Phylogenetic survey of soluble saxitoxin-binding activity in pursuit of the function and molecular evolution of saxiphilin, a relative of transferrin

LYNDON E. LLEWELLYN¹, PETER M. BELL²
AND EDWARD G. MOCZYDLOWSKI^{2*,3}

¹ Australian Institute of Marine Science, PMB 3, Townsville MC, Queensland 4810, Australia

^{2*} Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520–8066, USA
(Edward.Moczydlowski@Yale.edu)

³ Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06520, USA

SUMMARY

Saxiphilin is a soluble protein of unknown function which binds the neurotoxin, saxitoxin (STX), with high affinity. Molecular characterization of saxiphilin from the North American bullfrog, *Rana catesbeiana*, has previously shown that it is a member of the transferrin family. In this study we surveyed various animal species to investigate the phylogenetic distribution of saxiphilin, as detected by the presence of soluble [³H]STX binding activity in plasma, haemolymph or tissue extracts. We found that saxiphilin activity is readily detectable in a wide variety of arthropods, fish, amphibians, and reptiles. The pharmacological characteristics of [³H]STX binding activity in phylogenetically diverse species indicates that a protein homologous to bullfrog saxiphilin is likely to be constitutively expressed in many ectothermic animals. The results suggest that the saxiphilin gene is evolutionarily as old as an ancestral gene encoding bilobed transferrin, an Fe³⁺-binding and transport protein which has been identified in several arthropods and all the vertebrates which have been studied.

1. INTRODUCTION

The neurotoxin, saxitoxin (STX), and a large array of STX derivatives are produced by certain species of dinoflagellates in the marine environment and cyanobacteria in the freshwater ecosystem (Carmichael *et al.* 1990; Hall *et al.* 1990; Schantz 1986; Shimizu 1996). Consumption of toxic phytoplankton by filter-feeding organisms results in the accumulation and dispersal of STX through the food chain to animals which have been reported to include an ascidian, annelids, molluscs, crabs, fish, and ultimately mammals (Anderson & White 1992; Geraci *et al.* 1989; Gessner *et al.* 1996; Llewellyn & Endean 1989; Nagashima *et al.* 1984; Yasumoto *et al.* 1986). The human intoxication syndrome of paralysis and death resulting from unwitting consumption of STX-contaminated shellfish is commonly known as ‘paralytic shellfish poisoning’ (PSP). The neurotoxicity of STX is due to potent blockade of voltage-sensitive Na⁺ channels that mediate nerve and muscle action potentials (Ritchie & Rogart 1977). STX exerts half-maximal blockage of Na⁺ current at a concentration of 2–100 nM of STX, depending on the particular Na⁺ channel isoform (Guo *et al.* 1987). At the molecular level, the STX binding

site on Na⁺ channels has been localized to residues within a conserved sequence motif in four homologous domains of the α -subunit, which forms part of the ion-selective pore (Terlau *et al.* 1991).

While the molecular pharmacology of STX with respect to Na⁺ channels is well characterized, our group has been attempting to uncover the biological significance of a different high-affinity binding site for STX, which is located on a soluble protein named saxiphilin. This site was first recognized due to the finding of high-affinity binding activity for [³H]STX in tissue extracts and plasma of frogs and toads (Doyle *et al.* 1982; Mahar *et al.* 1991). A component exhibiting soluble [³H]STX binding activity was purified from plasma of the bullfrog, *Rana catesbeiana*, and found to correspond to a 91 kDa protein related to the transferrin family of Fe³⁺-binding proteins (Li & Moczydlowski 1991). Structural similarity of saxiphilin to transferrin is indicated by a high level of sequence similarity; e.g. 51% identity to *Xenopus laevis* transferrin and 44% identity to human serum transferrin (Morabito & Moczydlowski 1994). Serum transferrin and lactoferrin have a bilobed structure owing to the presence of two internally homologous domains of ca. 340 residues, the N-lobe and the C-lobe, that each contain a high affinity site for Fe³⁺ (K_D ca. 10^{–20} M) and the synergistic anion cofactor, HCO₃[–] (Baker & Lindley 1992). Bullfrog saxiphilin has the same internal

* Author and address for correspondence.

duplication as the transferrins, but has substitutions in nine out of ten highly conserved Fe^{3+} -site residues, thereby accounting for its lack of demonstrable Fe^{3+} -binding activity (Li *et al.* 1993; Morabito & Moczydlowski 1994). The single high-affinity binding site for STX (K_D ca. 0.2 nM) in bullfrog saxiphilin has been localized to the C-lobe, as determined by assay of a recombinant form of the protein in which the N-lobe has been deleted (Morabito *et al.* 1995). [^3H]STX binding to saxiphilin is also inhibited at low pH in a manner reminiscent of the pH-dependent release of Fe^{3+} by transferrin (Llewellyn & Moczydlowski 1994), a process which is important in the delivery of iron to eukaryotic cells by transferrin receptor-mediated endocytosis (Thorstensen & Romslo 1990).

Aside from the recognized role of serum transferrin in iron transport, transferrin and lactoferrin are also responsible for maintaining low levels of free Fe^{3+} in biological fluids, which inhibits the growth of Fe^{3+} -requiring microorganisms and protects against the potential toxicity of $\text{Fe}^{2+}/\text{Fe}^{3+}$ in the generation of hydroxyl free radicals (Crichton 1991). By analogy to this latter chemical defence function, it may be hypothesized that saxiphilin functions as a defence mechanism against STX intoxication, by sequestering any STX that an animal might acquire from microbial sources. Although comparatively little is known about the chemical ecology of STX in the freshwater environment, an argument for this hypothesis can be drawn from previous observations of tadpole mortality associated with STX production by the cyanobacterial species, *Aphanizomenon flos-aquae* (Ikawa *et al.* 1982), and the occurrence of saxiphilin in Ranid tadpoles and frogs (Mahar *et al.* 1991).

Another question concerns the molecular evolution of saxiphilin. Transferrins have thus far been identified in all classes of vertebrates, several insect species and an ascidian (Bartfeld & Law 1990; Jamroz *et al.* 1993; Kurama *et al.* 1995; Martin *et al.* 1984; Welch 1990). Sequence data imply that the gene duplication underlying the bilobed structure of modern transferrin occurred before the emergence of insects (Bowman *et al.* 1988; Bartfeld & Law 1990). Since saxiphilin contains this same internal duplication, a transferrin gene may have been the direct ancestral precursor of the saxiphilin gene. Evidence has recently emerged that saxiphilin is not the only example of a non- Fe^{3+} -binding member of the transferrin family. Fierke and her co-workers have identified a protein inhibitor of carbonic anhydrase (PICA) in the pig which is homologous to transferrin, but has substitutions of several key Fe^{3+} -site residues (Roush & Fierke 1992; Wuebbens *et al.* 1994). The examples of saxiphilin and PICA suggest that transferrin-like proteins may reflect a family of genes with diverse biological functions.

To pursue the function and evolution of saxiphilin, we conducted a phylogenetic survey of soluble [^3H]STX binding activity. The known pharmacological characteristics of this activity in the bullfrog provide a unique set of molecular criteria to identify the presence of saxiphilin-related proteins in other species. We hypothesized that if saxiphilin functions primarily in a defensive capacity against STX in-

toxication from microbial sources, it ought to be commonly expressed by animal species inhabiting ecosystems known to harbour STX-producing phytoplankton. Unexpectedly, we found putative saxiphilin-like activity in terrestrial and aquatic arthropods and in a wide variety of ectothermic vertebrates, including fish and amphibians from the aquatic environment as well as reptiles indigenous to semi-arid locales. The data imply that saxiphilin has an ancient origin in animal evolution, and that it may function in a process(es) of broadly-based biological significance.

2. METHODS

(a) *Materials*

[^3H]STX was purchased from Amersham International, purified, and standardized according to Moczydlowski *et al.* (1988). Several different lots of [^3H]STX used in this study had specific activities in the range of 20200–35100 cpm pmol $^{-1}$. STX was purchased from Calbiochem (La Jolla, CA). The following STX derivatives were generously provided by Dr Sherwood Hall (US Food and Drug Administration): decarbamoylsaxitoxin (dcSTX), neosaxitoxin (neoSTX), B1 and C1 (see figure 2*a* for structures). Stock solutions of these toxins were diluted in 1 mM citrate buffer, pH 5.0. The common buffers, Mops, Mes, Hepes, and Tris, and the anaesthetics, tricaine methanesulphonate and sodium brevitall, came from Sigma (St Louis, MO). The cation exchange resin, AG50W-X2, H $^+$ form, 100–200 mesh, was obtained from Bio-Rad (Richmond, CA). Other chemicals were reagent grade, and came from commercial sources.

(b) *Sources of animals and sample preparation*

Some species used in this study were purchased from Connecticut Valley Biological (Southampton, MA), Carolina Biological (Burlington, NC), and Charles Sullivan Co. (Nashville, TN). Various animals were collected in the vicinity of Mount Desert Island Biological Laboratory (Salisbury Cove, ME) and the Australian Institute of Marine Science (Townsville, Queensland, Australia). Numerous professional colleagues, listed in the acknowledgements, generously donated plasma and tissue samples from animals used in their own studies. Plasma samples from several species of sharks, dolphins and whales were obtained from the New England Aquarium (Boston, MA) through the assistance of Dr Don Anderson at the Woods Hole Oceanographic Institute (Woods Hole, MA). Lyophilized plasma from terrestrial mammals, birds, and the Thailand cobra (*N. n. kaouthia*), liver extract of the African lungfish (*P. aethiopicus*) and horseshoe crab (*L. polyphemus*) haemolymph were purchased from Sigma.

Live animals were handled humanely according to the guidelines of the Yale University Animal Care and Use Committee. Live amphibians and reptiles studied in the laboratory were anaesthetized with tricaine methanesulphonate or sodium brevitall and exsanguinated via the aortic arch with a syringe containing 100 μl of 0.1 mg ml $^{-1}$ heparin sulphate. Whole blood was centrifuged in an Eppendorf microfuge; the plasma was removed and stored frozen at -80°C for later assay. In our experience, saxiphilin-like [^3H]STX binding activity in whole plasma is very stable over the course of a year when stored frozen or lyophilized. This is in contrast to dilute solutions of purified bullfrog saxiphilin which are labile to repeated freeze-thawing (Llewellyn & Moczydlowski 1994). Animals which

were too small for convenient collection of blood or haemolymph were anaesthetized by hypothermia and processed for tissue extraction. Whole animal extracts were prepared by homogenization on ice with a Tissumizer homogenizer (Tekmar, Cincinnati, OH), using two 10 s bursts at 8000 rpm followed by two 10 s bursts at 24000 rpm in a buffer consisting of 10 mM of Mops-NaOH, pH 7.4, 0.3 M of sucrose, 5 mM of EDTA, 1 μ M of pepstatin, 1 μ M of aprotinin, and 100 μ M of phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 100000 g for 1 h to ensure complete removal of solid debris and particulate membranes that might contain STX binding sites associated with Na⁺ channels. The supernatant was decanted for assay and stored frozen at -80 °C.

(c) Measurement of [³H]STX binding

All measurements of [³H]STX binding were carried out at *ca.* 0 °C by incubation of assay solutions on ice. In the standard survey for soluble [³H]STX binding, 50–100 μ l aliquots of plasma samples or extracts were added to a solution with a final concentration of 20 mM of Mops-NaOH, pH 7.4, 200 mM of NaCl, 0.1 mM of EDTA, and approximately 5 nM of [³H]STX in a volume of 250 μ l, and incubated for at least 1 h. Control samples for the determination of non-specific binding also contained 10 μ M of STX. Duplicate aliquots of 100 μ l were processed for separation of bound and free [³H]STX on small columns of AG50W-X2 cation exchange resin, and quantitated by liquid scintillation counting, as previously described (Llewellyn & Moczydlowski 1994).

Assay of saturable binding behaviour for selected species was similarly performed by varying the concentration of [³H]STX in the assay from 0.1–26 nM. Data from such experiments were fitted to a one-site binding model using the EBDA and LIGAND equilibrium binding analysis programs from Biosoft (Cambridge, UK). Structure–activity relationships for STX derivatives were investigated by competitive binding titrations in which [³H]STX was held constant at 4.4 nM and the concentration of various unlabelled STX derivatives was varied. Data from such experiments were fitted to the Hill equation: $f = K_{0.5}^{n^1} / ([\text{toxin}]^{n^1} + K_{0.5}^{n^1})$, where f is the ratio of [³H]STX bound in the presence of competitor toxin compared to that without competitor toxin, $K_{0.5}$ is the competitor concentration at 50% inhibition, and n^1 is a pseudo-Hill coefficient. To perform a nonlinear least squares fit we used the curve fitting utility of Sigmaplot (Jandel, San Rafael, CA). Error estimates for $K_{0.5}$ given in table 3 were obtained from the Sigmaplot fitting routine.

Dependence of binding on pH was studied by a similar assay using 4.4 nM of [³H]STX in buffers ranging from pH 4–9. The pH was buffered with 20 mM of Tris, 10 mM of Mes, and 10 mM of acetic acid adjusted with tetramethylammonium hydroxide or HCl at nearly constant ionic strength, as described by Ellis & Morrison (1982). H⁺ titration data were analysed by fitting to the following function (similar to the one used for toxin competition): $f = [H^+]_{0.5}^{n^1} / ([H^+]^{n^1} + [H^+]_{0.5}^{n^1})$, where $[H^+]_{0.5}$ is the proton concentration at 50% inhibition.

Dissociation kinetics of [³H]STX were followed by pre-equilibration of samples with [³H]STX and assays of bound radioligand at various times after addition of 10 μ M of STX. Similarly, association kinetics under pseudo-first order conditions were followed by assays of bound radioligand at various times after addition of 4.4 or 8.8 nM of [³H]STX. Kinetic data were fitted either to a 'single exponential' function of time or to a 'sum-of-two exponentials' function of time as described in the text. Protein assays were performed according to the method of Cabib & Polachek (1984).

3. RESULTS

From previous biochemical characterization in the bullfrog, saxiphilin may be recognized by soluble high-affinity binding of [³H]STX, which is specifically displaced by excess unlabelled STX (Mahar *et al.* 1991; Llewellyn & Moczydlowski 1994). Binding of [³H]STX to saxiphilin is readily discriminated from binding to voltage-sensitive Na⁺ channels by its solubility in the absence of detergents, its presence in plasma and extracts of non-electrically excitable tissues, the lack of binding competition by 100 μ M of tetrodotoxin, and a distinctively slow time-course of dissociation. Thus, to screen various animal species for expression of saxiphilin, we assayed soluble [³H]STX binding in plasma or haemolymph samples where possible, or in whole tissue extracts of small vertebrates and invertebrates. The latter extracts were subjected to ultracentrifugation to eliminate the possibility of contamination by membrane-bound Na⁺ channels. Species selected for this study included representatives of all major vertebrate classes and invertebrate phyla. The survey sample consisted of animals indigenous to five continents (N. America, S. America, Europe, Asia, and Australia) with diverse habitats, including species that have been previously documented to acquire STX from blooms of toxic dinoflagellates.

Table 1 summarizes the effective concentration and specific activity (mg protein⁻¹) of soluble [³H]STX binding sites in samples from species which tested positive for saxiphilin. The level of detectable activity ranged from 0.6 nM effective concentration (pmol sites per ml of plasma or g of tissue extract) in a cockroach (*Periplaneta americana*) to *ca.* 5000 nM in the plasma of one individual specimen of the wood frog (*Rana sylvatica*). Species which tested positive include animals belonging to the major classes of ectothermic vertebrates and arthropods. The list of positive vertebrates includes teleost fish, amphibians (frogs, toads, salamanders and newts) and reptiles (lizards and snakes). For the arthropods, the low levels of activity measured in the cockroach, *Periplaneta*, and the water-flea, *Daphnia*, might be questionable since these measurements approached the lower limit of detection (*ca.* 0.1 nM [³H]STX binding sites). However, the finding of robust activity in an Australian centipede (*Ethmostigmus rubripes*), a North American isopod (sowbug, *Oniscus sp.*), a North American orb-weaving spider (*Araneus c. f. cavaticus*), and four different species of xanthid crabs (Llewellyn 1997), firmly establish the occurrence of saxiphilin-like activity in arthropods. Despite a concerted attempt (see table 2), we did not find many examples of insects containing saxiphilin activity. However, in addition to the positive cockroach species listed in table 1, we did observe robust activity (5.2 pmol g⁻¹) in an extract of damselfly nymphs obtained from Connecticut Valley Biological (Southampton, MA). Although we have not been able to obtain a precise taxonomic identification, such nymphs are the aquatic larval form of insects belonging to the family Calopterygidae.

Table 2 is a partial listing of species that were tested by the same method and found not to exhibit any

Table 1. [³H]STX binding in species found to exhibit saxiphilin-like activity

(Data are the results of a standard assay for binding of 5 nM [³H]STX as described in §2. Effective concentration of [³H]STX binding sites per ml of plasma or g of tissue is reported as the mean $\pm$ s.d. (n) where n is the number of individuals or determinations. Abbreviations: P, plasma; H, haemolymph; E, extract; F, freshwater species; M, marine species; n.d., not determined.)

species (common name and geographical origin)	[³ H]STX binding sites	
	pmol ml ⁻¹ plasma or g tissue	pmol mg ⁻¹ protein
teleost fish (except lungfish)		
<i>Hypostomus plecostomus</i> (catfish, S. America) E, F	20 $\pm$ 3 (4)	6.7
<i>Poecilia reticulata</i> (guppy, Americas) E, F	72 $\pm$ 9 (4)	15.8
<i>Anguilla rostrata</i> (eel, N. America) E, M, F	2.5 $\pm$ 0.8 (4)	n.d.
<i>Gambusia affinis</i> (mosquito fish, N. America) E, F	72 $\pm$ 8 (4)	3.2
<i>Pomacentrus</i> sp. (damsel fish, N. America) E, M	1.9 $\pm$ 0.1 (2)	0.1
<i>Apogon</i> sp. (cardinal fish, N. America) E, M	29 $\pm$ 1.3 (3)	1.0
<i>Danio rerio</i> (zebra fish, Asia) E, F	6.3 $\pm$ 0.3 (3)	0.3
<i>Protopterus aethiopicus</i> (lungfish, Africa) E, F	0.5 (1)	0.02
amphibians		
<i>Notophthalmus viridescens</i> (Eastern newt, N. America) E	1.5 $\pm$ 0.5 (11)	0.2
<i>Ambystoma tigrinum</i> (tiger salamander, N. America) P	76 $\pm$ 2 (3)	3.3
<i>Rana sylvatica</i> (wood frog, N. America) P	1590 $\pm$ 440 (18)	68
<i>Rana temporaria</i> (grass frog, Europe) P	669 $\pm$ 18 (3)	34
<i>Bufo marinus</i> (cane toad, S. America) P	49 $\pm$ 5 (8)	1.9
reptiles		
<i>Varanus rosenbergii</i> (goanna monitor lizard, Australia) P	326 $\pm$ 25 (4)	5.0
<i>Sceloporus poinsettii</i> (crevice spiny lizard, N. America) P	1100 $\pm$ 109 (6)	491
<i>Naja naja kaouthia</i> (Thailand cobra, Asia) P	223 $\pm$ 20 (3)	7.6
<i>Crotalus viridis viridis</i> (rattlesnake, N. America) P	2.4 $\pm$ 0.5 (3)	0.1
<i>Thamnophis ordinoides</i> (garter snake, N. America) P	133 $\pm$ 89 (4)	10.7
<i>Thamnophis sirtalis</i> (garter snake, N. America) P	486 $\pm$ 2322 (6)	19.8
arthropods		
<i>Daphnia</i> sp. (water-flea, N. America) E	0.8 $\pm$ 0.2 (2)	n.d.
<i>Oniscus</i> sp. (sowbug, N. America) E	8.2 $\pm$ 0.5 (8)	n.d.
<i>Ethmostigmus rubripes</i> (centipede, Australia) H	79 $\pm$ 18 (8)	2.2
<i>Araneus c. f. cavaticus</i> (orb web spider, N. America) E	2.9 $\pm$ 0.7 (7)	n.d.
<i>Periplaneta americana</i> (cockroach, N. America) E	0.6 $\pm$ 0.2 (3)	n.d.
<i>Lophozozymus pictor</i> (xanthid crab, Australia) H	111 $\pm$ 12 (2)	21
<i>Liomera tristis</i> (xanthid crab, Australia) H	97 $\pm$ 9 (2)	24
<i>Chlorodiella nigra</i> (xanthid crab, Australia) H	24 $\pm$ 5 (1)	6.3
<i>Actaeodes tomentosus</i> (xanthid crab, Australia) H	81 $\pm$ 22 (2)	37

detectable saxiphilin activity. We did not find evidence of saxiphilin-like activity in endothermic vertebrates, as represented by various birds or mammals, including dolphins and whales. Negative-testing reptiles included various turtles, crocodilians, a tiger snake, and the primitive tuatara of New Zealand. The common laboratory species, *Xenopus laevis* (African clawed frog), is the only amphibian tested to date that does not appear to express saxiphilin. Negative vertebrate marine species also include several fish, four sharks, the evolutionarily primitive coelocanth, and a lamprey.

Except for the arthropods, we did not detect saxiphilin activity in other invertebrate phyla. As shown in table 2, the negative phyla include ascidians, echinoderms, annelids and molluscs. In addition, the following invertebrate species are not shown in table 2, but they also tested negative: phylum Porifera (the sponges, *Xestospongia exigua*, *Lanthella basta*, *Jaspis stellifera*, *Perichera heteroraphis*, *Rhopaloides odorabile*), phylum Platyhelminthes (the flatworm, *Pseudoceros* sp.), phylum Cnidaria (soft corals, *Sarcophyton elegans*, *Simularia*, *dura*; sea anemone, *Actinia australis*), and phylum

Brachiopoda, (*Lingula* sp.). Of particular interest among negative species are the bivalve molluscs, *Saxidomus giganteus* and *Mytilus edulis*, which are known to accumulate STX from toxic marine dinoflagellates (Shimizu *et al.* 1978; Schantz 1986), but they do not appear to produce saxiphilin (table 2).

To test the presumption that soluble [³H]STX binding activity found across this diverse phylogenetic spectrum has properties similar to that of the previously characterized saxiphilin from *Rana catesbeiana*, samples from the following representative species were selected for further characterization: plasma of *Bufo marinus* (marine or cane toad), plasma of *Thamnophis sirtalis* (garter snake), lyophilized plasma of *Naja naja kaouthia* (Thailand cobra), an extract of whole *Gambusia affinis* (mosquito fish), and haemolymph of *Ethmostigmus rubripes* (an Australian centipede). Appropriate dilutions of these samples were titrated with various concentrations of [³H]STX in the range of 0.1–26 nM, and assayed for binding in the absence and presence of excess unlabelled STX (10 μ M) to assess total and non-specific binding, respectively. Samples from all five of

Table 2. *Species that tested negative for saxiphilin*

(This table lists species that did not contain a detectable amount (> 0.1 pmol ml⁻¹ sample) of [³H]STX binding activity in plasma (P), haemolymph (H), or soluble extracts (E). Several additional negative species are given in §3.)

phylum Vertebrata (super-class Agnatha)	<i>Asterius forbesii</i> (starfish) E
<i>Mordacia mordax</i> (lamprey) E	<i>Asterius vulgaris</i> (starfish) E
phylum Vertebrata (super-class Gnathostomes)	<i>Solaster endeca</i> (starfish) E
<i>Squalus acanthius</i> (dogfish shark) P	<i>Linckia laevigata</i> (starfish) H
<i>Ginglymostoma cirratum</i> (nurse shark) P	<i>Cucumaria frondosa</i> (sea cucumber) E
<i>Negaprion brevirostris</i> (lemon shark) P	<i>Echinarachnius parma</i> (sand dollar) E
<i>Somniosus microcephalus</i> (Greenland shark) P	<i>Holothuria atra</i> (holothurian) H
<i>Raja erinacea</i> (little skate) P	<i>Stichopus chloronatus</i> (holothurian) H
<i>Scomber scombrus</i> (Atlantic mackerel) P	phylum Annelida
<i>Cyclopterus lumpus</i> (lumpfish) P	<i>Gastrolepida clavigea</i> (annelid) E
<i>Pseudopleuronectes americanus</i> (flounder) P	<i>Eisenia fetida</i> (tiger worm) E
<i>Makaira indica</i> (marlin) P	<i>Eudrilus eugenia</i> (nightcrawler) E
<i>Arothron manilensis</i> (puffer fish) E	<i>Reteterebella queenslandia</i> (polychaete) E
<i>Latimeria chalumnae</i> (coelocanth) P	<i>Spirobranchus giganteus</i> (polychaete) E
phylum Vertebrata (class Amphibia)	phylum Arthropoda
<i>Xenopus laevis</i> (African clawed frog) P	<i>Limulus polyphemus</i> (horseshoe crab) H
phylum Vertebrata (class Reptilia)	<i>Penaeus monodon</i> (tiger prawn) H
<i>Elseya dentata</i> (snapping tortoise, Australia) P	<i>Cancer borealis</i> (Jonah crab) H
<i>Dermochelys coriacea</i> (leatherback turtle) P	<i>Pagurus sp.</i> (hermit crab) H
<i>Pseudemys scripta</i> (red ear turtle, N. America) P	<i>Homarus americanus</i> (lobster) H
<i>Caretta caretta</i> (loggerhead turtle) E	<i>Ocypode corimana</i> (ghost crab)
<i>Sphenodon punctata</i> (tuatara, New Zealand) P	<i>Artemia salina</i> (brine shrimp) E
<i>Notechis scutatus</i> (Australian tiger snake) P	<i>Uca vomeris</i> (fiddler crab) H
<i>Alligator mississippiensis</i> (alligator, N. America) P	<i>Callinassa australiensis</i> (marine yabby) H
<i>Crocodylus porosus</i> (crocodile, Australia) E	<i>Scylla serrata</i> (mud crab) H
phylum Vertebrata (class Aves)	<i>Manduca sexta</i> (tobacco hornworm moth) H
<i>Gallus gallus</i> (chicken) P	<i>Periplaneta australiensis</i> (cockroach) E
<i>Anser anser</i> (domestic goose) P	<i>Blaberus sp.</i> (cockroach) H, E
<i>Anas sp.</i> (duck) P	<i>Drosophila melanogaster</i> (fruit fly) E
<i>Columba livia</i> (domestic pigeon) P	<i>Cicada sp.</i> (cicada) E
<i>Meleagris gallopavo</i> (common turkey) P	<i>Oecophylla smaragdina</i> (green ant) E
phylum Vertebrata (class Mammalia)	<i>Camponotus pennsylvanicus</i> (carpenter ant) E
<i>Equus caballus</i> (horse) P	<i>Oncopeltus fasciatus</i> (milkweed bug) E
<i>Oryctolagus sp.</i> (rabbit) P	<i>Acheta domestica</i> (cricket) E
<i>Ovis aries</i> (domestic sheep) P	<i>Hippodamia convergens</i> (lady beetle) H
<i>Homo sapiens</i> (human) P	<i>Reticulitermes flavipes</i> (termite) E
<i>Rattus norvegicus</i> (rat) P	<i>Tenebrio molitor</i> (mealworm larvae) E
<i>Bos taurus</i> (cow) P	<i>Hyalophora cecropia</i> (silkworm) E
<i>Sus scrofa</i> (pig) P	<i>Papilio polyxenes</i> (black swallowtail butterfly) E
<i>Lagenorhynchus acutus</i> (white-sided dolphin) P	<i>Dermestes sp.</i> (dermestid beetle) E
<i>Globicephala malaena</i> (pilot whale) P	phylum Mollusca
<i>Delphinus delphis</i> (common dolphin) P	<i>Mytilus edulis</i> (blue mussel) H, E
<i>Balaenoptera acutorostrata</i> (minke whale) P	<i>Saxidomus giganteus</i> (butter clam) H, E
phylum Chordata (subphylum Ascidiacea)	<i>Aplysia californica</i> (sea hare) H, E
<i>Polycarpa sp.</i> (ascidian) E	<i>Spisula solidissima</i> (clam) E
<i>Didemnum molle</i> (ascidian) E	<i>Acmaea c. f. testudinalis</i> (limpet) E
<i>Polycarpa aurata</i> (ascidian) E	<i>Littorina litorea</i> (periwinkle) E
<i>Tunica mogula</i> (tunicate) E	<i>Donax deltoideus</i> (bivalve) E
phylum Echinodermata	<i>Vepricardium multispinosum</i> (bivalve) E
<i>Strongylocentrotus droebachiensis</i> (sea urchin) E	<i>Sepia plangon</i> (squid) E
<i>Acanthaster planci</i> (crown-of-thorns starfish) E	

these species exhibited a component of saturable, high-affinity binding, and a linear component of non-specific [³H]STX binding (figure 1). The binding titration data were analysed with the EBDA and LIGAND programs to derive an apparent equilibrium dissociation constant (K_D) for [³H]STX, based on the assumption of a single class of binding sites. The K_D values estimated by this approach were: 210 ± 29 pM (*B. marinus*), 15 ± 6 pM (*N. n. kaouthia*), 240 ± 60 pM (*T. sirtalis*), 39 ± 7 pM (*G. affinis*), and 10 ± 6 pM (*E. rubripes*). These K_D values, all less than 1 nM, fall in a

range where the accuracy of equilibrium analysis is limited by the ability to accurately determine extremely low concentrations of free [³H]STX ligands. However, the values indicate an equivalent or higher affinity than that previously reported for purified native ($K_D = 350 \pm 20$ pM) or recombinant ($K_D = 220 \pm 10$ pM) saxiphilin from *Rana catesbeiana*.

Although numerous organic molecules have been tested, derivatives of STX are the only class of compounds that we have ever observed to competitively displace [³H]STX binding to bullfrog saxi-

Figure 1. Saxiphilin-like activity in plasma, haemolymph or tissue extract of various species as demonstrated by titration of [^3H]STX binding. A fixed amount of soluble protein from various animal species was assayed for binding at the indicated concentrations of [^3H]STX as described in §2. Plasma samples and extracts were pre-equilibrated with [^3H]STX for 1–2 h before binding assay, except for *E. rubripes* haemolymph which was pre-equilibrated for 8 h. Total binding in cpm (○). Non-specific binding in the presence of 10 μM of STX (●). Data points are the means of duplicate determinations. Non-specific binding was fitted by a simple linear regression and total binding was fitted to a one-site model with K_D values given in the text. (a) *Bufo marinus* (cane toad) plasma, 72 μg protein ml^{-1} . (b) *Naja naja kaouthia* (Thailand cobra) plasma, 74 μg protein ml^{-1} . (c) *Thamnophis sirtalis* (garter snake) plasma, 103 μg protein ml^{-1} . (d) *Gambusia affinis* (mosquito fish) extract, 180 μg protein ml^{-1} . (e) *Ethmostigmus rubripes* (centipede) haemolymph, 360 μg protein ml^{-1} .

philin. To investigate whether the soluble STX-binding sites of the five test species share a common structure–activity relationship, a competition displacement assay was performed for four STX derivatives: neoSTX, dcSTX, B1, and C1 (figure 2a). As illustrated in figure 2, samples from all five test species exhibited monotonic displacement titration curves with Hill coefficients close to 1.0, characteristic of a single site or a homogeneous class of STX binding sites. In each case, the concentration of free STX required for 50%

Figure 2. Competitive inhibition of [^3H]STX binding by unlabelled STX and four different STX derivatives. (a) Chemical structure of naturally occurring STX derivatives used in this study: STX (○), $\text{R}_1 = \text{CONH}_2$, $\text{R}_2 = \text{H}$, $\text{R}_3 = \text{H}$; dcSTX (◆), $\text{R}_1 = \text{H}$, $\text{R}_2 = \text{H}$, $\text{R}_3 = \text{H}$; neoSTX (△), $\text{R}_1 = \text{CONH}_2$, $\text{R}_2 = \text{OH}$, $\text{R}_3 = \text{H}$; B1 (▽), $\text{R}_1 = \text{CONHSO}_3^-$, $\text{R}_2 = \text{H}$, $\text{R}_3 = \text{H}$; C1 (□), $\text{R}_1 = \text{CONHSO}_3^-$, $\text{R}_2 = \text{H}$, $\text{R}_3 = \text{OSO}_3^-$. (b) *Naja naja kaouthia* plasma, 40 μg protein ml^{-1} . (c) *Gambusia affinis* extract, 180 μg protein ml^{-1} . (d) *Ethmostigmus rubripes* haemolymph, 180 μg protein ml^{-1} . Data points are the mean of duplicate determinations. Solid curves are fits of the data to a Hill function given in §2. Best-fit values of $K_{0.5}$ for the various STX derivatives are listed in table 3.

displacement of specific [^3H]STX binding was very close to the concentration of free [^3H]STX in the assay (ca. 4 nM), in accordance with the relationship of Cheng & Prusoff (1973) for one-site binding. With respect to displacement by the four STX derivatives, the highest and lowest affinities were observed for dcSTX and C1, respectively. For the snake, fish, and centipede species, neoSTX exhibited higher affinity than B1 (table 3). However, for the toad, B1 exhibited slightly higher affinity than neoSTX. These results are in contrast to saxiphilin activity from Ranid frogs, where neoSTX has a 550-fold lower affinity than STX (table 3). It thus appears that the low intrinsic affinity

Table 3. Saxitoxin structure–activity relationships and pH dependence of [3 H]STX binding for various species

([3 H]STX binding to plasma or extracts was titrated with unlabelled STX derivatives or H^+ as in figures 2 and 3. IC_{50} values for STX derivatives, $pH_{0.5}$ values for 50% inhibition and pseudo-Hill (n^l) coefficients were determined by fitting data to a Hill function given in §2. Values in parentheses are ratios relative to STX for each species. Data for *Rana catesbeiana* are taken from Mahar *et al.* (1991) and Llewellyn & Moczydlowski (1994).)

species	IC_{50} (nM)					pH dependence	
	STX	dcSTX	NEO	B1	C1	$pH_{0.5}$	n^l
<i>Rana catesbeiana</i>	4.7 ± 0.7 (1.0)	11.1 ± 1.2 (2.4)	2640 ± 180 (560)	4.5 ± 0.4 (1.0)	730 ± 160 (155)	5.7	1.0
<i>Bufo marinus</i>	5.3 ± 0.9 (1.0)	10.8 ± 1.6 (2.0)	32.3 ± 2.4 (6.1)	19.0 ± 2.1 (3.6)	7540 ± 620 (1420)	4.4	1.1
<i>Thamnophis sirtalis</i>	4.0 ± 2.1 (1.0)	1.4 ± 0.2 (0.4)	6.0 ± 1.7 (1.5)	253 ± 25 (63)	3480 ± 190 (870)	5.5	1.4
<i>Naja naja kaouthia</i>	4.0 ± 0.4 (1.0)	1.7 ± 0.3 (0.4)	20.8 ± 1.7 (5.2)	118 ± 5 (30)	7540 ± 460 (1890)	5.4	2.1
<i>Gambusia affinis</i>	5.0 ± 0.3 (1.0)	7.7 ± 0.7 (1.5)	11.8 ± 0.8 (2.4)	179 ± 10 (36)	63000 ± 2500 (12600)	4.7	2.0
<i>Ethmostigmus rubripes</i>	5.0 ± 0.3 (1.0)	6.1 ± 0.9 (1.2)	8.5 ± 0.4 (1.7)	20.3 ± 1.2 (4.1)	2830 ± 120 (566)	5.4	3.2

Figure 3. pH dependence of [3 H]STX binding. Panel (a) shows results for *Bufo marinus* plasma ($\bullet$, 72 μ g protein ml^{-1}), *Naja naja kaouthia* plasma (∇ , 74 μ g protein ml^{-1}), and *Gambusia affinis* extract (Δ , 180 μ g protein ml^{-1}). Panel (b) shows results for *Thamnophis sirtalis* plasma ($\blacklozenge$, 41 μ g protein ml^{-1}) and *Ethmostigmus rubripes* plasma ($\circ$, 360 μ g protein ml^{-1}). Data points and error bars are the mean $\pm$ SEM of three experiments. Solid and dashed curves represent fits of the data to a Hill function of [H^+] given in §2. Best-fit values of $pH_{0.5}$ and n^l are listed in table 3.

for neoSTX in bullfrog saxiphilin is an exception rather than the rule, and that saxiphilin from many species binds neoSTX nearly as well as STX. For [3 H]STX binding activity examined in this study, in no case did we observe competitive displacement by 100 μ M tetrodotoxin, which is an Na^+ channel blocker with a very different structure from STX (Ritichie & Rogart 1977). This emphasizes the unambiguous pharmacological discrimination of saxiphilin from STX-binding sites of voltage-sensitive Na^+ channels, which are well known to exhibit competitive binding between STX and tetrodotoxin.

Previous studies showed that the pH dependence of [3 H]STX binding to bullfrog saxiphilin resembles the pH dependence of Fe^{3+} binding to serum transferrin (Llewellyn & Moczydlowski 1994). We previously suggested that this coincidence may reflect a common structural–functional relationship or a shared aspect of cellular physiology. To investigate whether this bio-

chemical property of saxiphilin activity is conserved in other species, equilibrium binding of [3 H]STX was measured as a function of pH for the five test species (figure 3). The pH titration curves of the two snake species, *N. n. kaouthia* and *T. sirtalis*, exhibited a similar pH dependence as that of *Rana catesbeiana* with half-maximal inhibition occurring near pH 5.5 (table 3). However, the Hill coefficient (n^l) derived for H^+ was higher than that previously observed for the bullfrog protein ($n^l = 1.0$), with $n = 1.4$ and 2.1 for *T. sirtalis* and *N. kaouthia*, respectively. [3 H]STX binding activity from *B. marinus* and *G. affinis* was less sensitive to H^+ inhibition. Complete titration curves for these latter two species could not be obtained due to the known limitations of the cation exchange column assay at pH values less than 4.5. The available data suggest $pH_{0.5}$ values of approximately 4.4 and 4.7 for *B. marinus* and *G. affinis*, respectively. The putative saxiphilin activity of the centipede species *E. rubripes* exhibited a $pH_{0.5}$ of 5.4 and a Hill coefficient of ca. 3.2, corresponding to a steeply sensitive inhibition with respect to [H^+] that may indicate more than one site of H^+ modulation.

The kinetics of dissociation and association of [3 H]STX were also investigated. Figure 4 shows representative data collected in these experiments for *B. marinus*, *N. n. kaouthia*, *G. affinis* and *E. rubripes*. In the case of the toad and the centipede, both the dissociation and association kinetics were well described by a simple exponential time-course. The derived dissociation rate constant for *B. marinus* from the experiment (figure 4a) at pH 7.4 was $k_{off} = 1.1 \pm 0.1 \times 10^{-5} s^{-1}$. The rates of [3 H]STX dissociation measured for plasma samples from two other specimens of *B. marinus* were $1.2 \pm 0.1 \times 10^{-5} s^{-1}$ and $1.3 \pm 0.2 \times 10^{-5} s^{-1}$, illustrating the consistency of these kinetic determinations from individual to individual. The association rate of the *B. marinus* sample was measured in the presence of 4.4 nM of [3 H]STX under near pseudo-first order conditions, where the concentration of ligand was about seven-fold greater than the number of total sites. Under these conditions, the time-course was so rapid that the early portion could not be resolved with the present methodology. However, the data can be used to calculate a lower limit for the bimolecular association rate constant of $k_{on} \geq 9.8 \times 10^6 s^{-1} M^{-1}$. The ratio of k_{off} to k_{on} provides an upper limit estimate for the

Figure 4. Time-course of dissociation and association for [^3H]STX binding compared for different species. (a,e) *Bufo marinus* plasma. (b,f) *Naja naja kaouthia* plasma. (c,g) *Gambusia affinis* extract. (d,h) *Ethmostigmus rubripes* haemolymph. Dissociation (a,b,c,d) or association (e,f,g,h) time-course of specific [^3H]STX binding was measured as described in §2. Data were obtained at pH 7.4 except for a, b and c, where dissociation at pH 7.4 (◆) is compared to that at various acidic pH values (○) as follows: (a) pH 4.3, (b) pH 5.3, and (c) pH 4.5. Data points are fitted (solid lines) to a single exponential function (a,d,e,f,h) or to a sum-of-two exponentials (b,c,g) as described in the text.

equilibrium K_D for *B. marinus* of 1.1 pM, which is an order of magnitude lower than that estimated (15 pM) from the equilibrium titration data of figure 1. As noted above, equilibrium K_D measurements in the range of 1 pM by standard Scatchard analysis with the available specific activity of [^3H]STX are technically unattainable. Thus, the K_D values estimated here by the kinetic approach are more likely to be closer to the true value.

The corresponding rate constants for *E. rubripes* were $k_{\text{off}} = 4.3 \pm 0.3 \times 10^{-6} \text{ s}^{-1}$ and $k_{\text{on}} = 5.2 \pm 0.7 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$, giving an estimated K_D of 0.8 pM. Figure 4h shows that the time-course of association for the centipede sample was fairly well resolved, being slower than that of the other species in figure 4. The dissociation rate of [^3H]STX from the centipede haemolymph sample corresponds to a halftime of 1.9 days, which is extraordinarily slow for the dissociation of a small organic molecule from a protein acceptor site.

The kinetic behaviour of the fish and snake samples was more complex than that of the toad and centipede. For these species, the time-course of [^3H]STX dissociation was better fitted by a sum-of-two exponentials rather than one exponential (figure 4). However, a major slow kinetic component of the dissociation

process comprised 77–92% of the decay for these three species, corresponding to the following k_{off} rate constants: $9.8 \pm 1.5 \times 10^{-6} \text{ s}^{-1}$ for *G. affinis*, $1.1 \pm 0.1 \times 10^{-4} \text{ s}^{-1}$ for *N. n. kaouthia*, and $1.3 \pm 0.1 \times 10^{-5} \text{ s}^{-1}$ for *T. sirtalis*. These latter slow components correspond to dissociation halftimes of 19.6, 1.7 and 14.8 h, respectively, all slower than the halftime of 1.3 h previously measured for bullfrog saxiphilin (Llewellyn & Moczydlowski 1994). The association time-course for the two snake species conformed well to a single exponential process, but the fish sample was better described by two components (figure 4g). For the cobra, *N. n. kaouthia*, and the fish, *G. affinis*, the association time-course was faster than a system governed by a bimolecular k_{on} of $10^7 \text{ s}^{-1} \text{ M}^{-1}$. Curiously, the association rate observed for the garter snake, *T. sirtalis*, was very well resolved since it was slower than that of any of the other species (data not shown), corresponding to a k_{on} of $3.2 \pm 0.2 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$. If we use a one-site equilibrium to approximate the behaviour of these systems, and consider only the major slow component of the dissociation time-course, then the ratios of k_{off} to k_{on} for the two snake species and the fish species yield the following effective K_D estimates: 6 pM, *N. n. kaouthia*; 40 pM, *T. sirtalis*; and 0.6 pM, *G. affinis*. Surprisingly, the approximate K_D values for

the five test species investigated most thoroughly in this study are all substantially lower than the K_D of ca. 200 pM previously measured for bullfrog saxiphilin.

With respect to the pH dependence discussed above, STX binding to bullfrog saxiphilin has also been shown to undergo allosteric modulation by H^+ as manifested by an increased rate of ligand dissociation at low pH (Llewellyn & Moczydlowski 1994). To investigate whether this functional property is conserved, we have also measured the dissociation time-course at pH 4.3–4.5 for three species. As shown in figure 4a–c, samples from *B. marinus*, *N. n. kaouthia*, and *G. affinis* all exhibited a substantially faster dissociation time-course at the lower pH. This effect is equivalent to a 3.6-fold, 5.6-fold and 9.1-fold enhancement of the slowest component of the dissociation reaction for *G. affinis*, *B. marinus* and *N. n. kaouthia*, respectively.

4. DISCUSSION

(a) Saxiphilin has an ancient origin in animal evolution

The properties of soluble [3H]STX binding activity characterized for the cane toad (*B. marinus*), garter snake (*T. sirtalis*), Thailand cobra (*N. n. kaouthia*), mosquito fish (*G. affinis*), and centipede (*E. rubripes*), leave little doubt that all five of these species contain a protein homologous to bullfrog saxiphilin. Although there are some subtle differences in binding kinetics, pH dependence, and structure–activity relationships of STX derivatives among the five species, these parameters have the characteristic biochemical and pharmacological signature of the purified saxiphilin protein from *Rana catesbeiana* (Mahar *et al.* 1991; Llewellyn & Moczydlowski 1994). The results thus imply that an STX binding site like that present in bullfrog saxiphilin is conserved in phylogenetic groups as diverse as arthropods and reptiles. This leads us to conclude that a gene coding for the saxiphilin protein is present and functionally active in both the arthropod and chordate phyla.

Of the major vertebrate classes, saxiphilin activity is readily detected in numerous fish, amphibians, and reptiles. The biogeographic distribution of animals found to express saxiphilin includes representatives from diverse habitats in Asia (e.g. *N. n. kaouthia*), Australia (e.g. *V. rosenbergii*), Europe (e.g. *R. temporaria*), Africa (e.g. *P. aethiopicus*), and the Americas (*R. sylvatica*), indicating that this phenotypic characteristic is not confined to a particular climatic zone. The probable existence of saxiphilin in a myriapod (centipede, *E. rubripes*), an isopod (sowbug, *Oniscus* sp.), an arachnid (orb web spider, *Araneus* c. f. *cavaticus*), crustaceans (water-flea, *Daphnia*; xanthid crabs, Llewellyn (1997)) and some insects (cockroach, damselfly nymph) further suggests that it may have first appeared during the emergence of invertebrates some 800 million years ago. Evolutionary speculations on its origin may be considered in light of the fact that bilobed, Fe^{3+} -binding, transferrin-like proteins are also present in insects (Bartfeld & Law 1990; Jamroz *et al.*

1993; Kurama *et al.* 1995). The occurrence of saxiphilin in arthropods is consistent with the possibility that it arose directly from an ancestral bilobed transferrin precursor and later acquired an insertion of 144 residues that is present in bullfrog saxiphilin (Morabito & Moczydlowski 1994; Morabito *et al.* 1995). Alternatively, it is possible that bilobed transferrin and saxiphilin both arose independently by gene duplication from a single-lobed, ca. 40 kDa transferrin precursor, such as that previously described in the urochordate ascidian, *Pyura stolonifera* (Martin *et al.* 1984).

The most striking finding of our characterization of [3H]STX binding activity from evolutionarily diverse species is the extraordinarily high affinity of the soluble STX binding site in some animals. While K_D 's in the range of 10^{-12} M are not uncommon for bioactive peptides, binding affinity in this range is relatively rare for small organic molecules; e.g. one of the strongest known interactions is that of biotin-avidin, K_D ca. 10^{-15} M (Gitlin *et al.* 1987). The binding energy for such protein–ligand interactions is generally derived from multiple non-covalent interactions between the functional groups of protein residues and atoms of ligand substituent groups. The STX molecule, with its divalent positive charge and six guanidino nitrogen atoms, offers a rigid scaffold with the potential ability to form multiple electrostatic and hydrogen-bonding interactions within a protein binding site. Another factor that may contribute to this picomolar affinity is the mechanical ability of transferrin proteins such as lactoferrin to capture their ligands (i.e. Fe^{3+}/HCO_3^-) within a cavity that closes, in the bound state, like a hinged jaw (Anderson *et al.* 1990), thus utilizing a protein conformational change to stabilize the bound ligand. Whatever the mechanism, the chemical specificity and evolutionary conservation of the STX–saxiphilin interaction makes it difficult to argue that this association is a completely fortuitous molecular affiliation unrelated to biological function. In this regard, it is evident that the STX–saxiphilin interaction in some species may be at least three orders of magnitude stronger than the typical nanomolar affinity of STX binding to the Na^+ channel (Ritchie & Rogart 1977), the site that mediates biological toxicity of STX.

(b) Constitutive expression of saxiphilin is exhibited by certain ectothermic animal species

The inference that a saxiphilin gene is widely distributed in the arthropod and vertebrate genomes leads to a series of questions raised by the negative observations of table 2, which imply the apparent absence of saxiphilin in sister taxa and possibly in whole classes of the vertebrate subphylum. For example, among the anuran subclass of Amphibia, why is saxiphilin readily detected in particular frogs and toads (e.g. *Rana catesbeiana*, *R. sylvatica*, *R. temporaria*, *Bufo marinus*), but not in the African frog (*Xenopus laevis*)? Similarly, among the class Reptilia, why is saxiphilin present in the subclass (Lepidosauria) containing the orders of lizards and snakes, but apparently absent in both the subclass (Archosauria)

that includes alligators and crocodiles and in the subclass (Anapsida) that includes turtles? One explanation may be that saxiphilin is a non-essential protein or that its function is readily served by other proteins. Alternatively, the pattern of disparate expression could indicate selective loss of a once functional gene. Another possible interpretation of the data of tables 1 and 2 is that saxiphilin expression is a character specific to certain ectothermic animals. One might speculate that the saxiphilin gene is preferentially utilized by cold-blooded animals that do not have metabolic control of their body temperature and was deactivated or lost in the evolutionary lineages leading to birds and to mammals.

Along these lines, it may be noted that the highest level of saxiphilin-like activity was observed in the wood frog, *Rana sylvatica* (having a mean activity of 1600 pmol ml⁻¹ plasma). This small frog species has the northernmost habitat range in North America and has developed special mechanisms to survive prolonged periods hibernating under a superficial layer of ground cover in a frozen state during the winter (Storey 1990). Although we certainly have not surveyed a sufficient number of species to permit a general conclusion, the present data lead us to wonder whether there may be a functional correlation between high levels of saxiphilin activity and particular species of ectothermic animals that tolerate a wide variation in body temperature.

On the other hand, an apparent lack of saxiphilin activity as measured by the present methodology does not necessarily prove that the saxiphilin gene is absent in the negative species of table 2. Indeed, it is possible that a functional gene is present and that we have failed to detect soluble [³H]STX activity for a number of reasons. For example, our survey results may reflect the underlying biochemical pharmacology of the STX-binding site. If, for instance, there existed an endogenous ligand that binds to saxiphilin in a competitive manner with STX, the failure to detect [³H]STX binding in a crude extract could be due to pre-existing saturation of the STX binding site with such a ligand. Alternatively, it may be that transcription of the gene is inducible and turned off in most animals under normal physiological conditions. Other possibilities are that saxiphilin is present in amounts below the detection limit of our assay (ca. 0.1 pM ml⁻¹), or that it is expressed only in certain tissues or cell types that are not well sampled by our typical assay of plasma or whole animal extracts.

Previous work showed that saxiphilin mRNA is transcribed in bullfrog liver and several other tissues (Morabito & Moczydlowski 1994). However, from the present results, saxiphilin does not appear to be a ubiquitous component of animal plasma-like serum transferrin in the vertebrates, which is found at a relatively constant concentration of approximately 2.5 mg ml⁻¹ (ca. 30 µM) in all adult specimens of vertebrate species that have been examined, excepting humans with the debilitating mutation of atransferrinanaemia (Welch 1990, 1992). Saxiphilin expression in some animals may be more like that of the locally expressed transferrin homologues, melanotransferrin

or lactoferrin, in vertebrates. The expression and secretion of these latter proteins in humans is mostly limited to the surfaces of melanoma cells and several other cell types in the case of melanotransferrin (Rose *et al.* 1986; Kennard *et al.* 1995); or to neutrophils and particular fluid secretions such as milk in the case of lactoferrin (Lönnerdal & Iyer 1995). The curious pattern of saxiphilin expression reflected in our results merits further investigation to determine what transcriptional and translational regulatory mechanisms may control its synthesis.

(c) *What is the biological function of saxiphilin?*

The simplest hypothesis for the function of saxiphilin is that it plays a defensive role against STX intoxication. However, this idea must be questioned since the most dramatic example of microbial STX production in nature occurs in conjunction with sporadic blooms of toxic marine dinoflagellates, and we did not find a consistent association of saxiphilin with species that may directly or indirectly be exposed to dinoflagellate toxins. In particular, molluscs such as *Mytilus edulis* and *Saxidomus giganteus* are known to bioaccumulate various STX derivatives (Shimizu *et al.* 1978; Schantz 1986), but do not exhibit evidence of saxiphilin-like activity. The Atlantic mackerel, *Scomber scombrus*, has been found to contain STX in its viscera, and such mackerel have previously been linked to the deaths of humpback whales (Clifford *et al.* 1993; Geraci *et al.* 1989). However, neither plasma from mackerel nor two whale species contained detectable saxiphilin activity (table 2). Similarly, most of the other marine invertebrates, fish, and mammals that we sampled (albeit a limited selection) tested negative for saxiphilin-like activity. Two interesting exceptions are high levels of saxiphilin activity found in the eel, *Anguilla rostrata* (table 1), which can adapt from saltwater to freshwater, and the finding of saxiphilin-like activity in certain species of marine xanthid crabs from Australia (Llewellyn 1997). Aside from these latter two possible cases, the present data do not indicate that saxiphilin plays a universal or even a common role in animal encounters with toxic marine dinoflagellates.

In the freshwater ecosystem, STX and various STX derivatives have so far been found to be produced by three genera of cyanobacteria: *Aphanizomenon*, *Anabaena* and *Lyngbya* (Ikawa *et al.* 1982; Carmichael *et al.* 1990; Negri *et al.* 1995; Carmichael *et al.* 1995). The occurrence of saxiphilin in many amphibians and small freshwater fish suggests that it may directly function or may be secondarily recruited as a mechanism of STX detoxification in some of these species.

In summary, this study has determined that soluble [³H]STX binding activity characteristic of saxiphilin has a broad phylogenetic distribution among arthropods and vertebrates whose nervous systems rely on STX-sensitive Na⁺ channels that function prominently in electrical excitability. The curious existence of the two distinct high-affinity binding sites for STX in many organisms raises the suspicion of a possible physiological relationship. Many questions regarding

the function, expression and molecular evolution of saxiphilin as a transferrin-related protein remain to be resolved. Further exploration of the evolutionary and biological significance of saxiphilin may unravel this biological mystery.

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