Cyclic imine toxins from dinoflagellates: a growing family of potent antagonists of the nicotinic acetylcholine receptors

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Abstract

We present an overview of the toxicological profile of the fast-acting, lipophilic macrocyclic imine toxins, an emerging family of organic compounds associated with algal blooms, shellfish contamination and neurotoxicity. Worldwide, shellfish contamination incidents are expanding; therefore, the significance of these toxins for the shellfish food industry deserves further study. Emphasis is directed to the dinoflagellate species involved in their production, their chemical structures, and their specific mode of interaction with their principal natural molecular targets, the nicotinic acetylcholine receptors, or with the soluble acetylcholine-binding protein, used as a surrogate receptor model. The dinoflagellates Karenia selliformis and Alexandrium ostenfeldii / A. peruvianum have been implicated in the biosynthesis of gymnodimines and spirolides, while Vulcanodinium rugosum is the producer of pinnatoxins and portimine. The cyclic imine toxins are characterized by a macrocyclic skeleton comprising 14–27 carbon atoms, flanked by two conserved moieties, the cyclic imine and the spiroketal ring system. These phycotoxins generally display high affinity and broad specificity for the muscle type and neuronal nicotinic acetylcholine receptors, a feature consistent with their binding site at the receptor subunit interfaces, composed of residues highly conserved among all nAChRs, and explaining the diverse toxicity among animal species.

Keywords: acetylcholine-binding protein, dinoflagellates, gymnodimines, marine phycotoxins, muscarinic acetylcholine receptor, nicotinic acetylcholine receptor, pinnatoxins, spirolides.


This is an article for the special issue XVth International Symposium on Cholinergic Mechanisms.
The cyclic imine toxins comprise a growing family of lipophilic organic compounds produced by some species of marine dinoflagellate microorganisms. These neurotoxins have been found in extracts from contaminated shellfish (mainly bivalves), from natural plankton assemblies, from clonal cultures of toxic dinoflagellates, and as resulting products of the shellfish metabolism (fatty acid acyl esters) (for reviews, see Molgó et al. 2007; Guéret and Brimble 2010; Stivala et al. 2015). Currently, the cyclic imine family of toxins is exemplified by 40 molecules (not taking into account the acylated esters) and comprises the prorocentrolides, spiro-prorocentrimine, gymnodimines, spirolikes, pinnatoxins, pteriatoxins, and portimine (Stivala et al. 2015). Spirolikes represent the largest family.

Cyclic imine toxins can accumulate in bivalve mollusks and are considered as emergent toxicants affecting food safety in the shellfish food industry. Although their potent neurotoxicity led concern on their potential risks to shellfish consumers, currently these toxins are not regulated.

This short review aims at providing an overview of the origin, toxicological profile, chemical structure, and mode of action of the lipophilic cyclic imine toxins, with particular emphasis on their specificity of interaction with muscle-type and neuronal nicotinic acetylcholine (ACh) receptors (nAChR), which are the main molecular targets involved in their toxicity. The nAChRs are prototypical cation-selective, ligand-gated ion channels that mediate fast neurotransmission in the central and peripheral nervous systems (reviewed by Albuquerque et al. 2000; Tsetlin et al. 2015). They belong to the Cys-loop superfamily of ligand-gated ion channels, and are formed by distinct combinations of five subunits that confer selectivity in pharmacological properties and cellular location (Corringer et al. 2000; Tsetlin et al. 2011).

Dinoflagellate species involved in the production of cyclic imine toxins

Several species of dinoflagellates, distributed worldwide in tropical, temperate, and cold marine waters, have been confirmed to be responsible for the production of cyclic imine toxins. Among the Prorocentrum species, P. lima, and P. maculosum have been linked to the production of prorocentrolides A and B and spiro-prorocentrimine (Torigoe et al. 1988; Hu et al. 1996b; Lu et al. 2001).

Gymnodimines A, B, and C have been shown to be produced by a species of dinoflagellates named Karenia selliformis (Miles et al. 2000, 2003; Haywood et al. 2004). However, other gymnodimine analogs like 12-methyl gymnadinone A and 12-methyl gymnadinone B are produced by the dinoflagellate Alexandrium peruvianum and A. ostenfeldii (Van Wagoner et al. 2011; Van de Waal et al. 2015; Strangman et al. 2016). Also, a new gymnodimine D was found in A. ostenfeldii clonal cultures isolated from the Northern Baltic Sea (Harju et al. 2016). The production of these various gymnodimine analogs indicates that common biosynthetic pathways are shared by distinct species of dinoflagellates.

The toxigenic dinoflagellate A. ostenfeldii and the closely related A. peruvianum have been reported to produce the large family of spirolikes (Cembella et al. 1999, 2000; Touzet et al. 2008; Borkman et al. 2012). However, the profile of toxin production seems to differ with environmental conditions, genetic factors, and the location where the dinoflagellates are collected (Otero et al. 2010; Gu 2011; Suikkanen et al. 2013; Almandoz et al. 2014; Kremp et al. 2014; Salgado et al. 2015). Alexandrium ostenfeldii/A. peruvianum appear capable of producing saxitoxin and analogs (Anderson et al. 2012; Hakanen et al. 2012; Van de Waal et al. 2015; Kremp et al. 2016; Savela et al. 2016) that bind and block voltage-gated sodium channels (Catterall 1980), yet the cyclic imine toxins appear as the primary toxic agent in most of the world regions. During blooms of A. ostenfeldii/A. peruvianum, morphological uniqueness of the dinoflagellates can no longer be used as the indicator of the presumed toxin produced, and should always be accompanied by the chemical characterization of the toxin profile to identify the type(s) of toxins.

Indistinguishable peridinoid dinoflagellate strains were reported to be responsible for the production of pinnatoxins E, F, and G in Australia, pinnatoxin G in Japan, and pinnatoxins E and F in New Zealand (Rhodes et al. 2010, 2011a,b; Smith et al. 2011). Then, identification of the dinoflagellate producers of pinnatoxins was associated with the discovery, in coastal waters of France, of a novel dinoflagellate species, Vulcanodinium rugosum (Nézan and Chomérat 2011), that produced mainly pinnatoxin G (Hess et al. 2013). The smallest cyclic imine toxin so far isolated from V. rugosum was named portimine (Fig. 1), which is distinguished by a five-membered cyclic imine ring and a macrocycle of only 14 carbon atoms (Selwood et al. 2013), while pinnatoxins contain a seven-membered cyclic imine ring. In contrast to other cyclic imine toxins, portimine exhibits very low acute toxicity in the mouse, but it is extremely toxic to human Jurkat T-lymphoma cells and mouse embryonic fibroblasts cells in culture. Cells treated with portimine display rapid caspase activation and phosphatidylserine exposure, suggestive of apoptotic cell death (Cuddihy et al. 2016). To the best of our knowledge, none of the known cyclic imine toxins has been reported to activate caspase-3 and initiate apoptosis, as portimine does. Among toxins produced by the dinoflagellate V. rugosum, neither pinnatoxin F nor pinnatoxin G were reported to induce apoptosis (Cuddihy et al. 2016).

Another pinnatoxin congener (pinnatoxin H) was purified from cultures of V. rugosum collected from the South China Sea (Selwood et al. 2014) and in the marine waters of Qatar, Arabian Gulf (Al Muftah et al. 2016). Vulcanodinium rugosum strains of various origins have been
reported to produce not only different pinnatoxins, but also
to differ from each other in partial large subunit rDNA,
internal transcribed spacer regions, and 5.8S rDNA
sequences (Rhodes et al. 2011b), suggesting a complex
variation in splicing options. At present, no definitive
evidence has been published regarding a dinoflagellate
origin of pteriatoxins.

Chemical structure
The general chemical structure of cyclic imine toxins
encompasses a macrocyclic ring of 14–27 carbon atoms,
and two highly conserved moieties: the cyclic imine group
(mainly found as a spiroimine) and the spiroketal ring
system. The cyclic imines are composed of a 5-membered
(portimine), 6-membered (gymnodimines, spiroprorocentrimine, prorocentrolides), or 7-membered rings (spiro-
lides, pinnatoxins, pteriatoxins); all are considered as
essential components for bio-activity. Amino-ketone
derivatives with an open imine ring, for example, spirolides
E and F (Hu et al. 1996a) or the amino-ketone form of
pinnatoxin A (Aráoz et al. 2011; Bourne et al. 2015) are
devoid of biological activity. The other features of the ring
system can be a simple tetrahydrofuran (in portimine and
gymnodimines) or a tetrahydropyran group (prorocentrolides
and spiroprorocentrimine), but also more complex 6,5-
(spirolides H and I), 6,6,5- (spirolide G), 6,5,5- (spirolides
A–F), or 6,5,6-spiroketal moieties (pinnatoxins and pteri-
atoxins), as shown in Fig. 1.

Several approaches for the synthesis of cyclic imine toxins
were reported during the last few years (Beaumont et al.
2010; for reviews, see Molgó et al. 2014; Stívala et al.
2015), which were focused mainly on pinnatoxin A
(McCauley et al. 1998; Sakamoto et al. 2004; Nakamura
et al. 2008; Stívala and Zakarian 2008; Aráoz et al. 2011),
pinnatoxins B and C (Matsura et al. 2006a), pteriatoxins
(Matsura et al. 2006b), and gymnodimine (Kong et al.
2009, 2011). A fragment of gymnodimine A containing the

6,6-spiroimine core induced significant inhibition of ACh-evoked nicotinic currents generated by the nAChRs, a feature revealing the critical role of this moiety for the biological activity of the toxin (Douroue et al. 2011). The unusual stability to hydrolysis of the spiromine group in pinnatinoxin A, which is related to the high oral toxicity of this phycotoxin compared to its congeners (Molgó et al. 2015), was demonstrated by in vitro experiments performed under conditions addressing stability and by computational studies (Jackson et al. 2012).

### Mode of action on muscle-type nAChRs

The nAChR from the Torpedo electric organ, a prototype of the vertebrate skeletal muscle nAChR, is a transmembrane heteropentameric molecule composed of four homologous subunits with a $\alpha_1\beta_1\gamma_0$ stoichiometry. Two binding sites with distinct binding affinities for the nicotinic agonists and competitive antagonists are located at the $\alpha$-$\gamma$ and $\alpha$-$\delta$ subunit interfaces. Functional studies revealed that nanomolar concentrations of gymnodimine A (Kharrat et al. 2008), 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C (Ariaoz et al. 2015), 20-methyl spirolide G (Couesnon et al. 2016), and pinnatinoxins E, F, and G (Hellyer et al. 2013) blocked, in a concentration- and time-dependent manner, muscle twitches evoked by nerve stimulation in isolated mouse or rat nerve-muscle preparations without affecting the directly elicited muscle twitches, a feature indicating that these phycotoxins alter neuromuscular transmission without affecting the excitation-contracting coupling process. This interpretation was confirmed by electrophysiological recordings performed at single neuromuscular junctions, which showed that gymnodimine A (Kharrat et al. 2008), 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C (Ariaoz et al. 2015), and pinnatinoxins E, F, and G (Hellyer et al. 2013), either reduced or completely blocked (depending on concentration) the amplitude of spontaneous miniature endplate potentials, an effect suggesting that the toxins impeded the interaction of ACh quanta with endplate nAChRs. In addition, the toxins blocked nerve-evoked endplate potentials without affecting the resting membrane potential of muscle fibers, so that endplate potentials could no longer reach the threshold for opening voltage-gated sodium channels in muscle fibers, and could not trigger a muscle action potential. These in vitro findings are consistent with in vivo studies in anesthetized mice showing that a local injection of toxins caused a dose-dependent block of the maximal compound muscle action potential amplitude, evoked by nerve stimulation. The effective doses needed to block 50% of the maximal compound muscle action potential amplitude ($ED_{50}$) were 1.7 ng/kg for 20-methyl spirolide G (Couesnon et al. 2016) and 6 ng/kg for 13-desmethyl spirolide C, which in turn was about 300-fold more active than gymnodimine A on an equimolar basis (Marrouchi et al. 2013). Overall, these studies indicated that these cyclic imine toxins potently block neuromuscular transmission on junctions expressing the mature muscle-type ($\alpha_1\beta_1\delta\gamma_0$) nAChR.

Further studies were carried out using Xenopus skeletal myocytes expressing the embryonic muscle-type $\alpha_13\beta_1\gamma_0\delta$ nAChR at their membrane surface, and the patch-clamp technique. Under these conditions, it was disclosed that gymnodimine A blocked nicotinic currents caused by short (5 ms) iontophoretic pulses of ACh, and that the block of ACh-evoked current exhibited no voltage dependency, and was persistent but reversible (Kharrat et al. 2008). Comparable results were obtained with 13-desmethyl spirolide C (Ariãoz et al. 2015).

To gain further insight into the interaction between cyclic imine toxins and nAChRs, studies were performed on Xenopus oocytes microtransplanted with purified electrocyte membranes prepared from the electric organ of the fish Torpedo marmorata that expresses muscle-type $\alpha_11\beta_1\gamma_0\delta$ nAChR. Microinjection of the purified electrocyte membranes to the oocyte cytosol allows rapid incorporation of native and functional $\alpha_11\beta_1\gamma_0\delta$ nAChR into the oocyte membrane (for reviews, see Miledi et al. 2006; Eusebi et al. 2009; Bourne et al. 2010). Using this approach and the two-microelectrode voltage-clamp technique, the actions of gymnodimine A, 13-desmethyl spirolide C (Ariãoz et al. 2009; Bourne et al. 2010), 13,19-didesmethyl spirolide C (Ariãoz et al. 2015), 20-methyl spirolide G (Couesnon et al. 2016), and pinnatinoxins A and G (Ariãoz et al. 2011; Bourne et al. 2015) were studied. None of the cyclic imines analyzed exhibited by themselves an agonist action on the $\alpha_11\beta_1\gamma_0\delta$ nAChR incorporated to the Xenopus oocyte membrane, but they decreased the peak amplitudes of the ACh-elicited nicotinic current in a concentration-dependent manner. The IC$_{50}$ for the various toxins studied are reported in Table 1. The 6,6-spiroimine synthetic analog of gymnodimine A also blocked ACh-evoked currents in Xenopus oocytes having incorporated the $\alpha_13\beta_1\gamma_0\delta$ nAChR (Douroue et al. 2011), but it was much less active than the native toxin.

Reversibility of antagonism of the nAChR was relatively fast with gymnodimine A and pinnatinoxin A, but it was extremely slow with 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C, and 20-methyl spirolide G; the latter compounds exhibited much higher potency than the other phycotoxins (see Table 1).

Competition binding studies revealed that the six cyclic imine toxins studied interact with high affinity with the $\alpha_11\beta_1\gamma_0\delta$ nAChR, and totally displaced [125]I-$\alpha$-bungarotoxin from its binding site with dissociation constants in the nanomolar or subnanomolar ranges, as reported in Table 2.

Several methods for detecting cyclic imine toxins have been set up to substitute the conventional mouse bioassay, which has several weaknesses including specificity, sensitivity, and ethical concerns (reviewed in Daneshian et al. 2013).
Table 1 Inhibition constants (IC50, nM) for the action of some cyclic imine dinoflagellate toxins on ACh-evoked nicotinic currents, recorded from oocytes micro-transplanted with the muscle-type α2β1γδ nicotinic ACh receptor, or expressing the human neuronal α7 or α4β2 nAChR subtypes (various subunit stoichiometries for the α4β2)

<table>
<thead>
<tr>
<th>Cyclic imine toxin</th>
<th>α2β1γδ (Torpedo)</th>
<th>α7 (human)</th>
<th>α4β2 (human)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-meSPX-Ga</td>
<td>0.36 (0.29–0.45)</td>
<td>0.48 (0.15–1.4)</td>
<td>2.1 (1.4–3.1)</td>
<td>Couesnon et al. (2016)</td>
</tr>
<tr>
<td>13,19-dimethylSPX-Ca</td>
<td>0.20 (0.16–0.26)</td>
<td>0.25 (0.24–0.27)</td>
<td>6.26 (4.7–8.3)</td>
<td>Aróz et al. (2015)</td>
</tr>
<tr>
<td>13-SPX-C Ba</td>
<td>0.51 (0.4–0.6)</td>
<td>0.18 (0.16–0.21)</td>
<td>3.9 (2.9–5.1)</td>
<td>Bourne et al. (2010)</td>
</tr>
<tr>
<td>GYM-Ac</td>
<td>2.8 (1.9–4.1)</td>
<td>0.9 (0.6–1.2)</td>
<td>30.4 (19.4–47.5)</td>
<td>Aróz et al. (2011)</td>
</tr>
<tr>
<td>PnTX-Ae</td>
<td>5.53 (4.5–6.8)</td>
<td>0.107 (0.086–0.132)</td>
<td>4.90 (3.97–6.06)</td>
<td>Bourne et al. (2015)</td>
</tr>
<tr>
<td>PnTX-AK</td>
<td>24,760 (9,771–62,750</td>
<td>182,500 (2,213–1,505,000)</td>
<td>&gt; 1,000,000</td>
<td>Hellyer et al. (2015)</td>
</tr>
</tbody>
</table>

Note: Data obtained with (α4)2(β2)2.

Table 2 Inhibition constants (Kf ± SEM, nM) obtained from competition binding assays at equilibrium for some cyclic imine toxins on various nicotinic ACh receptors, and comparisons with the nicotinic antagonists α-toxin, methyllycaconitine (MLA), and epibatidine

<table>
<thead>
<tr>
<th>Cyclic imine toxin</th>
<th>α2β1γδ (Torpedo)</th>
<th>α7-5HT3 (chick)</th>
<th>α3β2 (human)</th>
<th>α4β2 (human)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-meSPX-Ga</td>
<td>0.028 ± 0.005</td>
<td>0.11 ± 0.08</td>
<td>0.040 ± 0.001</td>
<td>3.6 ± 0.7</td>
<td>Couesnon et al. (2016)</td>
</tr>
<tr>
<td>13,19-dimethylSPX-Ca</td>
<td>0.017 ± 0.003</td>
<td>0.22 ± 0.06</td>
<td>0.51 ± 0.14</td>
<td>53 ± 25</td>
<td>Aróz et al. (2015)</td>
</tr>
<tr>
<td>13-SPX-C Ba</td>
<td>0.080 ± 0.002</td>
<td>0.53 ± 0.08</td>
<td>0.021 ± 0.005</td>
<td>0.58 ± 0.07</td>
<td>Bourne et al. (2010)</td>
</tr>
<tr>
<td>GYM-Ac</td>
<td>0.23 ± 0.08</td>
<td>0.33 ± 0.08</td>
<td>0.24 ± 0.09</td>
<td>0.62 ± 0.07</td>
<td>Kharrat et al. (2008)</td>
</tr>
<tr>
<td>PnTX-Ae</td>
<td>2.80 ± 0.03</td>
<td>0.35 ± 0.04</td>
<td>9.4 ± 1.9</td>
<td>15.6 ± 5.2</td>
<td>Aróz et al. (2011)</td>
</tr>
<tr>
<td>PnTX-AK</td>
<td>0.11 ± 0.04</td>
<td>0.72 ± 0.03</td>
<td>64 ± 2</td>
<td>101 ± 30</td>
<td>Bourne et al. (2015)</td>
</tr>
<tr>
<td>α-Toxin</td>
<td>0.011 ± 0.002</td>
<td>0.83 ± 0.12</td>
<td>0.034 ± 0.002</td>
<td>0.054 ± 0.011</td>
<td>Couesnon et al. (2016)</td>
</tr>
<tr>
<td>MLA</td>
<td>0.83 ± 0.12</td>
<td>0.034 ± 0.002</td>
<td>0.054 ± 0.011</td>
<td>Couesnon et al. (2016)</td>
<td></td>
</tr>
</tbody>
</table>

Note: Data obtained with (α4)2(β2)2.

2013). The new methods are based on competition assays where the cyclic imine toxins prevent the interaction of fluorescent- or biotin-labeled α-bungarotoxin with the α1β2γδ nAChR contained in purified Torpedo membranes (Vilarino et al. 2009; Fonfría et al. 2010; Otero et al. 2011; Rodríguez et al. 2011, 2013a; Aróz et al. 2012; Rubio et al. 2014). Also, a receptor-based detection method was developed using Torpedo nAChR, or Lymnaea stagnalis ACh-binding protein immobilized on the surface of carboxylated microspheres, and the competition of cyclic imines toxins with biotin-α-bungarotoxin for binding to these proteins (Rodríguez et al. 2013b). Available evidence indicates that both mature (α1β2γδe) and embryonic (α1β2γδ) muscle-type nAChRs are important targets for cyclic imine toxins, and are certainly responsible for the fast depression of respiratory neuromuscular transmission during acute toxicity assays in rodents (Munday et al. 2004, 2012).

Mode of action on neuronal types of nAChRs

Cyclic imine toxins are among the few organic compounds produced by dinoflagellates known to interact with the major neuronal nAChRs, as assessed by functional and ligand-binding assays. Vertebrate neuronal nAChRs comprise a varied population of receptors with assorted subunit assemblies of α2–α10 and β2–β4 subunits (Millar and Gotti 2009). The human α3β2 and α4β2 subtypes, which play a
predominant role in both pre- and post-synaptic functions in the central and peripheral nervous systems, may have variable stoichiometries of 2 αβ/β versus 3 α2β β subunits, with higher affinity ligand binding at the αβ subunit interfaces and lower affinity ligand binding at the αα subunit interfaces (Shahsavari et al. 2015).

Under voltage-clamp conditions, gymnodimine A, 13-desmethyl spiridole C, 13,19-didesmethyl spiridole C, 20-methyl spiridole G, or pinnatoxins A, E, F, and G do not activate the human homomeric α7 or heteromeric α4β2 nAChRs expressed in Xenopus oocytes, and therefore do not exert the agonist action typically observed with ACh or nicotine. In contrast, the toxins reduce the inward peak current elicited by ACh in both human α7 and α4β2 nAChR subtypes (Kharrat et al. 2008; Bourne et al. 2010, 2015; Aráoz et al. 2011, 2015; Hellyer et al. 2015; Couesnon et al. 2016). The IC50 values are reported in Table 1.

The action of cyclic imines was also studied using a fluorescence Ca2+ mobilization assay. In brief, nicotine binding to cell surface nAChRs caused a release of the intracellular calcium (monitored through its binding to the previously loaded calcium-sensitive dye, fluorescence imaging plate reader calcium 4) and thereby increased the cell fluorescence intensity measured with a fluorescence imaging plate reader. Neither gymnodimine A nor 13-desmethyl spiridole C at concentrations up to 10 μM displayed any calcium release from cells expressing the human α4β2 or α4β4 nAChRs (Hauser et al. 2012). The antagonistic blockade effect of gymnodimine A and 13-desmethyl spiridole C was also examined using the calcium-flux assay in cells expressing various subtypes of nAChRs by incubating the cells with the toxins for 30 min, and thereafter adding nicotine at an EC50 concentration. Under these conditions, the inhibition of nicotine-induced calcium-flux response, determined at each antagonist concentration, revealed the following rank order of potency for 13-desmethyl spiridole C: α7 > low sensitivity form of α4β2 > human α3β4 > high sensitivity form of α4β2 > rat α3β4, and for gymnodimine A: low sensitivity form of α4β2 > human α3β4 > α7 > high sensitivity form of α4β2 > human α3β4 > rat α3β4. The antagonism of the nicotine-induced calcium mobilization by both toxins was found to be not surmountable (Hauser et al. 2012). Furthermore, 13-desmethyl spiridole C and gymnodimine A inhibited nicotine-mediated dopamine release from rat striatal synaptosomes with similar high potency (IC50 = 0.2 and 0.3 nM, respectively) (Hauser et al. 2012).

Additionally, none of the toxins studied appeared to modify the desensitization kinetics of the α7 nAChR. The antagonistic activity of gymnodimine A on the human α7 and α4β2 nAChR subtypes was found to be readily reversible, whereas those of 13-desmethyl spiridole C and 20-methyl spiridole G were not abolished after a 30–60 min washout (Bourne et al. 2010; Couesnon et al. 2016). Pinnatoxin A activity on the human α7 nAChR was also reported to be slowly reversible, whereas on α4β2 nAChR it was rapidly reversible (Aráoz et al. 2011; Bourne et al. 2015).

Functional studies were also performed with the voltage-clamp technique on oocytes expressing the α4β2 nAChR in two stoichiometric forms [the low-affinity (α4β2)2, and high-affinity (α4β2)3] forms (Nelson et al. 2003). Both pinnatoxins F and G inhibited the ACh-evoked responses, yet with different potencies reflected in their IC50 values. As shown in Table 1, pinnatoxin F was more active on (α4β2)2 and (α4β2)3 nAChRs than pinnatoxin G. However, both pinnatoxins displayed similar potencies on the (α4β2)2 versus (α4β2)3 nAChRs (Hellyer et al. 2015). Thus, the lower sensitivity of the cyclic imine toxins toward the α4β2 nAChR, compared to the homomeric α7 nAChR, does not result from changes in the subunit stoichiometry and creation of an α–α interface, but rather from essential structural properties in the ligand-binding sites at the respective subunit interfaces.

The spiromine fragment seems essential for the functional blocking activity of pinnatoxin A, as the open-ring, aminoketone derivative of pinnatoxin A has no action on the various neuronal nAChR subtypes (Table 1).

A deeper insight into the interaction between cyclic imine toxins and neuronal nAChRs was obtained from competition binding experiments performed at equilibrium on membranes from cells expressing various nAChR subtypes and an α7-5HT3 chimera, and using radiolabeled probes and standard protocols (Servent et al. 1997). Gymnodimine A, 13-desmethyl spiridole C, 13,19-didesmethyl spiridole C, 20-methyl spiridole G, and pinnatoxins A and G totally displaced [125I]α-bungarotoxin from its binding site, thereby confirming that these phycotoxins interact with high affinity with the α7-5HT3 chimera. In addition, their property of displacing [3H]tetrahydroaminidealine binding from human α3β2 and α4β2 neuronal nAChRs highlights the broad capacity of cyclic imine toxins to interact with either of the homo- and hetero-pentameric forms of neuronal nAChRs.

The rank order of potency for gymnodimine A (from Ki values) was found to be chicken α7-5HT3 > human α3β2 > human α4β2 nAChR (Kharrat et al. 2008). In another study this pharmacological profile was confirmed and detailed, the order of potency for gymnodimine A being: α7, α6β3β4α5 > rat α3β4 > human α3β4, α4β4 > rat α4β2, human α4β2 (Hauser et al. 2012). Table 2 summarizes the dissociation constant (Ki) values of several cyclic imine toxins relative to distinct neuronal nAChRs subtypes, recorded under the same experimental conditions.

A comparable broad specificity toward the neuronal nAChR subtypes was also observed with 13-desmethyl spiridole C (Bourne et al. 2010). The rank order for inhibition by 13-desmethyl spiridole C was: α7 > α6β3β4α5 >> rat α3β4, α4β4, human α3β4 > human α4β2 > rat α4β2 (Hauser et al. 2012). The selectivity profile for pinnatoxin
A also exhibited a higher affinity for the human α7 compared to the human α3β2 and α4β2 nAChRs (Aráoz et al. 2011), and this was maintained for other pinnatoxins exhibiting the following order of potency: pinnatoxin F > pinnatoxin G > pinnatoxin E (Hellyer et al. 2015).

Interaction with mAChRs

Muscarinic ACh receptors (mAChRs) have been proposed in early studies to explain part of the acute toxicological mode of action of 13-desmethyl spirolide C in rats (Gill et al. 2003). Indeed, both transcriptional alterations for early injury markers (c-jun and HSP-72) and for mAChRs and nAChRs were revealed. The M1, M4, and M5 mAChR genes, as well as the α2 and β4 nAChR genes were altogether up-regulated, implying that both types of cholinergic receptors could be potential molecular targets for the 13-desmethyl spirolide C. Studies carried out in the human neuroblastoma cell line BE(2)-M17, expressing mAChR subtypes, reported that 13-desmethyl spirolide C inhibited ACh-induced Ca²⁺ signals, while the reversible competitive antagonist atropine diminished the inhibitory effect of the spirolide. Also, the spirolide at 0.5 μM reduced the [³H]N-methyl scopolamine specific binding to the cells by ca. 53%. Similar inhibition of [³H]quinuclidinyl benzilate binding (59%) was observed under the same experimental conditions. Such data suggested that the spirolide binds to the orthosteric binding site of mAChRs (Wandscheer et al. 2010). However, later competition binding assays, performed on membrane embedded mAChRs from TE671/RD clonal cells and rat cortices using radiolabeled [³H]quinuclidinyl benzilate and both gymnodi- mine A and 13-desmethyl spirolide C, failed to show any significant interaction with the mAChRs (Hauser et al. 2012).

Further work performed with CHO cells stably expressing each of the five human mAChRs subtypes revealed that pinnatoxin A (1 μM) had no significant action on [³H]N-methyl scopolamine binding to M1, M2, M3, and M4 mAChRs, whereas it displaced radiotracer binding to the M5 mAChR subtype by 35%, a value reflecting interaction in the low micromolar range (Aráoz et al. 2011). This property was not observed using the pinnatoxin A amino-ketone derivative. In a similar assay, 13-desmethyl spirolide C and 13,19-didesmethyl spirolide were found to interact with very low affinity (in the micromolar range) with the five mAChR subtypes, yielding affinities 3–4 orders of magnitude lower than those for the nAChR subtypes (Aráoz et al. 2015).

Structural studies

The absolute stereochemistry of gymnodimine A was unambiguously assigned from the crystal structure of the p-bromobenzamide derivative of the reduced form of gymnodimine A (Stewart et al. 1997), whereas the relative stereochemistry of 13-desmethyl spirolide C, except for one chiral center, has been determined using the ConGen molecular modeling method, from NOESY and ROESY NMR data (Falk et al. 2001). Later on, the crystal structures of acetylcholine-binding protein (AChBP) complexes with gymnodimine A (Bourne et al. 2010), 13-desmethyl spirolide C (Bourne et al. 2010), and pinnatoxins A and G (Bourne et al. 2015) unveiled the molecular determinants of toxin-binding selectivity. The soluble AChBPs, with their overall pentameric architecture and their amino acid residues forming a binding pocket for the nicotinic ligands at subunit interfaces, have been extensively used as functional and structural surrogates for the ligand-binding domains of the nAChRs and have provided valuable information on how ligands interact with the various nAChR subtypes (Brejc et al. 2001; Cellé et al. 2004; for a recent review, see Shahsavari et al. 2016).

In addition to the overall structural features of the subunits, the aromatic side chains that form the ligand-binding pocket at the subunit interfaces are well conserved in the nAChR family, with greater variability for residues at the complementary [or (−)] face than the principal [or (+)] face of each interface. In fact, the binding pocket of AChBP possesses all the functional residues identified in the nAChR ligand-binding domain. Hence, the ligand-binding pocket encompasses a nest of five electron-rich aromatic side chains provided by residues Tyr93, Trp147, Tyr188, Tyr195 on the (+) face and residue Tyr55 on the (−) face of the interface.

In each complex, the toxin is imbedded within this aromatic nest contributed by loops C and F on opposing faces of the subunit interface and display exquisite shape and chemical complementarity with the ligand-binding pocket (Fig. 2). The orientation and conformation of the toxin carbon skeleton, with its long axis roughly aligned parallel with the pentamer five-fold axis, ideally position the protonated cyclic imine donor, similar to the anabaseines (Talley et al. 2006; Hibbs et al. 2009), to be within H-bond distances to the carbonyl oxygen of Trp147 (loop B). At the apical side of the interface, the tetrahydrofuran ring (gymnodimine A) or bulky and more rigid bis-spiroacetal ring system (13-desmethyl spirolide C, pinnatoxin A) abuts against the tip of loop C to localize the binding interface. At the opposing subunit face, the variable terminal γ-butyr-o lactone (gymnodimine A) or cyclohexene (13-desmethyl spirolide C, pinnatoxin A) rings promote additional interactions including the conserved Tyr93 from the (+) face. In fact, pinnatoxin A contains a bulky bridged 5,6-bicyclocetal substructure instead of a smaller allylic alcohol linker found in gymnodimine A and 13-desmethyl spirolide C. This unique substructure in pinnatoxins (also found in pteriatoxins) extends radically from the interfacial binding pocket to interact with the sequence-variable loop F and governs nAChR subtype selectivity (Bourne et al. 2010, 2015).
In addition to the crystal structures of AChBP–toxin complexes, additional complexes between different nAChR subtypes (human α7, α4β2, α3β2, and α12β1) and pinnatoxin A (Arãoz et al. 2011), 13-desmethyl spirolide C (Arãoz et al. 2015), and 13,19-didesmethyl spirolide C (Arãoz et al. 2015) generated by in silico molecular docking, provided complementary information for the identification of key residues responsible for the differences in binding affinities and subtype specificities that were determined experimentally (Fig. 2) (Arãoz et al. 2011, 2015).

Conclusion

In conclusion, the globally distributed and chemically well-characterized cyclic imine toxins, from toxic dinoflagellate species, represent a novel source of potent antagonists of muscle- and neuronal-type nAChRs. The distinctive chemical signature of these phycotoxins is related to the presence of a cyclic imine moiety in their structure, and their toxicological profile is predominantly associated to their specific interaction with the nAChRs.

Taking advantage of the competitive binding of cyclic imine toxins to nAChRs, several tests have been developed to detect spirolides, gymnodimines, and pinnatoxins in contaminated shellfish with better accuracy than the broad spectrum mouse bioassay. These tests are important in the food safety field, because shellfish represents a rich food resource that may be contaminated by toxins produced by toxic dinoflagellates.

Substantial progress has been obtained on the characterization of the dinoflagellates producing the cyclic imine toxins, but the genes involved in their production, and the pathways leading to the biosynthesis of the various families of toxins remain, at present, elusive. Furthermore, the ecological factors favoring dinoflagellate blooming need to be determined.

Shellfish regularly contain variable amounts of cyclic imine toxins in their edible tissues and can transfer these phycotoxins through the marine food chain. Although cyclic imine levels are not regulated, it has become a matter of concern to assess the risks for human health. Thus, a consensus is emerging that further studies should be conducted to enhance our understanding of the gastrointestinal absorption, tissue disposition, and crossing of the blood–brain and placental barriers. Also, more information is needed on the environmental distribution and risks of chronic exposure to these phycotoxins.

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References


Cyclic imine toxins acting on nicotinic receptors


