrane further, making the membrane potential decrease (Albert 2002). Further depolarization of the plasma membrane opens more sodium channels and more sodium ions enter the cells. This occurs until the net electrochemical flow of sodium ions is zero and the cell is in resting state. The sodium channels remain open at this point and two cellular mechanisms occur to stop the influx of sodium ions.

One mechanism is the opening of another voltage-gated channel, the potassium channel and the second is the inactivation of the sodium channel (Albert 2002). Normally, the voltage gated sodium channels stay inactivated in the late phase after activation until the channels return their shapes to a closed configuration when membranes begin to repolarize (Baden 2005).

Brevetoxins and the Inhibition of the sodium channel inactivation:

When the sodium channels remain in an open conformation continuously when not needed, the delivery of nerve impulses with message throughout nerve and skeletal muscle cells is distorted. This inappropriate opening of the channels under which they are supposed to be closed leads to an abnormal potential difference across an axon membrane. When no message is delivered and too many sodium ions enter the neurons, cell death may occur.

Brevetoxins are produced naturally by *Gymnodinium breve*, the first to be identified as case of NSP in North America. When aggregated in high cell density to form blooms, *G. breve* toxins have killed invertebrates, fish, birds, marine mammals. Toxins that became aerosolized cause respiratory illness in people who breathe them into the lungs (Potera 2007). *G. breve* are found in the continental shelf and they require very low levels of nutrients. However, *G.breve* blooms are carried inshore by currents. In the past, *G. breve* were believed to be found only in the Gulf of Mexico from Yucatan to Texas coast, but recent studies have shown that they have been found more prevalent in the east coast of Florida and farther north (Tibbets 1998).

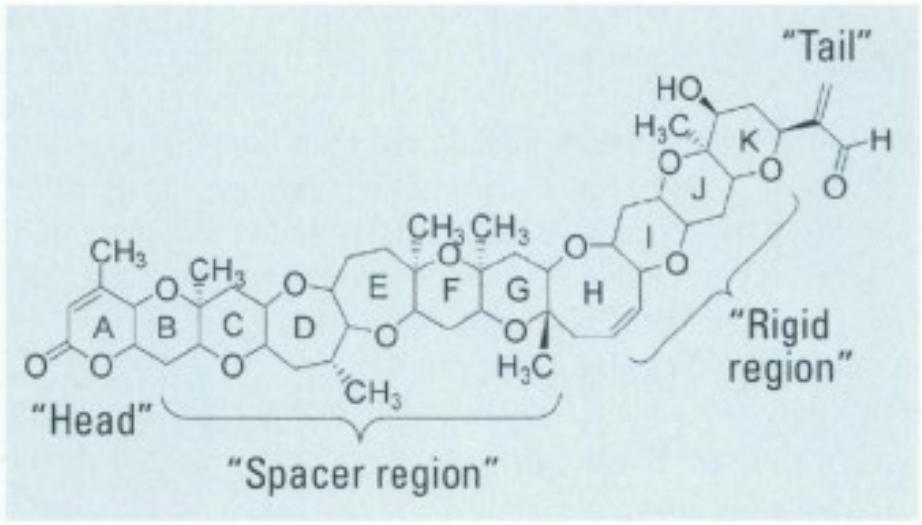
Brevetoxin is a lipid soluble, hydrophobic suite of nine ladder-like polycyclic ethers. There are two types of brevetoxins, brevetoxin A (Pb-Tx-1) and brevetoxin B (Pb-Tx-2). It was

not until the 1970's that two forms were purified using high-performance liquid chromatography (HPLC) and their structures examined (Baden 1983). The structure of brevetoxin B was determined in 1981 (Lin et al. 1981). In 1986, brevetoxin A was described as having the same feature as brevetoxin B but slightly different in the polyether backbone (Shimizu et al. 1986). Both Brevetoxin A and brevetoxin B possess a lactone functionality group, known as the head A-ring, a spacer region of relatively rigid polyether rings that from a ladder-like structure, and an identical very reactive αβ-unsaturated aldehyde side chain,

$-CH_2-C(=CH_2)CHO$. However, brevetoxin A and B differ in the different head lactone groups and the spacer region, each possessing a different number and different size of ether rings. Brevetoxin B is the only known toxin with rings of five, six, eight, and nine members in a single molecule that exist in nature. Various publications analyze and study the effects of altering the certain regions of the brevetoxin molecules. The studies included changing the lactone functionality, altering the side chain, and even a combination of both (Abraham et al. 2005). However, even today PbTx-1 and Pbtx-2 are still the most toxic toxins and none of the derivatives of brevetoxins synthetically produced is found to be more toxic than the parent molecules (Baden 2005). All active brevetoxins are found to share three common features in order to be fully active at their binding site on the voltage gated sodium channel.

Figure 3: Structure of Brevetoxin and the active regions

(Baden 2005)



In order for brevetoxin to have a complete expression of activities, three distinct features must be present. There is a cyclic ester lactone indicated as A-ring which is the "Head." The "Spacer region," denoted as B-G ring and the "Rigid region" consisting of four relatively rigid carbon rings denoted as H-K ring (Gawley et al. 1995). An A-ring acts an electrophile which is the first to orient between domain III and IV of the a-subunit. The B-G ring varies in the number of rings and sizes and has a limited flexibility which separates the binding region from the activity region. The H-K ring is the region to be involved in the actual binding at site 5 of the α-subunit of the voltage gated sodium channel (Baden 2005). Binding to this site changes the channel voltage sensitivity and results in an inappropriate opening of the channel and hinder

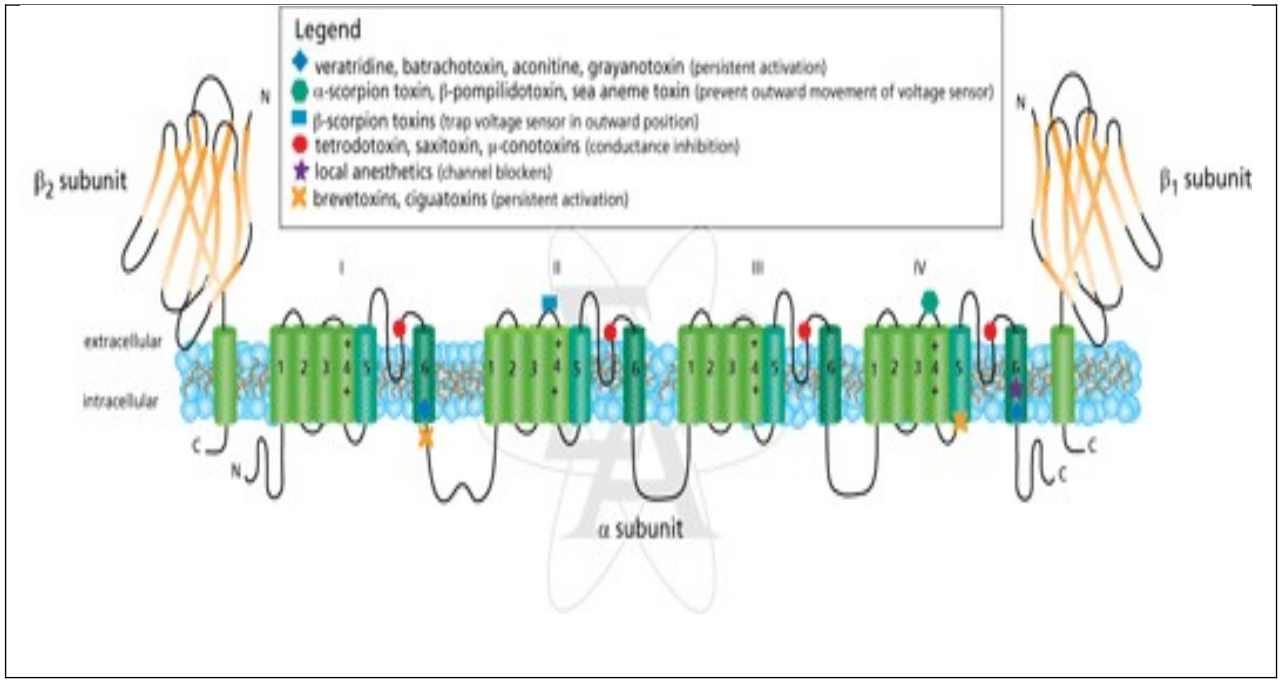
channel inactivation (Van Dolah 2000). Ongoing research has discovered five new brevetoxins that are found in cultures and in the field. Their structure varies having a shortened side chain while others have natural ketone brevetoxin, and also a new brevetoxin of Pbtx-2 backbone lacking any side chains. Alhough they all share the common feature of competitively binding at site 5 on the sodium channel, their toxicology is unknown (Bourdelais and Baden 2004).

Conformation of voltage gated sodium channel (VGSC)

The sodium channel main α-subunit is a single polypeptide glycoprotein chain of more than 1800 amino acids. The subunit is made up of four homologous domains and each domain consists of six transmembrane α-helices, a total of 24 transmembrane α-helcies in the chain (Heinemann 1992). Within a domain, some helices are neutral in charge, one helix is positively charged, and others are hydrophobic. The S4 helix is known as the "voltage sensor," where changes in the membrane potential result in an allosteric change of this helix. Channel configuration from closed to open is based on the allosteric realignment of all four S4 helices (Baden 2005).

Figure 4: Voltage gated sodium channels: VGSC

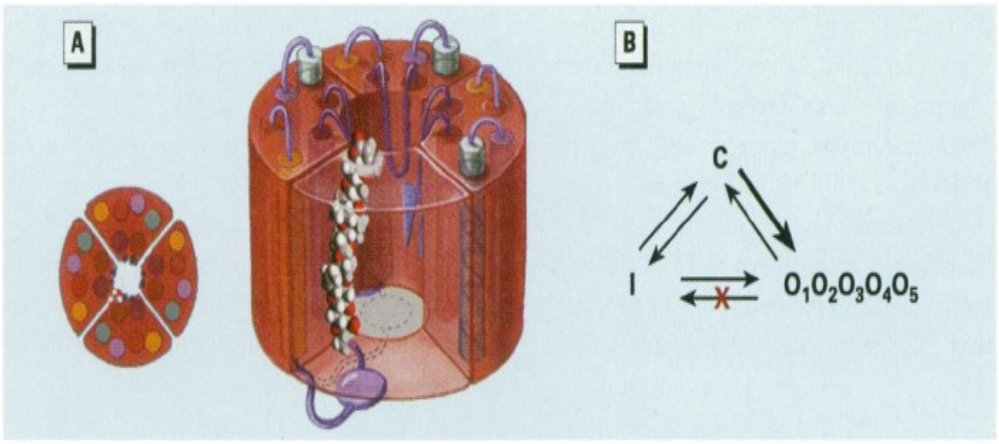
(Sigma-Aldrich Co.)



A theoretical mechanism of the interaction between brevetoxins and the voltage gated sodium channel was proposed by a group of scientists in 1995. They hypothesized that brevetoxin binding is oriented as "head-down" between the α-helices of domains III and IV of the VGSC (Figure 5). This "Head" group serving as an electrophile initially binds to the α-subunit and leads to the alteration of the VGSC configuration. The allosteric realignment of the helices results in a change from a closed channel to an open channel configuration. All naturally found brevetoxins alter the normal function of VGSC when the nerve impulse occurs. The opening configuration is favored when the activation is shifted to more negative potentials, a longer mean open time induced by the persistence of an open channel configuration and an inhibition to inactivation (Baden 2005).

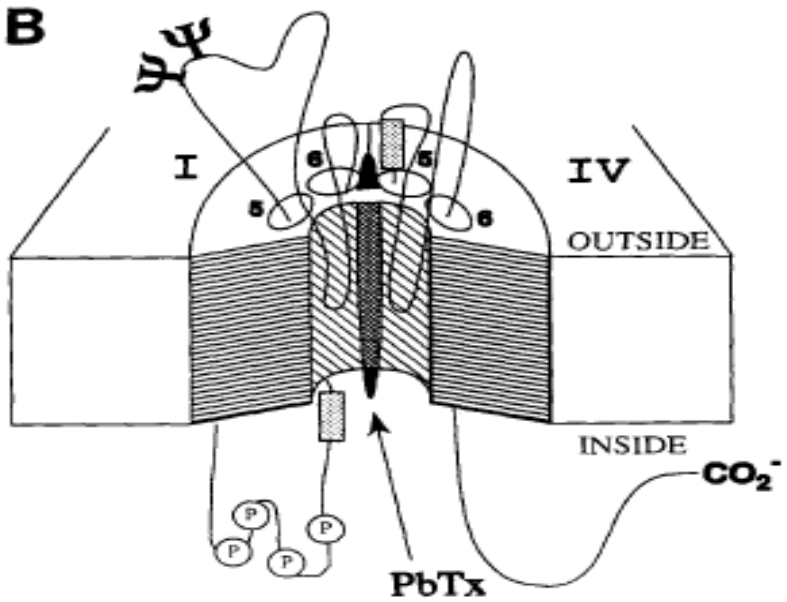
Figure 5: A proposed mechanism

(Poli et al. 1986, Trainer et al. 1994 and Gawley et al. 1995)



A study conducted in 1994 by Trainer using antibody recognition of rat brain sodium channels showed that the high affinity binding of brevetoxin requires a native conformation of the sodium channel and the neurotoxic receptor site 5 is found in active form on the solubilized purified sodium channel. Figure 6 shows the folding of four homologous domains bringing domain I and IV closer together and this forming a transmembrane pore. A molecule of brevetoxin interacts with the transmembrane segments S6 and S5 of domain I and IV respectively. This interaction of the transmemebrane segments domain I and IV contributed to the formation of the brevetoxin receptor site. This study shows that brevetoxin receptor site is formed from distinct segments of the primary sequence that interact with each other to form the high affinity receptor site in the folded structure of the a-subunit.

Figure 6: Model of breveotxin binding site on the sodium channel a-subunit. (Trainer 1994)



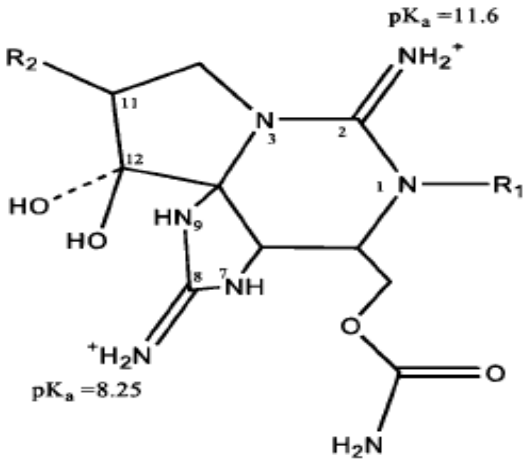
Voltage gated sodium channels are critical for normal CNS functioning and abnormal gating of these ion channels may lead to symptoms of NSP. In severe cases, young children experience seizures and unconsciousness (Steidinger 1998). Recent studies showed that when pregnant mice received an aerosolized radioactive form of brevetoxin-3, the toxin and its by-products were later detected in fetuses, uterine and placental tissues, and in the stomachs of nursing pups of brevetoxin-exposed mothers. This indicated that pregnant and nursing women not only inhale the toxin but also pass the toxin to their offspring (Potera 2007). Research on the interaction between brevetoxin and TRPVI channels may lead to development of effective therapies for thermal and pain sensation (Cuypers 2007). Current studies on brevetoxin levels in mouse plasma showed brevetoxin binding to HDLs. This association of brevetoxins and HDLs provides a new foundation for understanding the delivery process of brevetoxin throughout the blood and the removal process of the toxin from tissues. Brevetoxin, a lipophilic toxin, is most likely to be partitioned in the blood and associated with carrier proteins that bind and transport nonendogenous hydrophobic agent. Here the study evaluated the role of plasma carrier protein, lipoproteins with brevetoxins to develop more effective therapeutic treatment for intoxication of brevetoxins (Woofter 2005).

Saxitoxins

Saxitoxin is a heterocyclic guanidines possessing two positively charged 1,2,3 guanidinium groups. The compound consists of several functional groups, with an amide, two

OH- groups and the two R-groups (side chains) of simple hydrogens. Below in Figure 7 is the structure of saxitoxin and its analogs.

Figure 7: Saxitoxins and its analogs. (Choudhary 2002)



	R_1	R_2
STX	H	H
GTX2,3	H	SO_4^-
NeoSTX	OH	H
GTX1,4	OH	SO_4^-

Saxitoxin is also a non-peptide toxin produced by the dinoflagellates species, *Alexdanrium spp. Gymodinium spp.* and *Pyrodinium spp. Alexandrium* grows rapidly and contaminates shellfish causing Paralytic Shellfish Poisoning (PSP). PSP is most prevalent along the coastline of the United States producing the most harmful algal problems (Anderson 1997). PSP occurs frequently from Main to Massachusetts, and farther south to New Jersey. However

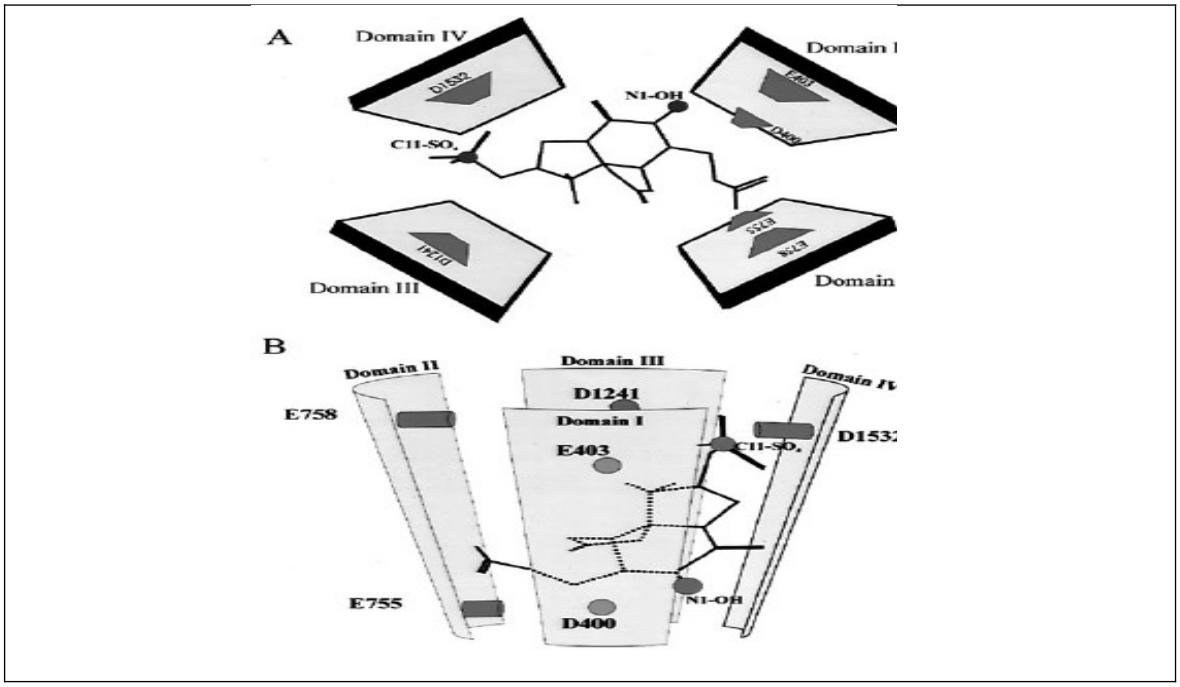
in 1990's, PSP was detected in the West Coast (Tibbetts 1998). Before the 1970s, PSP was endemic to North America, Europe and Japan, and today, PSP is found in South America, Australia and Southeast Asia. The first outbreak of PSP in South America occurred in 1972 as subsequent outbreaks occurred in 1981 and 1989 (Benavides 1995). Outbreaks also occurred in the Indo-Pacific areas. The first reported outbreak of PSP occurred in 1972 in Papua New Guinea and spread to the Philippines and Malaysia in 1980s (Furio 1996). *Alexandrium* are found to grow in relatively pristine waters. The threat of PSP is caused by intoxication of saxitoxin and can be fatal in humans. PSP has been associated with deaths of birds, humpback whales and fish (Van Dolah 2000). This toxin has a high affinity to bind to the voltage gated sodium channel, interfering with the channels normal conductance and leading to blockage of neuronal pathways. In 1990 through NMR (Nuclear Magnetic Resonance) analysis, saxitoxins were found to be synthesized through an unexpected pathway involving arginine, S-adenosylmethionine, acetate and other uncharacterized cellular metabolites (Shimizu et al 1990). Dinoflagellates producing saxitoxins and its derivatives require multiple enzymes. Most interesting is that enzymes that modify the saxitoxins were found in shellfish and bacteria (Plumley 1997).

The mechanism by which saxitoxins bind to the channel has been debated. Recently, the docking of saxitoxins and its derivative on the voltage gated channel has been explained. Saxitoxins bind to the P-loop (pore-forming loop) which is an extracellular loop between the fifth and sixth transmembrane segments of each sodium channel domain. It was experimentally determined that neosaxitoxins, a derivative of saxitoxins have an additional –OH group at the NI position of the 1,2,3 guanidinium which interacts with domain I and IV of the sodium channel (Choudnary 2002).

Another derivative of saxitoxins, Gonyautoxin 2,3 and Gonyautoxin 1,4, are C-11 sulfated. The localization of sulfate is evaluated by its interaction with all carboxyl groups from each of the four domains known to affect site 1 toxin binding, where saxitoxin has high affinity for site 1 and binds to it. The C-11 group was found to be located closest to domain IV and the OH group at the NI position of the 1,2,3 guanidiniumlay closest to domain I and the 7,8,9 guanidinium group orient towards the selectivity filter of the sodium channels. These three interactions fix the orientation for STX with respect to the channel. The proposed mechanism of saxitoxin localization in its channel binding site showed the orientation of saxitoxin in the outer vestibule, which is an area involved in channel gating and selectivity (Choudnary 2002).

Figure 8: Docking of saxitoxin derivative in the outer vestibule and the coupling of C-11 sulfate and NI-OH localization to domain IV and I

(Choudnary 2002)



At the molecular level, saxitoxins bind to the sodium channel at the specific site and localize to the residues within a conserved sequence motif in a-subunit four homologous domains (Terlau 1991). This showed that saxitoxin containing the two guanidinium groups is specific in protein binding. Binding is specific to the voltage gate sodium channel and blocks sodium influx in neuron cells. Relaxed muscular smooth muscle, depresses the rate the normal action potential of cardiac muscle. This leads to central nervous system and peripheral nervous system dysfunctions (Kao 1993). Saxitoxin neurotoxicity results from the effective blockage of the voltage sodium channels that mediate nerve and muscle action potentials (Llewellyn 1997).

By studying naturally occurring dinoflagellates saxitoxins and brevetoxins, other similar toxins and the molecular pathways these toxins use, researchers continue to develop new ways of synthesizing these complex toxins in the laboratory and manipulating them for biomedical applications. These powerful neurotoxins affect a wide variety of species in the ocean; affect

humans by targeting the voltage sodium gated channels as blockers (saxitoxins) and activators (brevetoxins). Hence, further research in saxitoxons and brevetoxins have advanced the understanding of the sodium channels and neurons in animals and humans. This will be beneficial to develop more effective anesthetics and better understanding of these molecular channels. Moreover, new therapies can be developed to treat toxin poisoning. This will help in controlling and treating outbreaks of PSP and NSP when they occurred.

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