Sodium channel mutation leading to saxitoxin resistance in clams increases risk of PSP

V. Monica Bricelj, Laurie Connell, Keichi Konoki, Scott P. MacQuarrie, Todd Scheuer, William A. Catterall & Vera L. Trainer

Institute of Marine Biosciences, National Research Council, Halifax, Nova Scotia B3H 3Z1, Canada
School of Marine Sciences, University of Maine, Orono, Maine 04469, USA
Department of Pharmacology, University of Washington, Seattle, Washington 98195, USA
NOAA Fisheries, Northwest Fisheries Science Center, Seattle, Washington 98112, USA

Bivalve molluscs, the primary vectors of paralytic shellfish poisoning (PSP) in humans, show marked inter-species variation in their capacity to accumulate PSP toxins (PSTs) which has a neural basis. PSTs cause human fatalities by blocking sodium conductance in nerve fibres. Here we identify a molecular basis for inter-population variation in PSP resistance within species, consistent with genetic adaptation to PSTs. Softshell clams (Mya arenaria) from areas exposed to red tides are more resistant to PSTs, as demonstrated by whole-nerve assays, and accumulate toxins at greater rates than sensitive clams from unexposed areas. PSTs lead to selective mortality of sensitive clams. Resistance is caused by natural mutation of a single amino acid residue, which causes a 1,000-fold decrease in affinity at the saxitoxin-binding site in the sodium channel pore of resistant, but not sensitive, clams. Thus PSTs might act as potent natural selection agents, leading to greater toxin resistance in clam populations and increased risk of PSP in humans. Furthermore, global expansion of PSP to previously unaffected coastal areas might result in long-term changes to communities and ecosystems.

PSP, caused by human consumption of shellfish that feed on toxic algae, is a public health hazard and causes severe economic losses globally due to bans on harvesting of contaminated shellfish and the need for costly monitoring programmes. PSP-producing dinoflagellates (for example Alexandrium spp.) cause toxic blooms (‘red tides’) in North America and worldwide. PSTs block conduction of the nerve impulse by interfering with the voltage-dependent increases in sodium-ion conductance that generate the action potential in nerve and muscle fibres, leading to neuromuscular paralysis. Large differences in PST accumulation between bivalve species in vivo have been associated with in vitro differences in sensitivity of isolated nerves to saxitoxin (STX), the most potent PSP, and the related tetrodotoxin (TTX). STX and TTX bind to a single site in the outer pore of the Na\(^{+}\) channel, formed by the amino-acid residues in the outer-pore loops located between the S5 and S6 segments of each of the four homologous domains (I–IV) of the \(\alpha\)-subunit. Could differences in amino acid sequence in the receptor site for STX and TTX in Na\(^{+}\) channels cause differences

**Figure 1** Responses to PSTs in two M. arenaria populations. **a**, Percentage of clams that burrowed after 24 h of exposure to A. tamarense (strain PR18b or CCMP115) or I. galbana (T-iso) (\(n = 2\) tanks). **b**, Mortality after 16 days of toxification with strain PR18b (determined by visual inspection (dead clams removed) (tank 1) and by removal of all clams every 2 days (live clams reintroduced in sediment) (tank 2)). **c**, Tissue toxicity of live clams after 16 days of toxification of resistant BF and sensitive LE clams (burrowers and non-burrowers at 24-h, respectively). All results are means ± s.e.m.
letters to nature

in toxin sensitivity and accumulation in shellfish and thereby contribute to the risk of PSP? We have probed this question with a combination of behavioural, physiological and molecular biological approaches.

*Mya arenaria* is a commercially important native species with wide latitudinal distribution in Atlantic North America, from the Gulf of St Lawrence to Chesapeake Bay. We collected clams from two sites with contrasting histories of PSP (Fig. 1a inset): Lepreau Basin, Bay of Fundy (BF), where annual, recurrent toxic blooms of *Alexandrium* spp. occur in summer, and the Lawrencetown estuary (LE), Nova Scotia, an area with no record of PSP. Burrowing activity provided an index of susceptibility to toxins in clams. After exposure to toxic *A. tamarense* cells for 24 h, most clams (a mean of 86%) from LE were unable to re-burrow after deployment at the sediment surface, whereas only 10% of clams from the BF population were compromised (Fig. 1a). Burrowing was not affected by exposure to non-toxic *Isochrysis galbana* (T-Iso) or to *A. tamarense* strain CCMP115, which is of negligible toxicity. Therefore burrowing incapacity in susceptible clams is toxin-induced and is attributed to muscle paralysis. PSTs also prevented siphon retraction in susceptible clams. Sublethal effects of PSTs on burrowing and siphon retraction in a high-energy, intertidal habitat, where clams can become exposed at the sediment surface, may cause indirect mortalities through desiccation and predation.

Longer-term laboratory exposure to toxic *A. tamarense* cells resulted in high differential mortalities between the two populations (Fig. 1b). Mortalities of LE clams were consistently higher (26–42% after 16 days of toxin exposure) than those of BF clams held under identical conditions (2% or less). LE clams also showed significantly lower feeding rates on toxic cells and decreased metabolic rates during toxification. This individual variation in fitness-related traits provides a basis for natural selection.

To compare their toxin accumulation capacity, dominant phenotypes from the two populations were exposed to toxic *A. tamarense* for 16 days. Resistant BF clams (burrowers after 24 h of toxification) attained a mean toxicity significantly (fivefold) higher than sensitive LE clams (non-burrowers) (Fig. 1c, equal to a toxin concentration of 657 and 112 nmol g$^{-1}$ respectively). Toxicities in both groups exceeded the regulatory safety level (80 µg STX equivalents per 100 g) and increased linearly at rates of 771 ($r^2=0.96$) and 113 µg STX equivalents per day ($r^2=0.80$) in resistant and sensitive clams, respectively (data not shown), resulting in more than an order of magnitude difference in toxicity among individuals after 2 weeks. *M. arenaria* field populations can attain toxicities (∼9,600 µg STX equivalents per 100 g) comparable to those determined in this study and accumulate even higher toxin levels in the viscera.

*In vitro* exposure of isolated cerebrocervical nerve trunks of naive (toxin-free) clams from BF and LE to serially increasing STX concentrations also revealed large (more than 100-fold) differences in sensitivity to STX. Two clams from each population were exposed to STX concentrations of 2, 3 and 4 STX equivalents per 100 g STX equivalents per day ( respectively) and the Lawrencetown estuary (LE), Nova Scotia, an area with no record of PSP. Burrowing activity provided an index of susceptibility to toxins in clams. After exposure to toxic *A. tamarense* cells for 24 h, most clams (a mean of 86%) from LE were unable to re-burrow after deployment at the sediment surface, whereas only 10% of clams from the BF population were compromised (Fig. 1a). Burrowing was not affected by exposure to non-toxic *Isochrysis galbana* (T-Iso) or to *A. tamarense* strain CCMP115, which is of negligible toxicity. Therefore burrowing incapacity in susceptible clams is toxin-induced and is attributed to muscle paralysis. PSTs also prevented siphon retraction in susceptible clams. Sublethal effects of PSTs on burrowing and siphon retraction in a high-energy, intertidal habitat, where clams can become exposed at the sediment surface, may cause indirect mortalities through desiccation and predation.

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*In vitro* exposure of isolated cerebrocervical nerve trunks of naive (toxin-free) clams from BF and LE to serially increasing STX concentrations also revealed large (more than 100-fold) differences in sensitivity to STX between and within populations (Fig. 2a; compare results at 4 and 400 µM STX). Nerves from most clams in the LE population with no history of PSP (69%) experienced full block of the action potential at 33 µM STX, whereas only 10% of BF clams held under identical conditions (2% or less) achieved full block of the action potential at 33 µM STX. The half-maximal inhibitory concentration (IC$_{50}$) for WT was 11.2 ± 1.5 µM. In contrast, 1,000-fold more TTX (10 µM) blocked only ∼20% of the current through *E. coli* channels (Fig. 4a, bottom) and the IC$_{50}$ increased about
3,000-fold to 35 ± 9 μM (Fig. 4c). Because STX is more potent, an order of magnitude less toxin was required to obtain a similar blocking effect. STX at 1 nM blocked 40% of WT current, and 10 nM STX blocked it nearly completely (IC\(_{50}\) = 1.7 ± 0.2 nM). In contrast, a 1,000-fold higher STX concentration (1 μM) blocked only 25% of the E945D current (IC\(_{50}\) < 2.7 ± 0.6 μM; Fig. 4d). Thus, the E945D substitution made the Nav1.2a channel about 1,500-fold or 3,000-fold less sensitive to STX and TTX, respectively, and a similar decrease in affinity is expected in the \(M.\) arenaria Na\(^+\) channel (see Supplementary Discussion). This is a surprisingly large reduction in binding affinity resulting from simply shortening of the side chain at position 945 by one methylene group without changing the negative charge of the carboxyl group. These results imply that this side chain is rigid in the outer vestibule of the channel and emphasize that even a small conformational change in the receptor site can result in a large decrease in the binding affinity of these toxins to Na\(^+\) channels by altering the spatial relationships between ion-pair-forming and hydrogen-bond-forming partners.

Our results show that marked differences in whole-animal and...
nerve susceptibility to PSTs and in toxin uptake capacity in *M. arenaria* result from a single mutation in domain II of the Na⁺ channel pore region. These observed differences correspond to inter-population differences in the history of exposure to toxic blooms in the environment. The adaptive value of resistance to PSTs (increased survival) was also shown experimentally. Our results support the conclusion that *M. arenaria* populations in PSP-affected areas undergo genetic adaptation to toxins through selective mortality or reduced fitness of sensitive individuals. In this respect *M. arenaria* differs from Pacific butter clams, *Saxidomus giganteus*, in which PSTs function as an anti-predator defence.

A neural basis for inherited toxin resistance was shown in cattle ticks and house flies, in which a single Na⁺ channel mutation confers resistance to pesticides (pyrethroids and DDT)26–28. Resistance to TTX in pulifer fish *Taikifugu (Fugu) pardalis*, which can accumulate high concentrations of TTX in tissue without adverse effects, has been attributed to a single mutation in the skeletal-muscle Na⁺ channel29,30, yet natural selection for resistance was not shown in this species. In terrestrial ecosystems, marked geographic variation in resistance to TTX in populations of garter snakes (*Thamnophis sirtalis*) has coevolved with that of its prey23 (the toxic newt, *Taricha* sp.). Thus, different selective pressures (predation or toxin blooms) can lead to geographic differentiation in resistance to natural toxins within species. The increased accumulation of toxin in resistant *M. arenaria* points to this resistance mutation as an important risk factor for human PSP resulting from the consumption of this species. Our findings raise the possibility that other bivalve species might harbour similar mutations, thus allowing further understanding of the molecular basis of toxin resistance across shellfish species.

**Methods**

**Whole-animal observations**

Juvenile clams (~30–47 mm shell height), collected at a time of year when they contained no PSTs, were acclimated (16°C, 30% salinity) for at least 2–3 weeks before experiments. Clams were toxified, as described previously31, in aquaria containing coarse sand 10 cm deep, at a constant (100 cells ml⁻¹) bloom concentration of *A. tamarense* (PIR18b from the Gulf of S. Lawrence; toxicity 60–90 pg STX equivalents per cell), maintained by cell delivery with a peristaltic pump32. Burrowing response was measured with previously described protocols19 after exposure of clams to equal biovolume concentrations of either toxin or toxin-free controls. For the measurement of survival, 40–50 clams from each population (BF and LE) were held together in each of two experimental tanks exposed to whole-cell toxin clamp as described previously20. The extracellular solution contained (in mM) 140 NaCl, 5.4 CaCl₂, 1 MgCl₂, 10 Hepes (pH 7.35) at ~23°C. The intracellular saline contained (in mM) 189 N-methyl-D-glucamine (NMDG), 1 NaCl, 4 MgCl₂, 10 Hepes, 8 bic(2-aminophosphonic acid)-N,N,N',N'-tetraacetic acid (BAPTA), 25 Tris-phosphocreatine, 2 NaATP, 0.2 NaGTP and 40 Hepes, pH 7.35. TTX was obtained from Calbiochem, and STX standard (480 μM STX in 0.1 M acetic acid) was provided by CRMPL.

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**Electrophysiology of transfected Na⁺ channels**

Human taf 201 cells were transfected with cDNAs encoding WT or E945D mutant Nav1.2a channels by calcium phosphate precipitation and Na⁺ currents were recorded by whole-cell voltage clamp as described previously20. The extracellular solution contained (in mM) 140 NaCl, 5.4 CaCl₂, 1 MgCl₂, 10 Hepes (pH 7.35) at ~23°C. The intracellular saline contained (in mM) 189 N-methyl-D-glucamine (NMDG), 1 NaCl, 4 MgCl₂, 10 Hepes, 8 bic(2-aminophosphonic acid)-N,N,N',N'-tetraacetic acid (BAPTA), 25 Tris-phosphocreatine, 2 NaATP, 0.2 NaGTP and 40 Hepes, pH 7.35. TTX was obtained from Calbiochem, and STX standard (480 μM STX in 0.1 M acetic acid) was provided by CRMPL.
also of *Listeria monocytogenes* and switches a cell death pathway of the infected macrophages from necrosis to apoptosis. Our data indicate that the *Ipr1* gene product might have a previously undocumented function in integrating signals generated by intracellular pathogens with mechanisms controlling innate immunity, cell death and pathogenesis.

It is estimated that about one-third of the human population on the planet has been infected by virulent *M. tuberculosis*\(^1\). Susceptibility to clinical tuberculosis is known to be influenced by environmental factors such as stress, malnutrition, concomitant infections (for example HIV) or senescence\(^2,3\). Although genetic variation within host populations is also known to affect resistance and susceptibility, individual genes responsible for innate immunity to the pathogen have been elusive. In susceptible individuals, progression of lung tuberculosis often leads to the formation of characteristic necrotic ‘cavities’ that destroy significant portions of the lung. Beyond their life-threatening clinical consequences, these lesions are essential for the efficient transmission of *M. tuberculosis* in aerosols. Because tuberculosis in humans is transmitted primarily by the respiratory route, the ability to cause lung disease is considered a key aspect of the pathogen’s virulence strategy and ensures its evolutionary success. Therefore, understanding pathogenic mechanisms that are employed by virulent *M. tuberculosis* during lung tuberculosis in susceptible individuals is essential for developing effective prevention and treatment strategies\(^4,5\). However, detailed mechanistic studies of pathogenesis of lung tuberculosis and its genetic control have been limited by the fact that in mouse models of *M. tuberculosis* infection, necrotic lesions in the lungs are rarely found unless the mouse is rendered systemically immunodeficient.

C3HeB/FeJ inbred mice are extremely susceptible to virulent *M. tuberculosis* and develop a marked lung pathology, which leads to their rapid death after infection\(^6,11\). We generated a congenic mouse strain C3H.B6-sst1 (sst1\(^6\)) carrying the C57BL/6J-derived resistant allele at the *sst1* locus on the C3HeB/FeJ genetic background. The survival time of the sst1\(^6\) congenic mouse infected either with a high dose of intravenous *M. tuberculosis* (Fig. 1a) or with a low dose of *M. tuberculosis* by the respiratory route (Fig. 1b), relative to their sst1\(^5\) counterparts, is significantly lengthened, indicating a profound effect of the locus on anti-tuberculosis immunity. However, the shorter survival of the C3H.B6-sst1 (sst1\(^5\)) mice, in comparison with the resistant parental strain C57BL/6J (B6), indicates that the sst1\(^5\) locus is responsible for a significant portion, but not all, of the tuberculosis resistance phenotype of the B6 mice.

The specific effect of the sst1\(^5\) locus on the progression of tuberculosis was related to a more efficient control of *M. tuberculosis* multiplication, primarily in the lungs, after both respiratory challenge by aerosol (Fig. 1c) and systemic intravenous infection (Supplementary Fig. 2a). The development of large necrotic lung lesions within 4 weeks after intravenous infection, characteristic of sst1\(^5\) mice, was prevented in the presence of the sst1\(^5\) allele (Fig. 1d). After a low-dose aerosol infection, chronic tuberculosis infection ensued, and the sst1\(^5\) mice developed encapsulated necrotic lung lesions, in some cases reaching about one-third of the lung lobe (Fig. 1e), that resembled tuberculosis cavities in human lungs. Mycobacteria were present both extracellularly, within necrotic central areas surrounded by the fibrotic capsule, and within macrophages of the granuloma wall (Supplementary Fig. 1). In the sst1\(^6\) mice, lung lesions were much smaller and contained fewer infected macrophages.

Although the greatest effect of the sst1 polymorphism on the progression of tuberculosis was observed in the lungs, bone marrow transplantation experiments showed that bone marrow-derived cells, but not lung cells, were responsible for the effect of the sst1 locus (Supplementary Fig. 2b). It is known that T lymphocytes and macrophages are of major importance in host resistance to tuberculosis. We have found that, whereas T lymphocytes are functionally

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**Ipr1** gene mediates innate immunity to tuberculosis

**Hui Pan**\(^*\), Bo-Shiun Yan\(^*\), Mauricio Rojas\(^1,3\), Yuriy V. Shehzukhov\(^1,4\), Hongwei Zhou\(^1\), Lester Kolzik\(^1\), Darren E. Higgins\(^1\), Mark J. Daly\(^1\), Barry R. Bloom\(^1\) & Igor Kramnik\(^1\)

1 Department of Immunology and Infectious Diseases and
2 Physiology Program, Department of Environmental Health, Harvard School of Public Health, 667 Huntington Avenue, Boston, Massachusetts 02115, USA
3 Grupo de Inmunología Celular e Inmunogeética, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia
4 Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA
5 Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, USA

* These authors contributed equally to this work
† Present address: Department of Molecular Immunology, A. N. Belozersky Institute of Physical and Chemical Biology, Moscow State University, Vorobjovy Gory, Moscow, 119899, Russia

An estimated eight million people are infected each year with the pathogen *Mycobacterium tuberculosis*, and more than two million die annually\(^5\). Yet only about 10% of those infected develop tuberculosis. Genetic variation within host populations is known to be significant in humans and animals\(^6,7\), but the nature of genetic control of host resistance to tuberculosis remains poorly understood. Previously we mapped a new genetic locus on mouse chromosome 1, designated *sst1* (for susceptibility to tuberculosis)\(^1\). Here we show that this locus mediates innate immunity in *sst1* congenic mouse strains and identify a candidate gene, Intracellular pathogen resistance 1 (*Ipr1*), within the *sst1* locus. The *Ipr1* gene is upregulated in the *sst1* resistant macrophages after activation and infection, but it is not expressed in the *sst1* susceptible macrophages. Expression of the *Ipr1* transgene in the *sst1* susceptible macrophages limits the multiplication not only of *M. tuberculosis* but