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The Sophisticated Peptide Chemistry of Venomous Animals as a Source of Novel Insecticides Acting on Voltage-Gated Sodium Channels

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1. Introduction

Arthropods pests are considered as a global health treat since they are responsible for the transmission of several new and reemerging human diseases such as malaria, dengue and yellow fever (mosquitoes), Lyme disease, ehrlichiosis and tularemia (ticks). Furthermore, arthropods pests destroy 20-30% of the world's food supply every year. Evidently, modern agriculture still depends strongly on the use of insecticides. The majority of insecticides used today act on one of the following neuronal targets: acetylcholinesterase, the nicotinic acetylcholine receptor, the γ-aminobutyric acid (GABA)-gated chloride channel and the voltage-gated sodium channel (Na_V). This review will focus on Na_V channels as possible targets for insecticides.
For over 50 years, synthetic pyrethroids, which are analogous of the natural occurring insecticidal components of *Chrysanthemum* flowers, have been used as classical industrial pesticides. The public awareness of the health hazards and environmental damage, due to their low insect-selectivity, has complicated the use of these pesticides. More importantly, the intensive use of DDT and other pyrethroids has facilitated the development of resistance against these compounds. The so-called knockdown resistance, in which insects have reduced their sensitivity towards pyrethroids by point mutations in their target site, heralded the loss of major classes of insecticides. As a consequence, there is an urgent need for new, potent and insect-selective insecticides. A need that could be fulfilled by insecticidal neurotoxins derived from venomous animals.

2. The sodium channel structure and function

Voltage-gated sodium channels (Na_V channels) are transmembrane protein complexes constituted of an α-subunit of approximately 260 kDa which can be associated with up to four auxiliary β-subunits (β1-4) of 30 to 40 kDa. The pore-forming α-subunit alone is sufficient to obtain sodium current, however co-expression of β-subunits modifies expression level, kinetics and voltage dependence of channel gating (Yu & Catterall, 2003). The α-subunit is organized in four homologous domains (DI-IV). Each domain contains six putative transmembrane segments (S1-S6) connected by extracellular or intracellular loops

(fig. 1A). The S4 segments are the most conserved segments and they contain a basic residue, either lysine or arginine, in every third position. These positive charged S4 segments are believed to function as voltage sensors. They transport gating charges by moving outward upon membrane depolarization and as such initiating the voltage dependent activation which results in the opening of the channel. The selectivity filter and pore are formed by the transmembrane segments S5 and S6 together with the re-entrant segments that are part of the loop which connects the S5 and S6 of each domain. Folding of the domains in a clockwise orientation, in which domain I and IV are in close proximity of each other, leads to the formation of the outer vestibule and the selectivity filter (Catterall, 2000; Chanda & Bezanilla, 2002). The short intracellular linker that connects the domains III and IV contains a highly conserved sequence of three hydrophobic residues (isoleucine, phenylalanine and methionine) or IFM motif. Sodium channel inactivation is mediated by this hydrophobic motif since it serves as an inactivation gate crucial for causing fast inactivation by binding to a receptor. This inactivation gate receptor is located near or within the intracellular mouth of the sodium channel pore. It has been shown that several residues in the intracellular loop that connects IIIS4-S5 and in the loop connecting IVS4-S5 are contributing to the inactivation gate receptor (Dong, 2007; Yu & Catterall, 2003).

Nine different mammalian sodium channel isoforms have been cloned, characterized and functionally expressed. These sodium channel isoforms exhibit distinct expression patterns in skeletal and cardiac muscle tissues and in the central and peripheral nervous systems (Goldin, 1999). $Na_V1.1$, $Na_V1.2$, $Na_V1.3$ and $Na_V1.6$ are expressed in the central nervous system (CNS), whereas $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are predominantly expressed in the peripheral nervous system (PNS). $Na_V1.4$ is expressed in skeletal muscles, while $Na_V1.5$ is also known as the cardiac muscle isoform. The functional and pharmacological diversity of the mammalian Na_V channels is primarily resulting from the expression of multiple genes (Goldin *et al.*, 2000). The selective expression of different sodium channel genes significance the specialized function of sodium channels in various mammalian tissues and cell types (Yu & Catterall, 2003). Their specialized function results from the fact that each mammalian sodium channel α-subunit isoform features distinct electrophysiological properties such as unique gating kinetics (Dong, 2007; Goldin, 2001).

Voltage-gated insect sodium channels closely resemble their mammalian counterparts in electrophysiology, ion conductance and also in overall structure. However, they do differ in amino acid sequence and therefore in their pharmacological diversity and flexibility (Zlotkin, 1999). Furthermore, the insect-selective action of pesticides such as pyrethroids and DDT and moreover, the high specificity for either mammalian or insect Na_V channels displayed by neurotoxins from scorpion, spider and sea anemone venoms have evidenced the existence of a pharmacological distinction between mammalian and insect Na_V channels.

3. Structural comparison between mammalian and insect Na_V channels

Two genes putatively encoding for Na_V channels, *DSC1* and *para*, were isolated from *Drosophila melanogaster*. Later on, it was shown that DSC1 encodes for a Ca^{2+}-selective cation channel and not for a sodium channel. The first insect Na_V channel encoding gene, *para*, was identified from a genomic DNA library in studies using mutants with a temperature sensitive paralytic phenotype (Loughney *et al.*, 1989). The gene has been cloned and upon functional expression in *Xenopus laevis* oocytes it was demonstrated that *para* indeed encodes for a voltage-gated sodium channel ($DmNa_V1$) (Feng *et al.*, 1995; Warmke *et al.*, 1997).

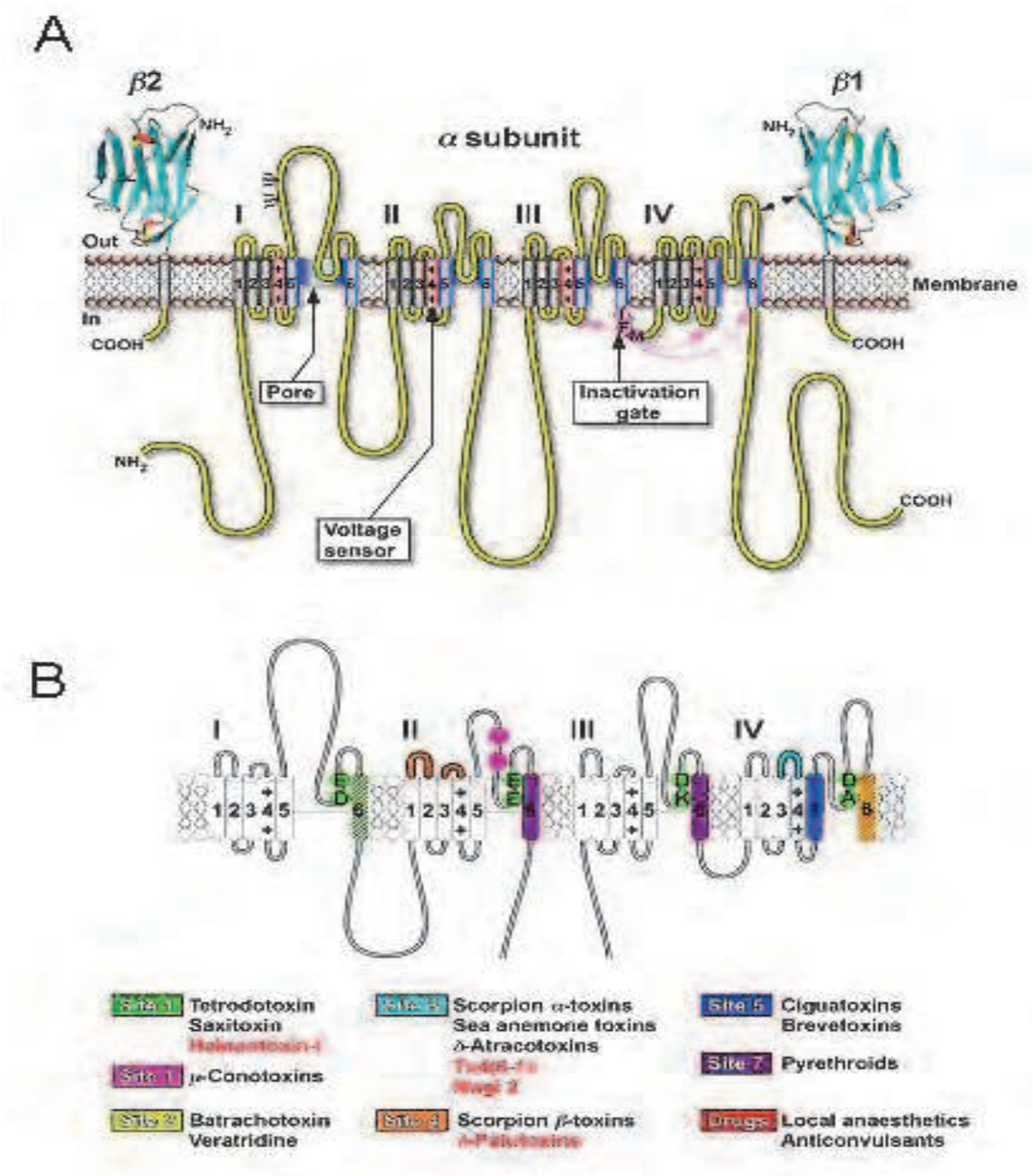

Fig. 1. Molecular structure and the neurotoxin receptor sites of Na_V channels. (A) Schematic two-dimensional representation of the functional α-subunit and the auxiliary β-subunits. (B) Identification of known neurotoxin receptor sites on Na_V channels. Green circles represent the outer (EEDD) and inner (DEKA) rings of amino acid residues that form the ion selectivity filter and the proposed neurotoxin receptor site-1. In the case of receptor sites 3 and 4, only areas where there is more than a five-fold increase in binding affinity are highlighted. Insect-selective spider toxins are highlighted in red text. Figures were adapted from (Nicholson, 2007).

Screening of the *Drosophila* genome revealed that no other genes were similar to *para*, suggesting that *para* is the only gene encoding the sodium channel in *Drosophila* and presumably also in other species (Littleton & Ganetzky, 2000). Electrophysiological recordings in different insect neurons have shown the presence of distinct sodium currents, suggesting the existence of sodium channels with differing properties (Defaix & Lapied, 2005; Grolleau & Lapied, 2000). Recent studies have shown that the occurring heterogeneity in sodium channel properties in insects is generated by extensive alternative splicing and RNA editing of the *para* gene transcript (Dong, 2007). Thus, it seems that insects rely on alternative splicing and RNA editing to produce a functional diversity of sodium channels, whereas mammals depend on multiple sodium channel genes to generate channels with unique gating properties (Dong, 2007; Yu & Catterall, 2003). An increasing number of othologous *para* genes have been identified in agricultural and medical important insect species (Soderlund & Knipple, 2003). Unfortunately, only partial cDNA clones could be obtained in most cases. Up to date, the full length cDNA clones for only 3 orthologous *para* genes have been identified: in the housefly *Musca domestica* (Vssc1), the German cockroach *Blattella germanica* ($BgNa_V$) and the mite *Varroa destructor* ($VmNa_V$) (Dong, 1997; Ingles *et al.*, 1996; Wang *et al.*, 2003). All 3 genes generate voltage-dependent sodium currents when heterologously expressed in oocytes (Du *et al.*, 2009; Ingles *et al.*, 1996; Tan *et al.*, 2002b). A functional characterization of 20 $BgNa_V$ splice variants demonstrated that, similar to *para*, RNA editing and alternative splicing results in an astonishing diversity of Na_V channels with distinct expression levels and varying kinetics of gating.

An insecticidal counterpart of the mammalian auxiliary β-subunit has also been identified in the Drosophila genome. The temperature-induced paralysis locus E (*TipE*) encodes for a small transmembrane protein consistent of two transmembrane segments which are connected by a large extracellular loop and intracellular amino and carboxyl termini (Dong, 2007; Feng *et al.*, 1995). *TipE* increases the sodium peak current, alters the kinetics of fast inactivation and changes the pharmacology of $DmNa_V1$ when it is co expressed with this para α-subunit (Warmke *et al.*, 1997). Furthermore, TipE plays an important assisting role in the trafficking of the α-subunit from the endoplasmic reticulum to the membrane and the incorporation in the membrane (Moore *et al.*, 2000).

Four *TipE* homologs have been characterized in *D. melanogaster* (*TEH1-4*) (Derst *et al.*, 2006). Similar to TipE, coexpression of TEH1-3 in oocytes results in increased sodium peak currents. Furthermore, it was shown that TEH1 shifts the voltage-dependent inactivation and it alters the rate of recovery from inactivation of $DmNa_V1$. TEH1 is only expressed in the central nervous system while TEH2-4 are widely expressed in both neuronal and non-neuronal tissues (Derst *et al.*, 2006). This might imply that these TEH auxiliary subunits are involved in specific regulation of sodium channels in wide variety of insect cells (King et al., 2008). An orthologous *TipE* gene has been cloned from the *Musca domestica* (Vssc β) (Lee *et al.*, 2000). Up to date, no ortholog has been identified in the German cockroach. However, it has been shown that co-expression of TipE with $BgNa_V$ or with Vssc1 in both cases resulted in an enhanced expression in oocytes (Lee *et al.*, 2000; Tan *et al.*, 2002a). These results suggest that the auxiliary subunits are functionally conserved among different insect species. A recent study has shown that that the channel affinity of the conotoxin MrVIB, a mammalian sodium channel blocker isolated from the cone snail *Conus marmoreus*, is strongly influenced by the co expression of β-subunits (Wilson *et al.*, 2011).

Their high conservation among different insect species, their wide expression in distinct tissues together with the observation that they are involved in the neurotoxin-channel

interaction raises the intriguing question whether the insect auxiliary subunits might represent interesting new phyla-selective targets for neurotoxin insecticides.

4. Rationale for the use of insect Na_V channels as targets for insecticides

Na_V channels mediate the increase in sodium permeability during the initial rapidly raising phase of the action potential making them a crucial component in the generation and propagation of action potentials in neurons and most electrically excitable cells. Because of this key role in the excitability of biological systems, Na_V channels are one of the foremost targets of venomous animals. These venoms are complex cocktails that have evolved to paralyze or to kill arthropod preys. Mutations and positive selection have lead to an optimization of the venom compounds which resulted in toxins able to act highly specific and very potently upon their target.

Further justification for the use of Na_V channels as targets for the development of novel insecticides is delivered by the pharmacological flexibility of Na_V channels. This arises for instance from the existence of a large number of sodium channel binding sites (fig; 1B). To date, seven neurotoxin binding sites have been identified, potentiating a broad diversity of insecticidal targets. Although the identification and characterization of the distinct receptor sites were mainly determined using vertebrate preparations, similar receptor sites have been shown for insect neuronal membranes (Gordon *et al.*, 2007). The large number of Na_V channel binding sites is much more than thus far described for other possible ion channel targets such as Ca_V and K_V channels. Several studies using insect-selective toxins from different venomous animals have underlined that even though there is an overall structural similarity with the mammalian isoforms, insect Na_V channels do exert a distinct pharmacological diversity compared to their mammalian counterparts. Furthermore, the allosteric coupling of the binding sites provides cooperative aspects which possess far-reaching practical, economical and ecological agro-technical implications (Nicholson, 2007; Zlotkin, 1999). As such, a synergistic mixture, constituted of 2 insecticides acting on distinct but allostericly coupled sites, will allow a significant decrease in the required dosages and concentrations of the 2 constituting insecticides and thus reducing production costs. Moreover, the combinatorial use of insecticides will delay the onset of resistance (Nicholson, 2007; Zlotkin, 1999).

It should be critically noted that there is no evidence to reason why Na_V channel are a more suitable target for developing new insecticides compared to other ion channels such as Ca_V or K_V. Given the fact that Na_V and Ca_V channels belong to the same superfamily of structural related voltage-gated ion channels, it is most likely that insect Ca_V channels present a similar broad panel of potential binding sites for insecticide development. Certainly since at least 3 distinct classes of insect Ca_V channels have been identified (Jeziorski *et al.*, 2000). However, due to a lack of functionally cloned insect Ca_V channels and a limited number of accessible and well-characterized insect neuron preparations, does the number of potential new insecticides acting on Ca_V channels remain scarce (King *et al.*, 2008).

5. Toxins isolated from venomous animals acting selective on insect Na_V channels

Venomous animals such as scorpions, spiders and sea anemones have become medicinal chemists of unprecedented skills by evolving their peptide chemistry and

neuropharmacology in order to develop components that insure complete shut down of the nervous system of their arthropod prey. Peptide neurotoxins that target ion channels are abundantly represented in these venoms. Specifically toxins acting on Na_V channels provide venomous animals the possibility to induce a rapid paralysis upon envenomation. These toxins, thanks to their insect selectivity, are potential new lead compounds for the development of a novel generation insecticides. All insect-selective neurotoxins characterized to date are selectively binding to three of the seven known neurotoxin sites on insect Na_V channels. Therefore these sites can be considered as potential insecticide targets (King *et al.*, 2008).

5.1 Spider venoms

Over 42,000 species of spiders have already been described worldwide (Platnick, 2000). They are divided in 110 families which are classified in the order Araneae within the Arachnida class, a group that also includes scorpions, mites and ticks. Araneae comprise three suborders: the non-venomous *Mesothelae*, the *Mygalomorphae* or tarantulas and the *Araneomorphae*.

Only a minority of spider species are capable of inflicting clinically significant or sometimes fatal envenomations. These species include the *latrodectism* or widow spiders, the *loxoscelism* or recluse spiders and certain *mygalomorph* such as the Australian funnel web and mouse spiders (Billen *et al.*, 2008; Isbister & White, 2004). Among the large number of species described, only of about 100 species has the venoms been studied. Venom composition is highly species-specific and varies depending on factors such as sex, nutrition, natural habitat and climate (Kuhn-Nentwig *et al.*, 2004; Mebs, 2002). Spiders have optimized their venoms as complex, chemical mixtures containing a variety of biological active substances serving the general purposes for both attacking (killing or paralyzing prey) and protecting (defending against competitors) (Mebs, 2002; Vassilevski *et al.*, 2009). These venoms can be divided into two groups based on the character of their function: neurotoxic and necrotic or cytolytic. This review will only focus on the neurotoxic group and more specific on the neurotoxins acting on Na_V channels. The complete molecular diversity of spider venoms has recently been very well reviewed in (Vassilevski *et al.*, 2009).

Spider neurotoxins channels are low molecular weight polypeptides. Even though peptides devoid of disulfide bridges have been reported, the majority of these neurotoxins are small disulfide rich peptides, containing 6 to 12 cysteine residues (Billen *et al.*, 2008; Pimenta & De Lima, 2005). Spider neurotoxins which target Na_V channels are in general peptides compromising 31 to 41 amino acid residues which are cross-linked by three to four disulfide bridges. Depending on their different cysteine arrangements and structural characteristics, these peptides can be divided into two distinct structural motif families: the inhibitory cysteine knot (ICK) motif and the disulfide-directed β-hairpin (DDH) scaffold (fig. 2) (Craik *et al.*, 2001; Norton & Pallaghy, 1998).

Both panels schematically represent the structural motifs of the cystine-knot folding and possible addition of the third β-sheet. β-sheets are shown as gray arrows and disulfide bridges connecting cysteine residues are shown as dark gray lines with roman numerals. The dark arrow (β1) in the right panel represents the additional β-sheet not always present in ICK spider toxins. Figure was adapted from (Nicholson, 2007).

The majority of spider toxins contain the ICK motif. It is interesting to note that peptides with the ICK motif can be found in very diverse sources such as animals, plants, fungi and even viruses. The following arrangement of disulfide bonds is observed in all peptides

belonging to this structural family: C^1-C^4, C^2-C^5, C^3-C^6. These ICK containing peptides are further characterized a β-hairpin and by the presence of a so called 'knot: the first two disulfide bridges (C^1-C^4, C^2-C^5) form a spatial ring which is penetrated by the third disulfide bridge (C^3-C^6) (Craik *et al.*, 2001; Vassilevski *et al.*, 2009). Even within a structural motif family are the biological activities diverse. Spider neurotoxins possessing the ICK motif can target proton-gated, voltage-gated or mechanosensitive channels while others exert a haemagglutination activity, protease inhibition or AMP activity (Bulet & Stocklin, 2005). The DDH scaffold is believed to be the ancestral motif from which the ICK motif has evolved. This structural motif is characterized by an arrangement of disulfide bridges as follows: C^1-C^3, C^2-C^4. Such a disulfide pattern implies that there are only two mandatory disulfide bridges that form the bulk of the hydrophobic core and that there is a formation of β-hairpins which are stabilized by these two conserved disulfide bonds (Vassilevski *et al.*, 2009).

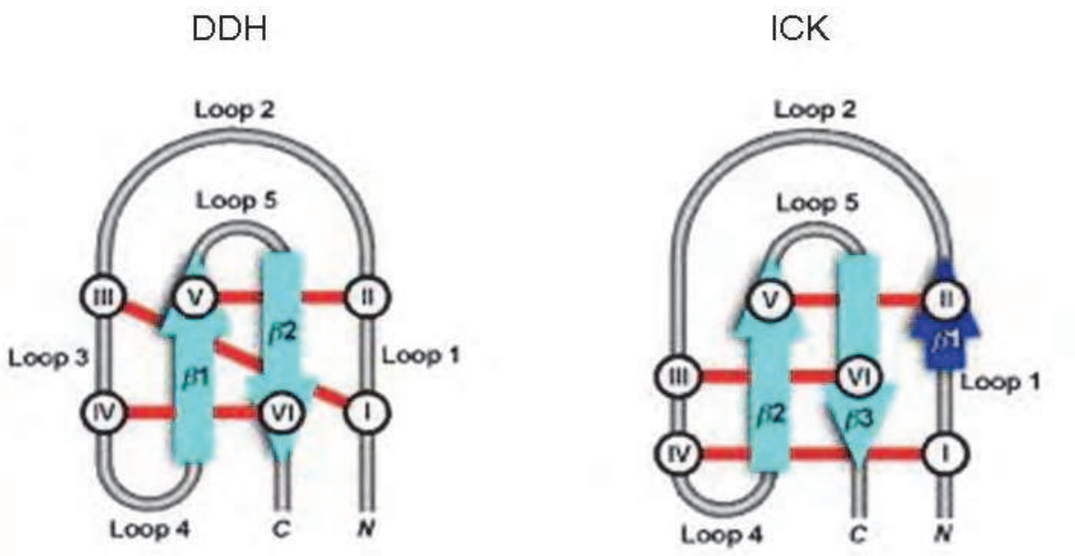

Fig. 2. Structural motifs found in insect-selective spider toxins.

Spider neurotoxins interact with Na_V channels either by binding to site 1, blocking the sodium current or by modulating the kinetics of gating by binding to site 3 and 4 (King *et al.*, 2008). Furthermore, a Na_V channel targeting family of 56-61 residue insecticidal polypeptides has been isolated from the primitive weaving spider *Diguentia canities*. Their exact interaction site with the sodium channel still remains unknown although binding studies have shown that it is unlikely that they bind to site 3 (Nicholson *et al.*, 2004). Further electrophysiological studies are awaiting in order to elucidate the binding site of these interesting insecticidal spider neurotoxins (Nicholson, 2007).

Environmental stress and evolution has led to hypermutational optimization of spider toxins resulting in mini-libraries of toxin variants allowing spiders to target slightly different versions of the same ion channel or receptor in distinct insect species (Gilles *et al.*, 2002). Therefore spider venoms contain pre-optimized insecticidal toxins, which are readily available for investigation to isolate potential lead compounds in the search for new insecticides (Nicholson, 2007).

5.2 Scorpion venoms

Scorpions belong to the most ancient group of animals on earth as they have been roaming this planet for more then 400 million years. More than 1500 different species have been described and they are classified in the order Scorpiones within the Arachnida class (Bosmans & Tytgat, 2007b). Approximately 50 species are known to cause clinical significant injuries out of which 25 are potentially fatal to humans. A scorpion sting might be trivial causing local pain only, but may also produce a very complex symptomatology of envenoming such as neurological, respiratory and cardiovascular collapse. Almost all of the lethal scorpions, except the *Hemiscorpius* species, belong to the family of the *Buthidae*. Dangerously venomous scorpions tend to have lean, delicate pincers, thin bodies and thick tails, as opposed to the large, bulky pincers, thick bodies and thin tails possessed by the non-lethal scorpions (Mebs, 2002). The lethal members of the *Buthidae* family include the genera of *Androctonus* (Northern Africa to Southeast Asia), *Centruroides* (Southwest of Northern and Central America), *Leiurus* (Middle East and Northern Africa), *Mesobuthus* (Asia), *Parabuthus* (Southern and Western Africa), *Buthus* (Mediterranean) and *Tityus* (Caribbean, Central and Southern America).

Scorpion venoms are multi component mixtures containing an unprecedented molecular diversity of pharmacologically active components such as enzymes, nucleotides, lipids, mucoproteins, biogenic amines, polypeptides and other unknown substances. The most characterized components of scorpion venoms are neurotoxins which have evolved towards a specific and specialized bioactivity aimed at efficient and successful capture of prey or defense against predators. Scorpion neurotoxins specifically target voltage-gated ion channels such as Na_V, K_V or Ca_V and other cellular receptors such as the ryanodine receptor (Zamudio *et al.*, 1997). Similar to spiders, is the composition of scorpion venom highly differing between species and are factors such as sex, habitat and local conditions determining for venom specificity (Bosmans & Tytgat, 2007b; Possani *et al.*, 1999). For example, scorpion neurotoxins targeting Na_V channels are much more represented in the venom from scorpions belonging to the dangerous *Buthidae* as compared to the venom of harmless species such as *Pandinus*. Moreover, the overall toxicity of scorpion venom to humans has mainly been attributed to the activity of neurotoxins affecting Na_V channels (Martin-Eauclaire *et al.*, 2005).

In general, scorpion neurotoxins acting on Na_V channels are single chain polypeptides composed of 58-76 amino acids and cross-linked by four disulfide bridges. They posses a highly conserved core formed by an α-helix and two to three strands of β-sheet structural motifs, stabilized by the three intermolecular disulfide bridges (Bosmans & Tytgat, 2007b). The scorpion structural motif families compromise the βαββ family and the βααββα family which are al stabilized by the four disulfide bridges (Mouhat *et al.*, 2004). Both structural motif families belong to the structural cysteine-stabilized α-helix and β-sheet (CSαβ) superfamily. Peptides belonging to the CS superfamily exhibit relatively diverse biochemical and biological functions. However, in most cases are these peptides sharing a common function as defenders of their host, as seen in animals (e.g. scorpion toxins), plants (e.g. defensins) and microorganisms (e.g. antifungal defensins). The extensive distribution of this common motif throughout diverse organisms highlights that this relatively stable and versatile scaffold has the potential to tolerate insertions, deletions and substitutions within the structure (Zhu *et al.*, 2005).

According to their pharmacological profile, scorpion neurotoxins acting on Na_V channels can be divided into two major groups: α-toxins and β-toxins. Upon binding at neurotoxin

receptor site 3, α-toxins modulate the Na_V channel by slowing down the fast inactivation. β-toxins modulate the activation process by binding to neurotoxin site 4. According to their preference for mammalian or insect Na_V channels, are α-toxins further subdivided into three groups: (i) the classical α-toxins with a preference for mammalian Na_V channel isoforms; (ii) the α-like toxins which are active on both the insect and mammalian Na_V channels; (iii) the insect α-toxins which are capable of discriminating with high affinity between insect and mammalian Na_V channels and thus serve as potential candidates for the development of insecticides (Billen *et al.*, 2008; Gordon *et al.*, 2007; Gurevitz *et al.*, 1998).

Similar to the α-toxins are the β-toxins also classified into 3 groups according to their pharmacological properties, exemplified by their preference for mammalian or insect sodium channels: (i) Mammalian-selective β-toxins are highly toxic to mammals (Martin *et al.*, 1987); (ii) β-like toxins are capable of competing for binding sites on both insect and mammalian Na_V channels; (iii) Insect-selective β-toxins fail to exert any affinity whatsoever for mammalian sodium channels, even in very high concentrations (de Dianous *et al.*, 1987). Exactly this complete lack of mammal activity combined with their strong insect specificity and potency makes these insect-selective β-toxins interesting lead compounds in the design of new insecticides (Gurevitz *et al.*, 2007). The insect-selective β-toxins can be further subdivided into excitatory and depressant toxins according to the symptoms they evoke *in vivo*. Injection of excitatory toxins induces a fast repetitive activity of motor nerves which results in a reversible contraction paralysis (Billen *et al.*, 2008; Zlotkin *et al.*, 1985). These excitatory toxins differ from the other β-toxins because there is one disulfide bridge differently located and furthermore they do display extra secondary structural elements (Gurevitz *et al.*, 1998; Rodriguez de la Vega & Possani, 2005). The depressant toxins cause a transient contraction followed by a slow depressant and flaccid paralysis (Karbat *et al.*, 2007; Zlotkin *et al.*, 1991). Remarkably, insect-selective depressant β-toxins are given the opportunity to affect mammalian channels when these channels are excited by a long preconditioning depolarizing prepulse. This potential prerequisite to affect mammalian Na_V channels possibly contributes to the observed lack of toxicity of depressant toxins towards mammals.

Predictions suggest that up to 100 000 distinct polypeptides are present in all known scorpion species. At this moment, only 1% thereof has been biochemical and functional characterized (Possani *et al.*, 1999). The high number of yet unknown peptides together with the strong phyla specificity exerted by their neurotoxins validates scorpion venoms as a promising source for novel Na_V channel targeting insecticides.

5.3 Sea anemone venoms

Sea anemones are ocean dwelling, solitary animals belong to the phylum Cnidaria. There are over 1400 species described, enclosing more then 45 families which are grouped into the order Actiniaria within the Anthazoa class. Sea anemones are widely distributed around the world as they can be found from the poles to the equator. Most sea anemones live in tidal zones and in shallow water. Sea anemones generally do represent a serious risk for humans as stings usually only cause mild reactions such as inflammation, pain and edema. However, certain tropical species can deliver a more painful sting in which case often also necrosis is observed (Oliveira *et al.*, 2009). Sea anemone venoms are a known pharmacological treasure of biological active compounds. The venom can be divided into two proteic groups of compounds: (i) pore-forming toxins such as hemolysins and actinoporins; (ii) neurotoxins (Beress & Beress, 1975). These neurotoxins are acting upon a

diverse panel of ion channels such as TRPV1, Na_V, K_V and acid-sensing channels (Diochot *et al.*, 2003, 2004). Of these different toxins, those that target Na_V channels are the best studied group with more then 100 known toxins (Bosmans & Tytgat, 2007a). In contrast, no more then 12 K_V channel toxins have been characterized to date. Since the beginning of last century sea anemones have been studied with an increasing interest. Although a number of sea anemone toxins have been isolated and characterized, these animals remain poorly studied in comparison with other venomous animals such as scorpions, spiders, cone snails or snakes. Based on structural differences and activity profile, the sea anemone toxins targeting Na_V channels have been subdivided into 3 groups or types (Honma & Shiomi, 2006). Type 1 and 2 toxins are polypeptides composed of 46-49 amino acid residues, stabilized by the connection 6 cysteine residues forming 3 disulfide bridges. Both type 1 and 2 toxins are believed to have evolved from the same ancestral gene (Ishida *et al.*, 1997). Consequently, type 1 and 2 toxins share the conservation of the six half-cysteines as well as several other residues thought to play a role in biological activity. Nevertheless, from a immunological point of view are these toxins distinguishable since no cross-reactivity occurs (Norton, 1991). Type 1 toxins are characterized by a core of four strands of anti-parallel β-sheets connected by two or three loops (Salceda *et al.*, 2007). Type 3 sea anemone toxins comprise 4 peptides. These toxins contain 30-32 amino acid residues, stabilized by 4 disulfide bridges (Honma & Shiomi, 2006). All 3 types of sea anemone Na_V channel toxins interact with site 3, herby altering the inactivation kinetics.

The validation of sea anemone as a potential source for new neurotoxin derived insecticides based on the existence of crustacean selective peptides in these sea anemones might seem peculiar. However, from an evolutionary point of view, it is understandable why we can encounter potential insecticidal toxins in the venom of sea anemones even when insects and sea anemones will never encounter one another (Bosmans & Tytgat, 2007a). It has been proposed that the 'Pancrustacean' taxon comprises all crustacean and hexapods (Bosmans & Tytgat, 2007a). It should herby be noted that the class Insecta is comprised in the phyla Hexapoda which is a subphylum of the phylum Arthropoda. A monophyletic 'Pancrustacea' taxon has been supported by many molecular studies (Nardi *et al.*, 2003; Shultz & Regier, 2000). In these studies is most of the subphylum Crustacea paraphyletic with respect to insects. Therefore it can be concluded that insects are descendents from crustacean ancestors and thus by definition can crustacean-selective toxins be considered as insect-selective toxins. Based on these peculiar evolutionary and pharmacological arguments have sea anemone venoms earned there status of promising source for new pesticides.

6. Neurotoxin receptor sites of Na_V channels as molecular targets of insect-selective toxins

6.1 Insect-selective inhibiters of the sodium conductance: site 1 *toxins?*

The amino acid residues that form the neurotoxin receptor site 1 are primarily located in the pore loop which is formed by the membrane dipping part of the connecting loop between S5 and S6 of each domain (Catterall *et al.*, 2007). This site is occupied by the water soluble heterocyclic guanidine tetrodotoxin (TTX) which is isolated from the tissue of at least 40 different species of puffer fish but it can also be found in mollusks, crabs, octopus and frogs (Catterall *et al.*, 2007; Hwang *et al.*, 1991). TTX exerts its strong toxicity and high fatality, also towards humans, by binding within the inner pore of the channel, physically occluding the ion pathway. Based on their sensitivity to TTX have the mammalian isoforms been divided

into TTX-sensitive ($Na_V1.1$-$Na_V1.4$, $Na_V1.6$ and $Na_V1.7$) or TTX-insensitive ($Na_V1.5$, $Na_v1.8$, $Na_V1.9$) (Narahashi, 2008).

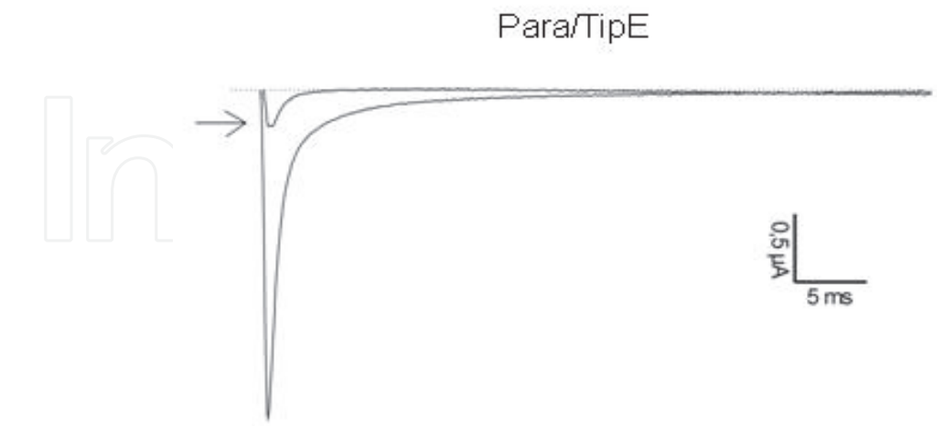

Fig. 3. Effects of site 1 toxins on insect Na_V channels. Panel represents the effects of site 1 toxins on insect Na_V channels expressed in *Xenopus laevis* oocytes. The arrow indicates the steady-state condition after application of site 1 toxin. Binding upon site 1 causes an inhibition of the sodium conductance (indicated by the arrow). Currents were evoked by 100 ms depolarizations to 0 mV, from a holding potential of -90 mV.

Fig. 4. Hainantoxin-I. Sequence and structural model of the insect-selective spider toxin Hainantoxin-I which is folded accordingly the ICK motif (PDB identification number: 1NIX).

Besides TTX, it has also been shown that µ-conotoxins, neurotoxin peptides isolated from Cone snail species, bind to a micro site within the neurotoxin receptor site 1 (Stephan *et al.*, 1994). Even though these µ-conotoxins are known to be highly selective and potent towards mammalian sodium channel isoforms, little investigation has been done regarding their activity towards insect sodium channels. No insect selective µ-conotoxin has been described yet. However, a recent study has reported that the δ-conotoxin TxVIA, which binds to receptor site 6 on Na_V channels, shows insecticidal activity when injected into lepidopteran (cabbage moth) and dipteran (house fly) larvae, suggesting that Cone snail neurotoxins

might have an interesting insecticidal potential (Bruce *et al.*, 2011). For example, the terrestrial molluscan crop pest, *Deroceras reticulatum* or grey field slug, is the most damaging molluscan crop pest in the UK. Annual applications of pellets containing over 250 tonnes of active ingredients are estimated to cost approx. 34 million euro per annum (Bruce *et al.*, 2011). As such it can be reasoned that Cone snail species, which have other mollusks as natural competitors, could be a yet unexplored source of neurotoxin-derived novel insecticides.

6.1.1 Site 1 toxins from spider venom

Huwentoxins and hainantoxins, isolated from the Chinese bird spiders *Ornithoctonus huwena* and *Ornithoctonus hainana* respectively, all belong to the same family of spider toxins. They are constituted of 33-35 amino acids, cross-linked by 3 disulfide bridges and folded according to the ICK motif. Both huwentoxins and hainantoxins are found to target Na_V channels (King *et al.*, 2008). Hainantoxin-I (HNTX-I) causes an inhibition of the sodium conductance without alteration of channel inactivation kinetics or of the voltage dependence of steady-state activation (Billen *et al.*, 2008). HNTX-I stabilizes the channels in the inactivated state as demonstrated by the observed hyperpolarizing shift in the voltage dependence of steady-state inactivation. Electrophysiological studies have shown that HNTX-1 displays a 15 fold-selectivity for insect Na_V channels over the mammalian channel $rNa_V1.2$. HNTX-I does not show any affinity for other mammalian Na_V channel isoforms and is therefore an interesting lead in the development of insecticides (Li *et al.*, 2003).

Huwentoxin IV and hainantoxin III-V are also capable of reducing the sodium conductance but unlike HNTX-I show these toxins affinity for both mammals and insects (Xiao & Liang, 2003). These spider toxins possibly exert there activity through an interaction with site 1 although this remains to be determined.

It has been claimed that this group of toxins is the first family of spider polypeptides capable of selectively blocking Na_V channels by occupying site 1. However, studies have evidenced that huwentoxin IV binds at site 4, trapping the voltage sensor of domain II in its inward position rather than interacting with site 1 or a distinct binding site within the extracellular pore region. Characterization of the huwentoxin IV interaction revealed that this toxin fails to induce any modification on the activation and steady-state inactivation, making it electrophysiological distinguishable from the previously described HNTX-I induced effects (Xiao *et al.*, 2008). Therefore competitive radioligand binding studies are required to confirm for each of these toxins the exact interaction site with the voltage-gated sodium channel. Notwithstanding this lack of structural data, it can be concluded that these spider toxins represent the first family to selectively block the sodium conductance and, moreover, HNTX-1 can be seen as the first insect-selective Na_V channel inhibiting peptide isolated from spider venom (Nicholson, 2007).

To date, no insect-selective neurotoxins capable of inhibiting the sodium conductance have been isolated from the venoms of scorpions or sea anemones.

6.2 Insect-selective gating modifiers of inactivation: site-3 toxins

The neurotoxin receptor 3 is mainly localized at the extracellular loop connecting the segments S3 and S4 from domain IV. It is believed that other parts of the channel such as the extracellular loops between the S5 and S6 of domain I and IV also contribute significantly to channel recognition and binding of site 3 toxins (Bosmans & Tytgat, 2007a). The voltage sensors of each domain will normally move outward under influence of the electric field

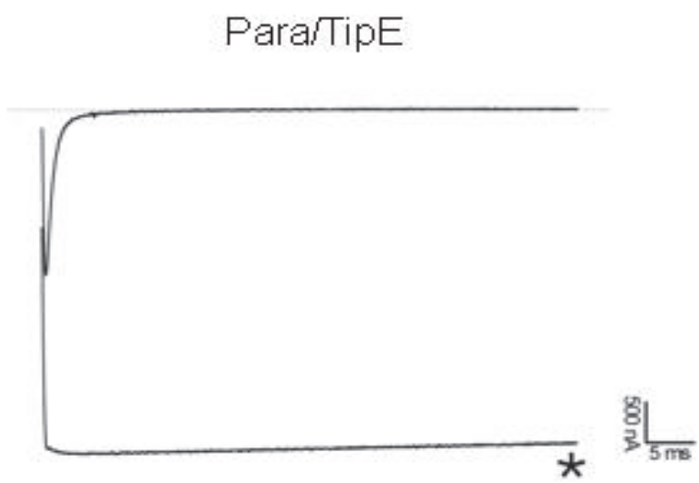

Fig. 5. Effects of site 3 toxins on insect Na_V channels. Panel represents the effects of site 3 toxins on insect Na_V channels expressed in *Xenopus laevis* oocytes. The asteriks indicates the steady-state condition after application of site 3 toxin. Binding upon site 3 causes such an extreme slowing down of inactivation that channels simply do not inactivate anymore. The result is a massive influx of Na^+ ions. Currents were evoked by 100 ms depolarizations to 0 mV, from a holding potential of -90 mV.

when the membrane is depolarized (Hille, 2001). Upon binding at site 3 these toxins trap the voltage sensing segment S4 of domain IV in its inward position. As such they prevent the normal outward movement of these voltage sensors and herewith the conformational changes necessary for fast inactivation (Catterall *et al.*, 2007).
Summarized, toxin binding at neurotoxin receptor 3 affects the coupling of activation and inactivation, resulting in a slowing down or inhibition of the fast inactivation (fig. 5). Several studies have demonstrated the functionally relevant structural differences in insect and mammalian receptor site 3 regions (Gordon *et al.*, 1996). Although more structure-function work is necessary to completely unravel these structural differences, these phyla depending differences do support the arguments to target site 3 in the search for new Na_V channel acting insecticides (Cohen *et al.*, 2006).

```
Magi 2        CMGYDIECNENLPCCKHRKLECVETSGYWWYKRKYCRPIK

Tx4(6-1)      CGDINAACKEDCDCCGYTTACDCYWSKSCKCREAAIVIYTAPKKKLTC
PnTx4(5-5)    CADINGACKSDCDCCGDSVTCDCYWSDSCKCRESNFKIGMAIRKKF-C
PnTx4-3       CGDINAACKEDCDCCGYTTACDCYWSSSCKCREAAIVIYTAPKKKLTC
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Fig. 6. Insect-selective site 3 toxins isolated from spider venom.

Comparison of the amino acid sequences of insect-selective spider toxins which target site 3. Identical residues are boxed in grey, cysteines are shown in red.

6.2.1 Site 3 toxins from spider venom

Several insect-selective spider neurotoxins are known to bind at site 3. Six spider neurotoxins (Magi 1-6) have been isolated from the Japanese funnel-web spider *Macrothele gigas* (Corzo *et al.*, 2003). Magi 1-4 compete for site 3 on insect Na_V channels with the well characterized scorpion toxin LqhαIT in radioligand binding experiments. Magi 2, which is composed of 40 amino acid residues and posses the ICK fold, shares little sequence homology with any other spider toxin. It is an insect-selective toxin as it induces paralysis in insects while it is devoted of any activity on mammals. Like other spider toxins acting on site 3, Magi 2 still awaits delineation of its structure-function relationship in order to elucidate the key residues involved in the insect-selectivity and potent channel modulation of this toxin (Nicholson, 2007). The venom of the South American 'armed' spider *Phoneutra nigriventer* has been extensively studied as this spider accounts for the majority of spider envenomations in Brazil (Borges *et al.*, 2009). Several insecticidal peptides have been isolated from the venom of *P. nigriventer*. One of them, a 48 residue long polypeptide with 5 disulfide bonds called PnTx4(6-1), was shown to exhibit its insecticidal activity through potent modulation of Na_V channels. Moreover, this toxin binds with high affinity to site 3 of insect channels but fails to bind at the mammalian Na_V channel isoforms $rNa_V1.2$ and $rNa_V1.4$, even in high concentrations (de Lima et al., 2002). Furthermore, two other toxins from *P. nigriventer*, PnTx4(5-5) and PnTx4-3, have shown to be highly insecticidal towards houseflies and cockroaches. At the same time, these toxins display no toxicity towards mammals. It has been shown that PnTx4(5-5) acts on NMDA-subtype of glutamate receptors and that PnTx4-3 inhibits glutamate intake possibly through interaction with Na_V channels (Borges *et al.*, 2009). Indeed, because of the high homology in sequence between these two toxins and PnTx4(6-1) it can be hypothesized that both toxin exert there insect-selective activity at least in part through an interaction with Na_V channels.

Other spider neurotoxins which bind to site 3 are the δ-atracotoxins, isolated from the venom of Australian funnel-web spiders. Although δ-atracotoxins modulate insect Na_V channels with high potency, they are of less interest for the development of new insecticides due to there almost equal affinity for both mammalian and insect Na_V channels (Nicholson *et al.*, 2004). Therefore this group of spider toxins will not be discussed in this review.

Magi 2 and PnTx4(6-1) are two examples of a growing group of potent insect-selective Na_V channel toxins from spiders, validating both spider venoms as source of insecticidal peptides and site 3 as a potential target of novel peptide derived insecticides.

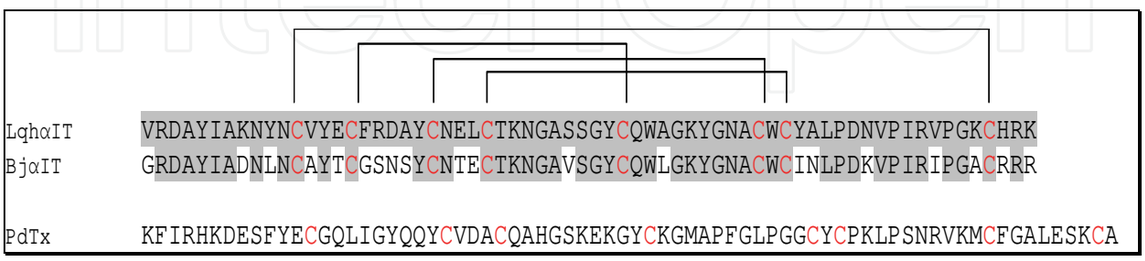

Fig. 7. Insect-selective site 3 toxins isolated from spider venom.

Comparison of the amino acid sequences of insect-selective scorpion toxins which target site 3. Identical residues are boxed in grey, cysteines are shown in red, disulfide-bonding pattern is indicated above sequences.

6.2.2 Site 3 toxins from scorpion venom

The insect α-toxins isolated from scorpion venoms are potently active on insects and at the same time importantly weak or not active on mammals. Mutagenesis studies and toxin crystal structure determinations have shed light upon the structural basis which provides these toxins the ability to discriminate between insect and mammal Na_V channels (Guan *et al.*, 2004). The insect α-toxin LqhαIT, isolated from the scorpion *Leiurus quinquestriatus hebraeus,* is the most potent and best characterized insecticidal scorpion toxin up to date (Gordon *et al.*, 2007). This peptide is 64 amino acids long and contains 8 cysteins. Similar to all scorpion neurotoxins acting on Na_V channels, LqhαIT belongs to the CSαβ structural superfamily (Tugarinov *et al.*, 1997). It serves as a marker toxin for site 3 and is widely used as radiolabeled ligand in binding studies.

More recently, BjαIT was isolated from the black scorpion *Hotentota judaica.* This toxin is an insect-selective toxin acting on site 3 of insect Na_V channels in nM range. Even a tenfold higher concentration displays only weak activity on the mammalian sodium channel isoform $Na_V1.2$ (Arnon *et al.*, 2005).

Phaiodotoxin is a new 72 amino acid long peptide with a disulfide bond pattern which is unique because of the position of the 2 cysteines forming the fourth bridge. This toxin, isolated from the Mexican scorpion *Anuroctonus phaiodactylus*, shares only 49% homology with any known scorpion toxin (Valdez-Cruz *et al.*, 2004). Interestingly, phaiodotoxin acts as an insect-selective toxin with a unique alteration of Na_V channel gating. It causes a negative shift in the voltage dependence of activation and, at the same time, a positive shift in the voltage dependence of inactivation. Both alterations of gating together results in an increased 'window current' by 225%, which is thought to be the cause of its high toxicity toward insects.

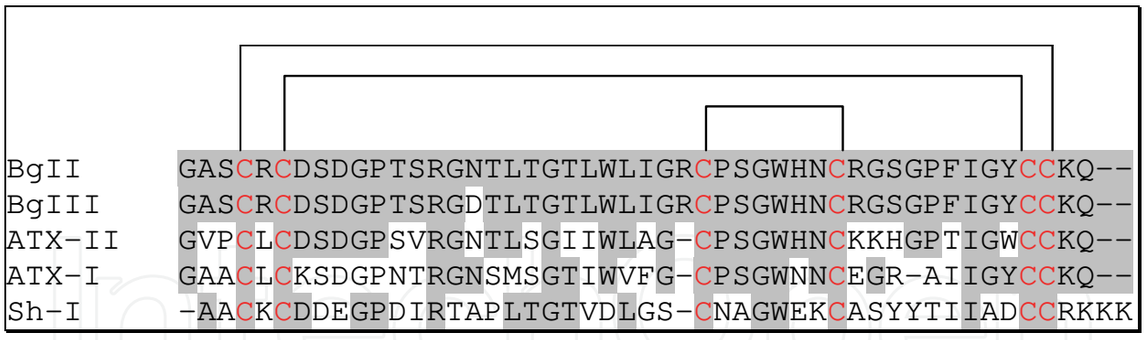

Fig. 8. Insect-selective site 3 toxins isolated from scorpion venom.

Comparison of the amino acid sequences of insect-selective sea anemone toxins which target site 3. Identical residues are boxed in grey, cysteines are shown in red, disulfide-bonding pattern is indicated above sequences.

6.2.3 Site 3 toxins from sea anemone venom

In comparison with scorpion and spider toxins, are sea anemone toxins poorly studied. However, their remarkable insect over mammalian specificity has been noticed already a long time ago (Schweitz, 1984). It was evidenced that the toxin ATX-I, isolated from the wax sea anemone *Anemonia sulcata*, could display preferential toxicity for crabs over mice (Norton, 1991). Since it has been described on a genetic level that there is a link between

crustaceans and insects, one could hypothesize that ATX-I consequently has potential as insect-selective toxin (Boore *et al.*, 1998). Another toxin from the same sea anemone, ATX-II is toxic to both insect and mammalian Na_V channels. However, its binding affinity for cockroach neuronal membranes is very high whereas its binding affinity for rat brain synaptosomes is low (Gordon *et al.*, 1996). Furthermore, ATX-II binds with extreme high potency to site 3 of insect Na_V channels, resulting in a strong slowing down of the fast inactivation (Warmke *et al.*, 1997). Sh-I from *Stichodactyla gigantea* and CgII from *Condylactis gigantea* were found to be moderately toxic to insects but were essentially non-toxic to mammals (Salgado & Kem, 1992).

Two toxins BgII and BgIII, both from *Bunodosoma granulifera*, have been extensively studied. These sea anemone toxins were studied for their activity on cloned mammalian and insect Na_V channels, dorsal root ganglia and rat brain synaptosomes (Bosmans *et al.*, 2002; Goudet *et al.*, 2001). Furthermore were these toxins also thoroughly tested in mice (Loret *et al.*, 1994). These studies revealed that both BgII and BgIII have a preference for insect channel. However, BgII has a 100-fold higher potency on insect channels compared to mammalian channels, BgIII only exert a 5-fold difference in potency of insect over mammal. This dissimilarity in insect-selectivity between both peptides is remarkable in this way that BgII and III only differ from each other in one amino acid, namely an aspargine to aspartate at position 16 (Bosmans *et al.*, 2002).

Another justification for the interest in sea anemone toxins as potential insecticides can be found in the fact that these toxins have a devastating effect on the inactivation of insect channels. Upon binding at site 3, sea anemone toxins cause such an extreme slowing down of inactivation that channels simply do not inactivate anymore (fig. 5) (Bosmans & Tytgat, 2007a).

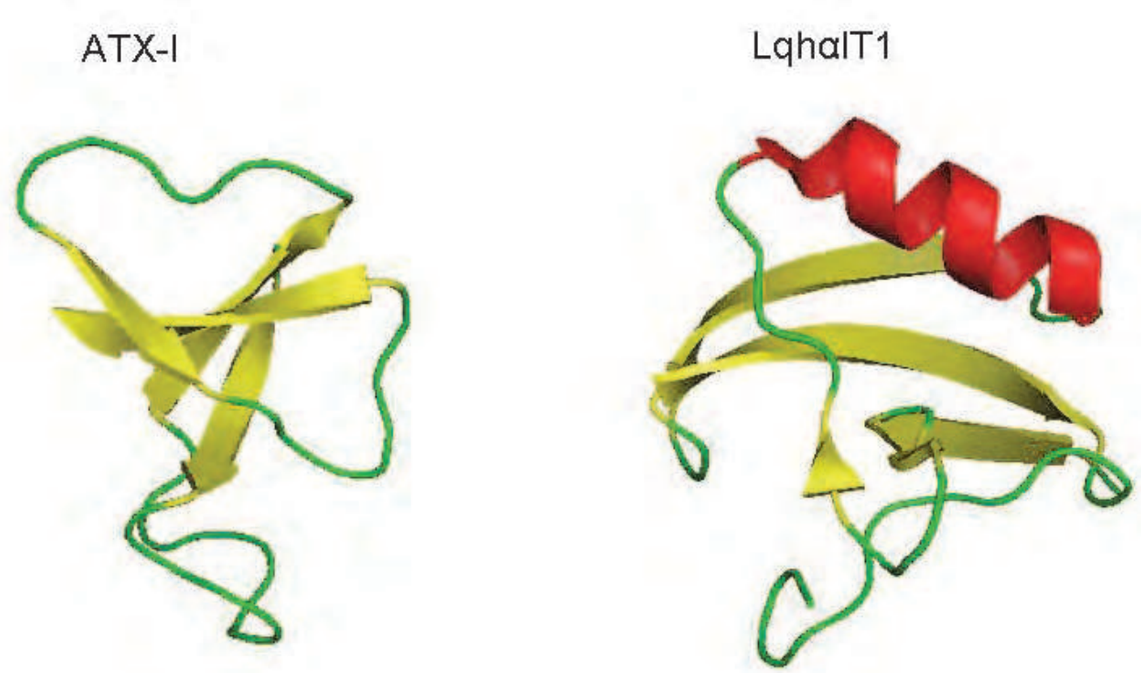

Fig. 9. Structural model of insect-selective site 3 toxins.

Insect-selective sea anemone toxins and insect-selective scorpion toxins, represented by ATX-I and LqhαIT1, respectively. Both toxins exhibit their insecticidal activity by binding upon site 3. PDB ID number:1ATX and 2YEO.

6.3 Insect-selective gating modifiers of activation: site-4 toxins

Toxin binding at site 4 causes a shift in the voltage dependence of activation towards more hyperpolarized membrane potentials and reduces the peak sodium current amplitude (Cestele *et al.*, 2006; Vijverberg *et al.*, 1984). The shift in voltage dependence of Na_V channel activating causes channels to open at, or close to, the resting potential. This increase in open channel probability leads to repetitive firing and consequently increases the influx of Ca^{2+} into the nerve terminals resulting in an increased frequency of miniature excitatory junctional potentials (King *et al.*, 2008). The alterations in channel gating are believed to be a direct result of toxin binding at site 4 leading to a trapping of the voltage sensor in its outward, activated position (Cestele *et al.*, 2001, 2006). The receptor site 4 has been primarily defined to specific residues in the extracellular loops connecting the S1-S2 and S3-S4 segments of domain II (Catterall *et al.*, 2007). However, using the scorpion β-toxin Tz1 (*Tityus zulianus*) it was shown that three residues in the pore loop of domain III are determining for the specificity of β-toxin for different sodium channel isoforms (Leipold *et al.*, 2006). A recent report showed that specific mutations in the voltage sensor of domain III enhance the binding of site 4 toxins to S4 of domain II providing evidence for the involvement of the domain III voltage sensor in the action mechanism of toxins acting on site 4 (Song *et al.*, 2011).

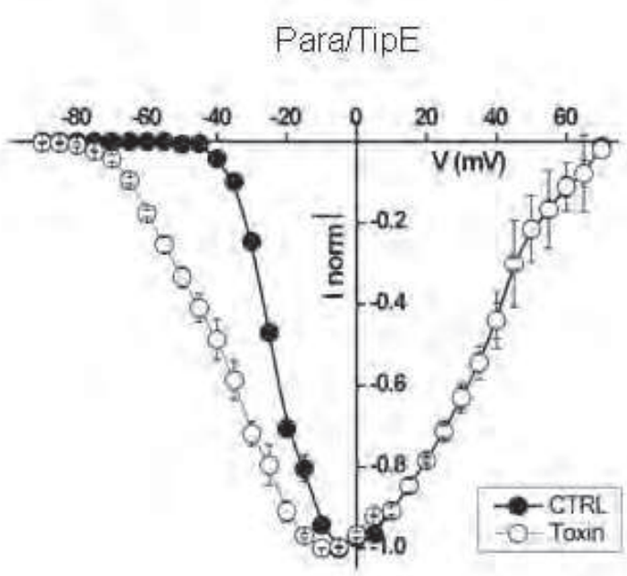

Fig. 10. Effects of site 4 toxins on insect Na_V channels. Panel represents the effects of site 4 toxins on insect Na_V channels expressed in *Xenopus laevis* oocytes. Toxin binding upon site 4 results in a shift in the voltage dependence of activation towards more negative membrane potentials, causing channels to open at potentials they normally remain closed. Currents were, from a holding potential of -90 mV, evoked by 100 ms depolarizations ranging from -90mv to 65 mV in 5 mV increments.

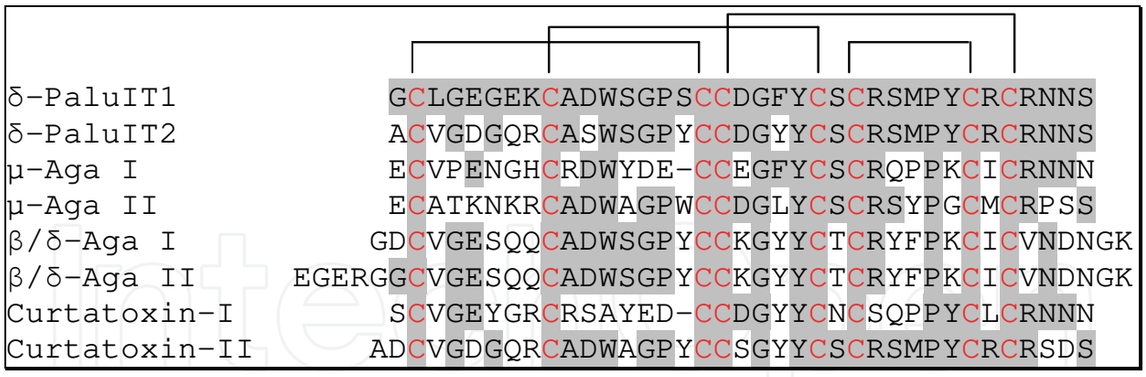

Fig. 11. Insect-selective site 4 toxins isolated from spider venom.

Comparison of the amino acid sequences of insect-selective spider toxins which target site 4. Identical residues are boxed in grey, cysteines are shown in red, disulfide-bonding pattern is indicated.

6.3.1 Site 4 toxins from spider venom

Insect-selective gating modifiers of activation have been isolated from spider venom. A highly selective site 4 toxin, Bs1 has been isolated from the venom of the Mexican theraposid spider *Brachypelma smithi.* Although with low potency, Bs1 could significantly shift the voltage-dependence of activation of insect channels whereas it did not affect mammalian Na_V channel isoforms (Corzo *et al.*, 2008). Another group of insect-selective site 4 spider toxins is constituted by the δ-palutoxins (*Paracoelotes luctuosus*), curtatoxins (*Hololena curta*), μ-agatoxins (*Agelenopsis aperta*) and the recently characterized β/δ-agatoxins (*Agelena orientalis*) (Billen *et al.*, 2010; Corzo *et al.*, 2000; Stapleton *et al.*, 1990). All peptides belonging to this group are structurally related as they are composed of 36-37 residues and cross-linked by 4 disulfide bridges forming an ICK motif (Nicholson, 2007). Little is known about the mechanism of action of the curtatoxin but the highly homologous μ- and β/δ-agatoxins and δ-palutoxins have been well studied. It was reported that the μ-agatoxins could shift the voltage-activation curve towards more hyperpolarized potentials. However, these toxins also slowed down the inactivation process of the sodium channels, resulting in a non-inactivating persistent current (Adams, 2004). The same observations were made for the β/δ-agatoxins and a thoroughly electrophysiological characterization of the action of these agatoxins was performed. It was concluded that agatoxins induce a bell-shaped voltage-dependent modulation of both the activation and the inactivation of insect Na_V channels, suggesting no strict correlation between the toxin binding site and its effect on channel gating (Billen *et al.*, 2010). The insect-selectivity of the δ-palutoxins was designated based on studies showing that these toxins potently modulate insect Na_V channels but fail to exert any affinity for the mammalian Na_V channel isoform $Na_V1.2$ (Ferrat *et al.*, 2005). Mutagenesis studies have confirmed that δ-palutoxins contain the same pharmacological determining key residues as the well studied site 4 toxin Bj-xtrIT. Furthermore, the δ-palutoxins compete with this depressant β-toxin Bj-xtrIT for site 4 but they fail to displace the binding of α-toxin LqhαIT from site 3 (Corzo *et al.*, 2005). Notwithstanding herewith, these toxins act as insect-selective modulators of sodium channels by slowing down the inactivation, a modulation typically seen upon toxin binding at site 3 (Corzo *et al.*, 2000). This remarkable difference in mode of action provides novel perspectives about the structural relatedness of receptor site

3 and 4. Therefore, the topological distinction between these two sites should be questioned. The structural belief that site 3 and 4 rather belong to one extended macrosite merit plausibility as the δ-palutoxins revealed that modulation of inactivation can be achieved by binding to a site which was until now, believed to be associated with alteration on channel activation (Nicholson, 2007).

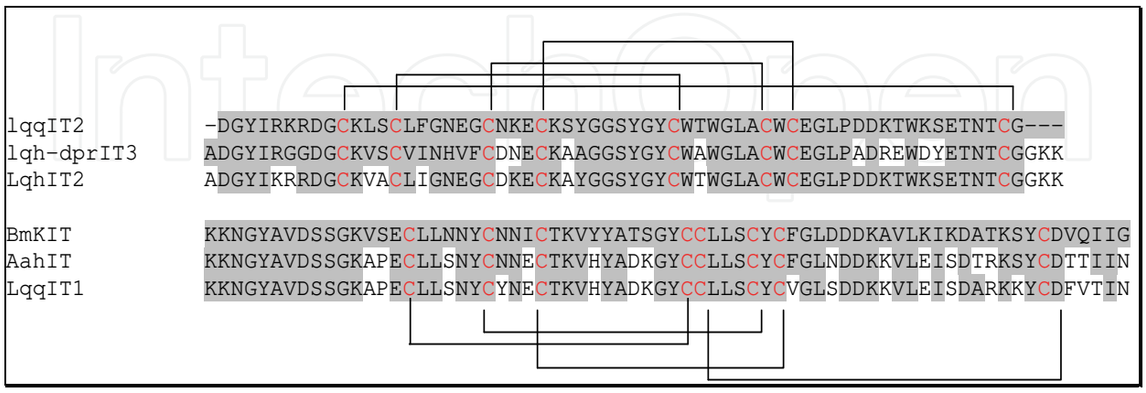

Fig. 12. Insect-selective site 4 toxins isolated from scorpion venom.

Comparison of the amino acid sequences of insect-selective scorpion toxins which target site 4. Identical residues are boxed in grey, cysteines are shown in red. The disulfide-bonding pattern is indicated above the sequences of the depressant toxins and under the sequences of the excitatory toxins, respectively.

6.3.2 Site 4 toxins from scorpion venom

Similar to spiders are scorpions also capable of producing toxins which recognize the neurotoxin receptor site 4. Toxins belonging to the class of insect-selective β-toxins are long chain peptides composed of 58 to 76 amino acids, cross-linked by four disulfide bridges. They belong to the structural superfamily of cysteine stabilized α/β motif containing proteins. The insect-selective β-toxins can be further subdivided into excitatory and depressant toxins according to the symptoms they evoke *in vivo*. AahIT from *Androctonus australis Hector*, LqqIT1 from *Leiurus quinquestriatus quinquestriatus,* Bj-xtrIT isolated from *Hotentota judaica* and BmKIT from *Buthus martensii* Karsch belong to excitatory insect-selective β-toxins (Billen *et al.*, 2008; Froy *et al.*, 1999; Zlotkin *et al.*, 1985). These excitatory toxins differ from the other β-toxins because there is one disulfide bridge differently located and furthermore they do display extra secondary structural elements (de la Vega & Possani, 2007). AahIT induces repetitive firing of action potentials as a result of activation of Na_V channels at lower membrane potentials, explaining the typical contractile paralysis observed in insects injected by excitatory insect-selective β-toxins such as AahIT.

The depressant toxins cause a transient contraction followed by a slow depressant and flaccid paralysis (Karbat *et al.*, 2007; Zlotkin *et al.*, 1991). Current clamp experiments have shown that peptides belonging to this group suppress the evoked action potentials as a result of strong depolarization of the membrane, causing an inability of axons to generate action potentials (Strugatsky *et al.*, 2005). Representatives of this group are LqqIT2 from *Leiurus quinquestriatus quinquestriatus*, BjIT2 from *Buthotus judaicus* and the highly potent and insecticidal toxins lqh-dprIT3 and LqhIT2, isolated from the scorpion *Leiurus quinquestriatus hebraeus* (Zlotkin *et al.*, 1993). It is interesting to note that LqqIT2 did not only cause a

hyperpolarizing shift in the activation of channels, it also affected the inactivation and the ion selectivity (Bosmans *et al.*, 2005). Therefore, the high insecticidal action of this toxin may be attributed to a combined modification of gating kinetics, resulting in Na_V channels that open at more negative membrane potentials and inactivate not normally and thus, display a significantly increased open probability. Remarkably, insect-selective depressant β-toxins are given the opportunity to affect mammalian channels when these channels are excited by a long preconditioning depolarizing prepulse. The same phenomenon is observed in the case of simultaneous binding of an α-toxin upon site 3 (Cohen *et al.*, 2007). As such, it can be seen that the presence of depressant β-toxins in the scorpion venom may still significantly contribute to the toxicity towards mammals.

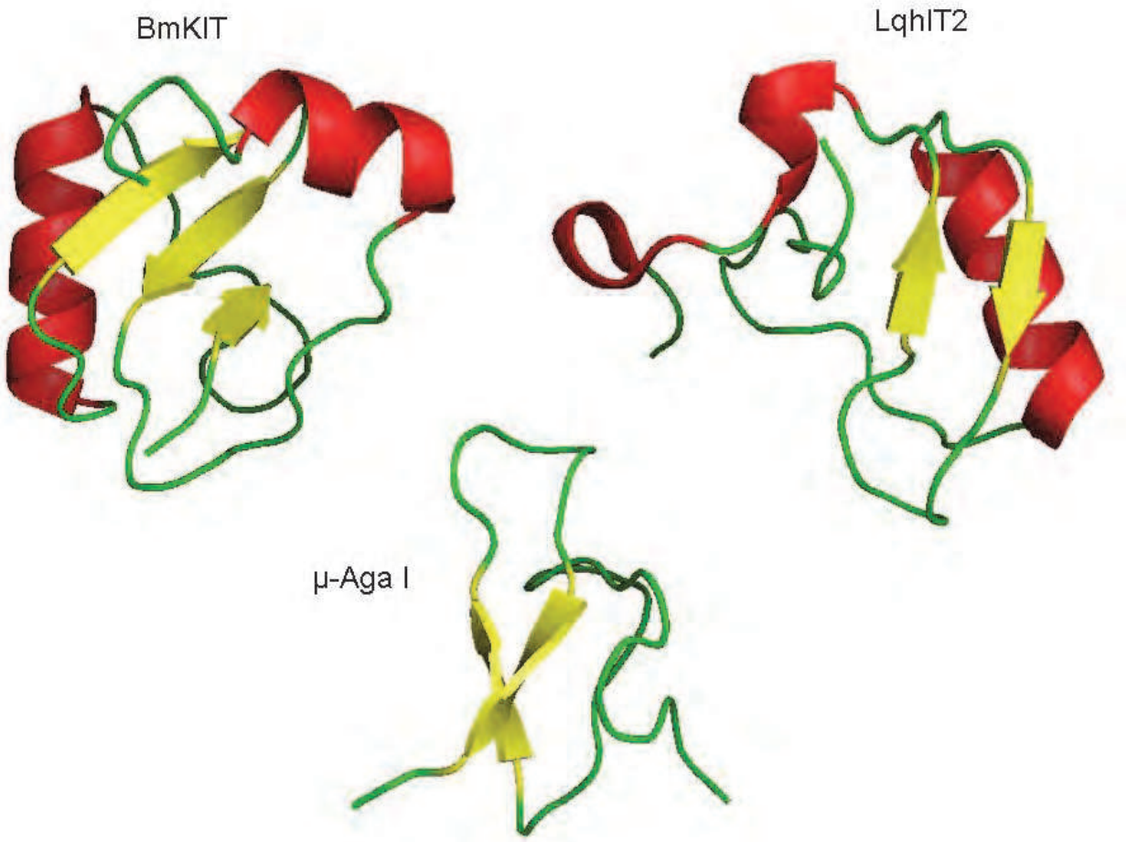

Fig. 13. Structural models of site 4 toxin.

Insect-selective spider and scorpion toxins, represented by μ-Aga1, BmKIT (excitatory toxin) and lqhIT2 (depressant toxin), respectively. Nevertheless these toxins are structurally differing from each other, they all do exert their insecticidal activity through binding upon site 4. PDB ID number: 1EIT, 1WWN and 2I61.

6.4 Insect-selective unidentified Na_V channel interactions: novel site toxins?

Although it has been determined for many spider toxins what the exact site of interaction with the insect Na_V channel is, there are still several insect-selective toxins awaiting further

structure-function and electrophysiological characterization. For example, the exact target site of ACTX-Hi:OB4219, a 38 residue long peptide isolated from the venom of the funnel-web spider *Hadronyche infensa* orchid Beach, has not yet been identified. Determination of its NMR structure revealed that this toxin contains 4 disulfide bridges and is folded accordingly the ICK motif with a triple-stranded antiparallel β-sheet (Rosengren *et al.*, 2002). Interestingly, the cysteine pattern and loop sizes of ACTX-Hi:OB4219 are identical to the μ-agatoxins and to other site 4 spider toxins such as the curtatoxins and δ-palutoxins. However, despite the identical fold is the sequence similarity between ACTX-Hi:OB4219 and these toxins very low. Therefore are electrophysiological and radioligand binding studies required to determine if ACTX-Hi:OB4219 is exerting its insecticidal activity by acting on Na_V channels and if so to identify the exact interaction site with the channel.

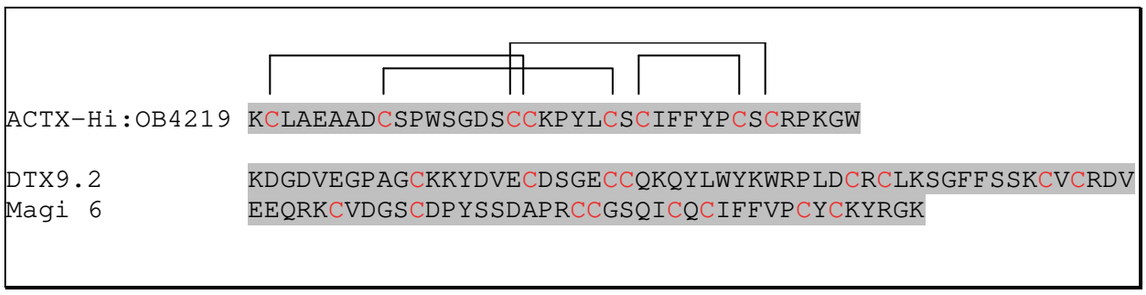

Fig. 13. Insect-selective toxins isolated from spider venom binding on unknown sites. Comparison of the amino acid sequences of insect-selective spider toxins which are still awaiting the designation of their binding site. Identical residues are boxed in grey, cysteines are shown in red. The disulfide-bonding pattern is indicated for ACTX-Hi: 0B4219.

DTX9.2, 10 and 11 constitute a family of 56-61 residue long insecticidal peptides, isolated from the venom of the primitive weaving spider *Diguentia canities* (Krapcho *et al.*, 1995). In vivo studies have shown that these toxins do not affect mice when injected intraperitoneal or intracerebroventricular. Because they do cause progressive spastic paralysis in tobacco budworms, can these spider toxins be considered as potential novel insecticides. DTX9.2 caused depolarizations of cockroach axons and repetitive potential discharges in housefly larvae neuromuscular and sensory nerve preparations. TTX blocked these actions, suggesting the involvement of Na_V channel in the mechanism of action of DTX9.2 (Bloomquist *et al.*, 1996). Radioligand binding studies have indicated that it is unlikely that DTX9.2 interacts with site 3 and thus are further structure-function studies necessary to identify the precise target of these interesting insect-selective toxins (Nicholson, 2007).

An ICK motif possessing peptide of 69 residues named Magi 6, from *Macrothele gigas,* induces flaccid paralysis in insects with an even higher potency than the earlier described site 3 toxin Magi 2. However, in contrast to Magi 2, does the site of action for Magi 6 remain unknown. Competitive binding studies indicated that Magi 6 does not compete for sites 3, 4 or 6, neither on insect or mammalian Na_V channels (Corzo *et al.*, 2003). The rapid and strong lethality of Magi 6 when injected in insects, is suggestive of a strong antagonist action on the insect nervous system. Therefore it seems that Magi 6 targets a, for now, unknown site on Na_V channels. Further structure-function studies are awaiting to determine if this is true for Magi 6 or that this toxin is rather interacting with another receptor.

7. The application of insect-selective toxins in the development of novel insecticides

There are 2 main issues that complicate further intensive use of classical industrial pesticides such as DDT and pyrethroids: firstly the growing public awareness of the health hazards and environmental damage caused by conventional agrochemical pesticides has resulted in a general public condemnation regarding the use of these agents (Dong, 2007; Hassan *et al.*, 1990). Secondly, the widespread use of the classical pesticides, together with their limited number of nervous system targets, has resulted in a far-reaching resistance among arthropod populations responsible for the transmission of many human diseases or among pest species involved in major devastation of crops (Nicholson, 2007; Zlotkin *et al.*, 2000). Together, both issues have led to a situation were the current demand of new pesticides is focusing on the one hand on insect-selective agents, minimizing the risk for human health and, on the other hand on potent agents, minimizing the influence on the environment.
Fulfilling this urgent need for new, potent and insect-selective insecticides by insecticidal neurotoxins derived from venomous animals implicates the requirement of formulations which provide these peptide toxins to reach their targets within the circulatory system of insects.
In nature, spiders bite and scorpions and sea anemones sting to deliver the venom directly in the bloodstream of their prey or offender, allowing the neurotoxins to get in direct contact with the Na_V channels. Peptide toxins and polypeptides in general do not penetrate the insect gut and are little to not at all resistant against the proteolytic environment of the insect digestive system. As such does the industrial use of these neurotoxins as insecticidal agents only has prospects depending on the development of means to guarantee a sufficient delivery of peptide toxins to their targets. Therefore a lot of effort has been done to improve the bioavailability of peptides in general and insecticidal peptide toxins specifically. Several strategies are used to overcome the difficulties associated with peptide bioavailability and thus improve the insecticidal potency of neurotoxins.
To implement the insecticidal capacity of polypeptides the following toxin modifications have been proven to increase the bioavailability: (i) Incorporation of the toxin in balcoviruses; (ii) Incorporation of toxins in plants; (iii) Chemical approaches to increase toxin stability and resistance against proteolysis.

7.1 Incorporation of insect-selective toxins in recombinant baculoviruses: biopesticides

One approach to develop environmentally friendly measures to face the highly resistant insect species is the release of baculoviruses that have been genetically engineered to express insecticidal neurotoxins. Exactly because of their safety and insect specificity are insect baculoviruses of great interest as enhancer of neurotoxin bioavailability. The infectivity of baculoviruses is limited to a few closely related species within a single insect family, favoring these viruses as environmental safe insecticides. Their great advantage arises from the fact that baculoviruses do not replace the natural predators, as is the case with chemical insecticides, but rather complements these predators (Zlotkin *et al.*, 2000). From an evolutionary point of view have these viruses adapted themselves for self-preservation and propagation (Zlotkin, 1999). These baculoviruses have developed a strategy as such to keep their host alive as long as possible allowing a maximal progeny production (Bloomquist *et al.*, 1996). This strategy of acting as slow as possible, and in the

mean time permitting the host insect larvae to feed continuously on the crops, is of course a major drawback in an efficient pesticide employment. However, this drawback has been overcome by genetically engineering of baculoviruses to potentiate their insecticidal activity and efficacy. The improved efficacy results from a significant reduced time that the viruses allow their host to feed itself (Bonning & Hammock, 1996). This enhancement of the insecticidal activity is achieved by engineering nucleopolyhedroviruses in such a way that their natural insect pathogenicity is combined with the expression of insect-selective neurotoxins (Gershburg *et al.*, 1998). As such a 30-40% improved insecticidal activity is achieved (Chejanovsky *et al.*, 1995).

The gene encoding for a insect-selective neurotoxin is subcloned into a transfer vector plasmid, which is then cotransfected with the parental baculovirus DNA into an appropriate insect cell line so that the neurotoxin encoding gene is transferred to the baculovirus by homologous recombination (Zlotkin *et al.*, 2000). Insect-selective neurotoxins from spiders, scorpions and sea anemones have been used to the construction of recombinant baculoviruses. Nevertheless, currently there is a limited use of recombinant baculoviruses expressing insect-selective neurotoxins mainly due the lack of well-characterized toxins to be selected from as potential candidates for incorporation into the baculovirus genome. Therefore it is still essential to explore the large pharmacological libraries contained within various venomous animals (Nicholson, 2007).

Even though the insecticidal efficacy of sea anemone and scorpion toxins is higher compared to spider toxins, it should be noted that **spider** Na_V channel toxins do posses some structural features which favors them as candidates for recombinant expression. Spider toxins are smaller in size and because of the smaller number of disulfide bridges, are they more likely to fold correctly. Furthermore, all characterized insect-selective spider toxins up to date are composed of the ICK motif. This conserved scaffold provides spider toxins a highly chemical stability and a strong resistance to a denaturizing and proteolytic environment (Nicholson, 2007; Norton & Pallaghy, 1998). Three spider toxins active on insect Na_V channels have been successfully incorporated into baculovirus genome: μ-Aga IV from *Agelenopsis aperta*, DTX9.2 from *Diguentia canities* and Ta1TX-1 from the hobo spider *Tegenaria agrestis* (Hughes *et al.*, 1997; Tomalski *et al.*, 1989). Genes encoding for DTX9.2 or Ta1TX-1 were inserted into the *Autographa californica* multiple nuclear polyhedrosis baculovirus (AcMNPV) (Krapcho *et al.*, 1995). AcMNPV is a baculovirus of wide interest as it is known to infect a variation of important lepidopterus pests (McCutchen *et al.*, 1991). The efficacy of the recombinant baculoviruses expressing these insect selective spider toxins were evaluated in three important lepidopteran insect pests, *Trichoplusia* ni Hubner, *Spodoptera exigua* and *Heliothis virescens.* It was demonstrated that both DTX9.2 and Ta1TX-1 expressing baculoviruses reduced the host feeding time up to 40% and caused a reduction in host survival time ranging from 18% to 25%. Interestingly, the DTX9.2 expressing virus stopped the feeding faster while the Ta1TX-1 virus killed the hosts faster, suggesting that DTX9.2 is more promising in effective reducing crop damage (Hughes *et al.*, 1997).

Similar results were obtained when the site 4 spider toxin, μ-Aga IV was expressed by AcMNPV. Infection of lepidopteran larvae with these recombinant baculoviruses resulted in a dramatic improvement of insecticidal efficacy, increasing killing time up to 50% (Prikhod'ko & Miller, 1996). The first insect-specific toxin gene to be transferred into a baculovirus was the highly insecticidal excitatory **scorpion** β-toxin AaIT (*Androctonus australis* Hector) (Stewart *et al.*, 1991). Lepidopterous larvae when infected with an AaIT expressing recombinant baculovirus such as BmNPV-AaIT, have a strongly reduced

survival time and hence damage a significant lower amount of host plants compared to wild type viruses. The expressed insect-selective neurotoxin found in the circulation of the infected insects is characterized by the following important properties: (i) Chemical identity with native toxin; (ii) The expressed toxin causes the same physiological symptoms in vivo as the injected native one. In the case of AaIT this is the typical contractile paralysis observed in insects injected by excitatory insect-selective β-toxin. (iii) Expressed neurotoxins display an enhanced affectivity, designated as toxin potentiation and depicted by the fact that the expressed toxin requires a much lower hemolymph titer than the injected native one to cause similar paralysis (Maeda, 1989). It was assumed that this potentiation was the obvious consequence of the insecticidal pharmacokinetic-targeting cooperativity of both the recombinant baculovirus and its expressed scorpion toxin AaIT. However, later studies have indicated that the toxin potentiation observed for expressed toxins is the outcome of two phenomena. Firstly, the continuously toxin expression by the virus infected tracheal epithelia, ramifying within the insect and as thus creating a baculovirus conduit, hereby constituting a pursuing overall supply of newly generated toxin to its specific target namely the insect Na_V channels. Secondly, the translocation of the toxin gene into the central nervous system of the insects provides the expressed toxin a free pathway to the target site which is inaccessible to the native toxin (Zlotkin *et al.*, 2000).

Several other insect-selective scorpion toxin have also been field-applicated in combination with baculoviruses. A comparative study was conducted in which the insecticidal efficacy of recombinant expressed depressant β-toxins was paralleled with this of excitatory β-toxins. This study revealed that the depressant toxin LqhIT2 displayed a higher insecticidal activity compared to its excitatory counterpart LqhIT1 (Gershburg *et al.*, 1998). The observed difference in activity between these toxins, isolated from the scorpion *Leiurus quinquestriatus hebraeus,* was surprising as excitatory β-toxins generate an immediate paralyzing effect whereas depressant β-toxins generate a delayed effect. The difference might be explained by pharmacokinetic differences resulting from a higher stability and thus increased bioavailability of depressant compared to excitatory β-toxins (Gershburg *et al.*, 1998).

Two insect-selective **sea anemone** toxins targeting Na_V channels have been used in the recombinant baculovirus strategy. The site 3 toxins As II, from the sea anemone *Anemonia sulcata* and Sh I isolated from *Stichodactyla helianthus*, displayed an effective ability to reduce crop damage upon expression by AcMNPV (Prikhod'ko & Miller, 1996). Furthermore, the construction of AcMNPV expressing both the site 3 toxin As II and the site 4 toxin μ-Aga-IV, resulted in a synergistic enhanced insecticidal activity, underlining the potentiating of insecticidal activity by coproduction of toxins targeting allostericaly coupled sites of Na_V channels (Nicholson, 2007).

7.2 Incorporation of insect-selective toxins in plants: transgenic plants

Another strategy in the development of potent insect-selective insecticides without burden for the environment is the incorporation of neurotoxins in the genome of plants.

It has been shown that the **spider** toxin ω-ACTX-Hv1a, from *Hadronyche versuta*, displayed remarkable insecticidal activity upon incorporation into the tobacco plant *Nicotiana tabacum* (Fletcher *et al.*, 1997). Although ω-ACTX-Hv1a is an insect-selective Ca_V and not a Na_V channel toxin, the insecticidal efficacy of ω-ACTX-Hv1a fusion proteins against important pest species of transgenic plants expressing this insect-selective spider toxin is a strong argument evidencing the efficiency of the transgenic approach. Nevertheless, no insect-selective spider neurotoxins targeting Na_V channels have been selected to be incorporated in

plants (Nicholson, 2007). However, the insect-selective spider toxin Magi 6, isolated from the Japanese spider *Macrothele gigas*, has been successfully expressed in transgenic tobacco. As described earlier is the exact target of this toxin still unknown, although its potent effects *in vivo* do suggest that Magi 6 is acting on a receptor within the nervous system (Corzo *et al.*, 2003). The incorporation of Magi 6 in transgenic plants conferred resistance to several insect pests and opens the possibility of employing this spider toxin to improve the resistance of diverse plants (Hernandez-Campuzano *et al.*, 2009).

Several **scorpion** toxins active on Na_V channels are successfully used as insecticide by application of the transgenic approach. A gene encoding for the high insecticidal scorpion toxin BmKIT from *Buthus martensi Karsch* was, in combination with a gene encoding for a insect-specific chinitase, from *Manduca sexta*, introduced into *Brassica napus* plants rendering these plants resistance against agricultural important pests (Wang *et al.*, 2005). Chitin is a major component is of the cuticle and gut epidermis of many lepidoptera. The chitinase gene is normally not expressed in insects during feeding periods but only in a narrow period prior to molting (Kramer *et al.*, 1993). Continuously exposure to chitinase might lead to malfunction because of chitin degradation and loss of structural integrity of the gut epidermis (Regev *et al.*, 1996). The loss of this absorption barrier in the gut may also enhance the bioavailability of the co expressed neurotoxin as the access pathway for these toxins to reach their targets is strongly facilitated (Wang *et al.*, 2005).

7.3 Improved toxin stability and resistance equals improved activity?

A general criticism upon peptide-based insecticides might be the fact that these Na_V channel targeting toxins despite their extraordinary insecticidal properties potentially suffer from generic problems encountered by all peptide-based drugs, such as short biological half-lives, proteolysis and poor absorption. However, in the last decade there has been an impressive number of studies focusing on the re-engineering of peptide toxins to address these biopharmaceutical problems (Halai & Craik, 2009). Even though the aim of application is different between clinical used drugs and insecticides, the properties of their general pharmacokinetic pathways are identical. As such can techniques used to improve pharmacological availability of peptide-based drugs be very useful to enhance the bioavailability of peptide-based insecticides.

One example of a very effective strategy to improve the drug-like properties of toxins is backbone cyclization, by either the addition of a linker to span the peptide termini or directly by head-to-tail linkage. The resulting cyclic peptides may have an improved binding efficacy but more over display an impressive decrease in susceptibility to proteolysis (Clark *et al.*, 2010). Many microorganisms are known to produce cyclic peptides, exemplified by the well known fungal product cyclosporine which has revolutionized organ transplant therapy because of its potent immunosuppressant activities (Clark *et al.*, 2010; Starzl, 1981). Furthermore, peptide cyclization is a widely used technique in the pharmaceutical industry in the development of a variety of drug design applications (Craik & Adams, 2007). For example, the conotoxin MII, isolated from the *Conus magus*, targets with high potency nicotine acetylcholine receptors (Shon *et al.*, 1997)). Cyclyzation of this toxin by adding a linker of seven amino acids resulted in maintenance of the structure of the native peptide and similar biological activity, but with an importantly enhanced resistance against proteolytic breakdown (Clark *et al.*, 2005). Cyclic peptides used in pharmaceutical applications are usual smaller than 12 amino acids which is noticeable shorter than most Na_V channel targeting neurotoxins described in this review. However, a large number of

naturally occurring disulfide-rich cyclic peptides have been discovered in plants and animals and all of these natural cyclic peptides share the remarkable feature of displaying an exceptional stability and a compact structure (Clark *et al.*, 2010). This suggests that cyclization of highly insecticidal neurotoxins from spiders, scorpions and sea anemone might be potentially useful to enhance their bioavailability by strongly increasing their stability and their resistance against proteolytic degradation.

For peptide toxins and in general for all proteins, several amino acids are sensitive for chemical degradation. Therefore can potential stability problems with peptide-based insecticides be avoided by replacing these residues, provided of course that substituting these residues does not lead to a loss of biological activity (Craik & Adams, 2007). Examples of amino acids susceptible to chemical degradation are deamidation of aspargine, oxidation of methionine and isomerization or cleavage of aspargine-proline bonds (Wakankar & Borchardt, 2006).

A widely used technique in the peptide chemistry is the substitution of $_{L}$-amino acids for $_{D}$-amino acids to improve the resistance against proteases. The use of $_{D}$-amino acids is particularly common in peptide-based drug design of cyclic peptides as described above (Schroeder *et al.*, 2004). An interesting approach to improve the activity of the *Conus striatus* toxin SIIIA is the incorporation of non-natural backbone spacers.

The use of flexible spacers such as amino-3-oxypenatnoic and 6-aminohexanoic acids to replace conformational constrained parts of the three disulfide bridges in SIIIA resulted in an enhanced activity (Green *et al.*, 2007). Since SIIIA is a highly potent inhibitor of mammalian Na_V channels and since it is folded accordingly the ICK motif which is shared by many insect-selective toxins, these observations of enhanced activity are of peculiar interest for the development of new insect-selective insecticides which act by inhibiting the sodium conductance such as HNTX-1.

Other approaches to improve bioavailability are the use of lipid tags on selected residues, terminal capping and peptide truncation. Capping involves C-terminal amidation because it is known that amidation leads to a reduced sensitivity to proteolytic breakdown by carboxypeptidases. Peptide truncation finds it application trough the rationale that residues lying outside the defined cystine framework, display an increased susceptibility to proteolytic or chemical degradation due to the fact that these residues are likely to be more flexible (Craik & Adams, 2007).

All together it can be seen that the increasing knowledge on bypassing the generic barriers insures that the exquisite potency and selectivity of insecticidal peptide toxins derived from venomous animals can be exploited as promising lead compounds in the development of novel peptide-based pesticides.

8. Conclusions and further directions

Arthropods are the most widespread and diverse group of animals worldwide. Even though only a small percentage of all arthropods are considered as pest species, there impact on human society is of great importance. Vector-borne human and veterinary diseases, in which pest species function as vectors for transmission, are of increasing concern to the general population and more specific to the public health. They represent a significant threat to the productivity, health, the normal lifecycle of humans, livestock, companion animals and wild life, and by generalization, to the viability of live on earth (Nicholson, 2007).

Pest species are responsible for major devastations of crops, destroying up to 20 % of the annual crop production worldwide (Nicholson, 2007). An increasing demand on crop production to feed the ever increasing world population, together with the fact that the majority of the world fertile land is being exploited raises a global insurmountable awareness for the urgent need of environmental compatible insecticides, capable of increasing crop production yields by decreasing the losses accountable by pest species. The increasing factors limiting the efficacy of conventional agrochemical pesticides are therefore worrying. Due to the wide usages and the narrow target range of classical agrichemicals, such as DDT and pyrethroids, arthropods have been submitted to a high degree of selection pressure, resulting in pest species with a far reaching resistance against these agents (Brogdon & McAllister, 1998). Consequently, the development of novel, potent and selective pesticides has become crucial for global welfare. Insect-selective neurotoxins might significantly contribute to the accomplishment of novel insecticides.

The range of organisms that is producing toxins for defense or for capturing their arthropod prey is remarkable diverse. Toxins isolated from the venoms of spiders, scorpions and sea anemones, as described in this review, are of particular interest because of their importantly high selectivity for insects over mammals, great potency and environmental friendly character. Spiders, scorpions and sea anemones have developed during their evolution a unique, complex pre-optimized combinatorial peptide library of neurotoxins which is now available as a valuable source of novel insecticides.

Voltage-gated sodium channels have gained great interest as target for future pesticides. This is mainly because of their important role in excitability, their pharmacological diversity exhibited in the large number of binding sites and their pharmacological distinctiveness as revealed by the ability of insect-selective neurotoxins to discriminate strongly between insect and mammalian Na_V channels (Zlotkin, 1999). A very important argument, favoring both neurotoxins and Na_V channels as key elements in the development of new insecticides is the existence of allostericly coupled sites as potentiating of the insecticidal activity. For instance, it has been shown that pyrethroids and scorpion toxins can operate synergistic with one another (Gilles *et al*., 2003). It was found that insects, which were first infected with a baculovirus expressing the scorpion toxin AahIT, were much more sensitive to the effects of pyrethroids then non-infected species (Sharkey *et al*., 1987). Interesting to note is the fact that pyrethroid-resistant strains do display an enhanced sensitivity towards AahIT and other site 4 toxins (Zlotkin, 1999). Furthermore it was also demonstrated that site 2, 3 and 5 toxins could enhance pyrethroid binding to locust neuronal membranes (Gilles *et al*., 2003). Using the toxin ATX II as a site 3 toxin representative, it was shown that sea anemone toxins could similarly enhance the potency of DDT and other pyrethriods (Bloomquist & Soderlund, 1988). All together these results do imply the interesting possibility to use the synergistic interaction of insect-selective Na_V channel toxins in order to not only overcome resistance but also to reduce the concentration of each toxin required to obtain an efficient control of pest species.

The synergistic action of insecticides should be seen beyond the boundaries of the Na_V channel toxins. Their exist many other interesting targets for insecticides such as Ca_V, K_V, GABA-gated Cl channels or other receptors such as nicotine acetylcholine receptors. Furthermore, baculoviruses, snowdrop lectine and chitinases have proven to be very useful in the battle against pest species whether this is because of their direct insecticidal properties or because they function as ideal transport vector for insect-selective toxins. It is recommendable to develop over the long term a strategy of applying synergistic mixtures

containing a variety of insecticidal substances acting on a broad range of different targets. The synergistic character of these mixtures will be the result of positive cooperativity between insecticidal agents acting on different targets and/or agents acting on allosterically coupled sites within the same target. Employment of such mixtures will significantly reduce the required doses of each insecticide and herewith not only their production cost but also their impact on the environment and public health.

Insect-selective Na_V channel toxins from spiders, scorpion and sea anemones can contribute importantly to these synergistic mixtures. They deserve to be lead compounds in the development of new insecticides thanks to the exquisite selectivity, unseen affinity and high potency displayed towards their target, the insect voltage-gated sodium channel.

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10. References

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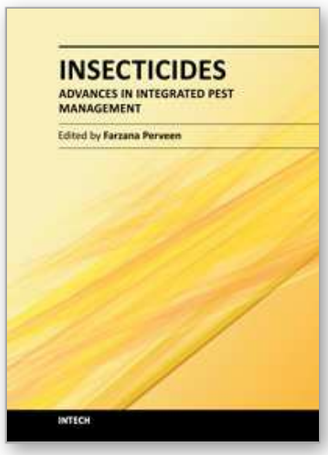

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This book contains 30 Chapters divided into 5 Sections. Section A covers integrated pest management, alternative insect control strategies, ecological impact of insecticides as well as pesticides and drugs of forensic interest. Section B is dedicated to chemical control and health risks, applications for insecticides, metabolism of pesticides by human cytochrome p450, etc. Section C provides biochemical analyses of action of chlorfluazuron, pest control effects on seed yield, chemical ecology, quality control, development of ideal insecticide, insecticide resistance, etc. Section D reviews current analytical methods, electroanalysis of insecticides, insecticide activity and secondary metabolites. Section E provides data contributing to better understanding of biological control through Bacillus sphaericus and B. thuringiensis, entomopathogenic nematodes insecticides, vector-borne disease, etc. The subject matter in this book should attract the reader's concern to support rational decisions regarding the use of pesticides.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

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