SURVEY OF SAXIPHILIN-LIKE ACTIVITY IN THE ANIMAL KINGDOM

Edward G. Moczydlowski[1,2], Lyndon E. Llewellyn[3], and Peter M. Bell[1]

[1]Departments of Pharmacology and [2]Cellular and Molecular Physiology,
Yale University School of Medicine, New Haven, CT 06520-8066
[3]Australian Institute of Marine Science, PMB 3, Towsnsville MC, Queensland 4810, Australia

Saxitoxin (STX) and a large variety of STX derivatives are guanidinium phycotoxins produced by various species of dinoflagellates in the marine environment and cyanobacteria in freshwater ecosystems (Shimzu, Y. *Annu. Rev. Microbiol.* 50: 431-465, 1996). These toxins are also present in numerous shellfish and fish due to consumption of toxic phytoplankton by filter-feeders and dispersal through the food chain. In humans and higher animals, STX acts as a paralytic agent due to blockage of voltage-dependent Na^+ channels (Na_V channels) of nerve and skeletal muscle. Na_V channels are integral membrane proteins that bind STX with high affinity ($K_D \simeq 2$ nM) at a single extracellular site.

Our group has previously described a different type of high-affinity binding site for STX that is associated with a soluble protein named saxiphilin. This site was originally identified by the finding of specific, high-affinity binding of [^{3}H]STX in plasma and soluble extracts of frog tissues (Doyle, et al., *Science* 215: 1117-1119, 1982; Mahar et al., *Toxicon* 29: 653-671, 1991). The purified saxiphilin protein from the bullfrog, *Rana catesbeiana*, has a molecular mass of 91 kDa and contains one binding site for [^{3}H]STX with a K_D of 0.2 nM (Li & Moczydlowski, *J. Biol. Chem.* 266: 15481-15487, 1991; Llewellyn, L. E., & Moczydlowski, E., *Biochemistry* 33: 12312-12322, 1994). The sequence of saxiphilin cDNA, cloned from bullfrog liver (Morabito & Moczydlowski, *Proc. Nat. Acad. Sci. USA* 91: 2478-2482, 1994) revealed that saxiphilin is a close relative of the transferrin family of Fe^{3+}-binding proteins (e.g., 51% identity to *Xenopus laevis* transferrin). However saxiphilin does not bind Fe^{3+} and transferrins do not bind STX (Li et al., *Mol. Pharmacol.* 44: 742-748, 1993), implying that these two proteins have different biological functions. STX binding to saxiphilin is also resistant to competitive inhibition by 100 μM tetrodotoxin, which distinguishes it pharmacologically from Na_V-channels. Since the physiological role of saxiphilin is unknown, we carried out a phylogenetic survey of saxiphilin activity in order to investigate its molecular evolution and seek clues to its function. In particular, we wished to determine whether saxiphilin occurs in animals that are known to acquire STX from toxic phytoplankton.

The survey was performed by assaying hemolymph, plasma, or tissue extracts from selected animal species for soluble high-affinity binding of [^{3}H]STX. The standard assay was performed by adding 50-100 μl aliquots of plasma samples or extracts to a solution with a final concentration of 20-mM Mops-NaOH, pH 7.4, 200 mM NaCl, 0.1 mM EDTA and 5 nM [^{3}H]STX in a final volume of 250 μl. Control samples for determination of the non-specific blank also contained 10 μM STX. After incubation on ice for 1 h , duplicate aliquots of 100 μl were processed for separation of bound and free [^{3}H]STX on mini-columns of a cation-exchange resin that retains free [^{3}H]STX as described previously (Llewellyn & Moczydlowski, *Biochemistry* 33: 12312-12322, 1994). Samples of hemolymph, plasma and tissue extracts were centrifuged at 100,000 g for 1 h to pellet membrane components and rule out contamination by STX-binding sites associated with Na_V-channels.

Species investigated in this study included representatives of all major classes of vertebrates and most invertebrate phyla including animals that are known to be exposed to STX from toxic blooms of dinoflagellates. Our general finding was that saxiphilin-like activity is broadly distributed among ectothermic vertebrates and arthropods. Among vertebrates, positive species include various teleost fish, amphibians, and reptiles. Positive arthropods include arachnids, myriapods and crustaceans. Selected examples of [^{3}H]STX-binding activity in pmol/ml hemolymph, plasma, or tissue extract are listed in Table 1. Detailed characterization of [^{3}H]STX-binding activity from representative species showed that the equilibrium and kinetic binding parameters, the pH-dependence, and the pharmacology of inhibition by STX derivatives were similar to that of native bullfrog saxiphilin as previously described (Mahar et al., *Toxicon* 29: 653-6712, 1991; Llewellyn, & Moczydlowski, *Biochemistry* 33: 12312-12322, 1994). These results imply that a gene analogous to that encoding bullfrog saxiphilin has an ancient origin in animal evolution and is present and functionally active in the arthropod and chordate phyla.

Table 1. [^{3}H]STX binding activity in species found to exhibit saxiphilin-like activity. Effective concentration of soluble [^{3}H]STX binding sites per ml plasma or g tissue is reprted as the mean ± SD (n) where n is the number of individuals or determinations.

Species (common name and origin)	[^{3}H]STX binding sites pmol/ml plasma or g tissue
Ethmostigmus rupripes (centipede, Australia)	79 ± 18 (3)
Araneus c. f. cavaticus (orb web spider, N. America)	2.9 ± 0.7 (7)
Gambusia affinis (mosquitofish, N. America)	72 ± 8 (4)
Danio rerio (zebrafish, Asia)	6.3 ± 0.3 (3)
Anguilla rostrata (eel, N. America)	2.5 ± 0.8 (4)
Ambystoma tigrinum (tiger salamander, N. America)	76 ± 2 (3)
Rana sylvatica (wood frog, N. America)	1590 ± 440 (18)
Scleroporus poinsetti (crevice spiny lizard, N. America)	1100 ± 109 (6)
Naja naja kaouthia (Thailand cobra, Asia)	223 ± 20 (3)

Our survey did not find evidence of saxiphilin in species that are knwn to be directly or indirectly exposed to dinoflagellate toxins. For example, the mollusks, *Mytilus edulis* (blue mussel) and *Saxidomus giganteus* (butter clam), are known to bioaccumulate various STX derivatives. However, these species lacked detectable levels of saxiphilin-like activity. Similarly, the Atlantic mackerel, *Scomber scombrus*, has been found to contain STX in its viscera and such mackerel have previously been implicated in the deaths of humpback whales (Geraci et al., *Can. J. Fish. Aquat. Sci.* 46: 1895-1898, 1989). However, we did not find evidence of saxiphilin-like activity in mackerel, nor in other marine fish, sharks and whales that we assayed. Also, we did not find any evidence of saxiphilin-like activity in birds or mammals.

In conclusion, this study has documented the presence of saxiphilin-like activity in numerous arthropods and ectothermic vertebrates. The wide evolutionary distribution suggests that saxiphilin functions in a process of broad biological significance. However, the apparent lack of saxiphilin in marine species that are known to accumulate STX from toxic dinoflagellates does not support the hypothesis that saxiphilin functions as a defense mechanism against STX intoxication. [This study was supported by grants to E. M. from NIH (GM-51172), USAMRMC (DAMD-17-93C-3069), and New Investigator Award (Milbury Fund, ESO3828-11) from MDIBL.]