Section 1

Molecular Mechanisms and Modes of Action of Microalgal Toxins

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The Mechanism of Action of Microalgal Toxins Interacting with Na_v and K_v Channels

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Introduction

Several species of marine microalgae produce biotoxins that are responsible for massive fish kills and seafood-related poisonings in humans (e.g., paralytic shellfish poisoning and ciguatera poisoning). The symptoms are often neurological and/or gastrointestinal and linked to an altered cellular excitability. Voltage-gated ion channels are transmembrane proteins that regulate and control the cellular excitability in the entire body and therefore serve as primary molecular targets for marine guanidinium and cyclic polyether toxins (Fig. 1 in McNabb 2014). Here we focus on the molecular

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Fig. 1. A. Schematic two-dimensional representation of the functional α -subunit of Na_v and K_v channels. B. Top view showing the receptor site 1 (red) on Na_v channels and receptor site 5 (green) on Na_v and K_v channels.

Color image of this figure appears in the color plate section at the end of the book.

aspect of the mechanism of action of saxitoxins (and tetrodotoxins), brevetoxins, ciguatoxins and gambierol on voltage-gated sodium (Na_v) and potassium (K_v) channels.

Voltage-gated sodium channels

Voltage-gated sodium (Na_v) channels are transmembrane protein complexes composed of an α -subunit of approximately 260 kDa which can be associated with up to four auxiliary β -subunits ($\beta 1$ -4) of 30 to 40 kDa (Catterall 2012). The pore-forming α -subunit alone is sufficient to obtain sodium current, but co-expression of β -subunits modifies the expression level, the kinetics and the voltage dependence of channel gating (Yu and Catterall 2003). The α -subunit is organized in four homologous domains (DI-IV). Each domain contains six transmembrane segments (S1-S6) connected by extracellular or intracellular loops (Fig. 1).

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 initiate 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 or P-loops 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 and Bezanilla 2002). The P-loops of each domain together form the narrow part of the pore which is involved in Na⁺ selectivity of Na_v channels. The P-loops contain a highly conserved sequence of multiple charged amino acids which are homologous in all Na_v channel isoforms known today. These residues are aligned in two rings which determine Na⁺ selectivity (Fig. 2). The inner ring or DEKA is formed by the Asp, Glu, Lys and Ala residues in DI, DII, DIII and DIV, respectively. The outer selectivity ring or EE(D/M)D is characterized by the residues Glu, Glu, Asp or Met and Asp of DI, DII, DIII and DIV, respectively (Stevens et al. 2011). The first crystal structure of a Na_v channel to be determined was the structure of a bacterial Na_v channel from *Arcobacter butzleri* in a closed-pore conformation with four activated voltage sensors at a 2.7 Å resolution (Payandeh et al. 2011). Analysis of the three-dimensional structure shows that the selectivity filter can be seen as a short, 4.6 Å wide, and water filled cavity in which four acidic side chains are surrounding the narrowest part of the ion conduction pathway. Therefore, the selectivity filter represents a high-field-strength anionic coordination structure, providing ion selectivity via direct interaction of Glu residue side chains with Na⁺ ions through partial dehydration of these ions (Payandeh et al. 2011). A more recent elucidated crystal structure of the marine alphaproteobacterium HIMB114 at 3.05 Å

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Fig. 2. Schematic representation of the Na_v channels outer vestibule, indicating critical residues of the inner and outer selectivity rings according to the Na_v1.4 numbering. Adapted from (Fozzard and Lipkind 2010).

resolution did confirm conservation of the Na⁺ selectivity architecture in Na_v channels (Zhang et al. 2012).

The short intracellular linker that connects the DIII and DIV contains a highly conserved sequence of three hydrophobic residues (isoleucine, phenylalanine and methionine) or the IFM motif. Sodium channel inactivation is mediated by this hydrophobic motif since it serves as an inactivation gate crucial for 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 IIS4-S5 and in the loop connecting IVS4-S5 are contributing to the inactivation gate receptor (Yu and Catterall 2003, Dong 2007).

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 sodium channel isoforms are often classified according to their TTX sensitivity. Na_v1.1–Na_v1.4, Na_v1.6 and Na_v1.7 are TTX-sensitive since they are inhibited at low nM concentrations of TTX. Na_v1.5, Na_v1.8 and Na_v1.9 are denoted as TTX-resistant since they require higher (μM) concentrations of TTX to be inhibited. The functional and pharmacological diversity of the mammalian Na_v channels results primarily from the expression of multiple genes (Goldin et al. 2000). The selective expression of different sodium channel genes subserves the specialized function of sodium channels in various mammalian tissues and cell types (Yu and 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 (Goldin 2001, Dong 2007).

Voltage-gated potassium channels

K_v channels are membrane-imbedded K⁺ selective pores that open and close in response to changes in membrane voltage, a property that is essential for controlling the cellular excitability of various physiological processes such as regulation of action potential duration and propagation in heart and neurons, and regulation of insulin secretion in beta cells. K_v channels belong to the 6TM structural class of K⁺ channels and are further divided in several subfamilies (K_v1–K_v12) according to amino acid sequence homology.

The official nomenclature for mammalian K_v channels is $K_vx.y$ where x denotes the subfamily and y the order of discovery (Chandy 1991).

In contrast to Na_v channels where the four “subunits” are expressed as a single polypeptide, K_v channels consist of four individual α -subunits (Fig. 1) which assemble to form a central ion conduction pore surrounded by four voltage-sensing domains (VSDs). Each α -subunit contains six transmembrane domains and an N- and C-terminus which are located at the intracellular side of the membrane. The first four transmembrane domains (S1–S4) form the four VSDs while S5–S6 domains (together with the intervening P-loop) form the single central pore domain following the tetramerization of α -subunits. The pore domain forms a permeation pathway which contains on the intracellular side a water-filled tunnel that opens into a water-filled central cavity. The outer tunnel is lined out by the P-loops forming the selectivity filter, which favors transport of K^+ over other ions. Each P-loop holds the TVGYGD-signature sequence of which the oxygen atoms of the carbonyl backbone orient towards the central axis of the pore, lining the selectivity filter.

K_v channels are able to sense voltage across the cell membrane and react by opening and closing the ion conduction pore. Changes in voltage are detected by the voltage sensor domain (VSD), where S4 stands out because it contains regularly spaced (every third residue) charged Arg or Lys residues. A depolarization will cause the VSD to move from the “resting” to the “activated” state. This rearrangement of the VSD is accompanied with the movement of charges in an electric field that can be detected electrically as a nonlinear capacitive current, also called “gating current” (Bezanilla 2008). Once all four the VSDs are activated the concerted step can occur that leads to the opening of the intracellular S6 gate, allowing the flux of K^+ ions through the pore (Fig. 5A).

The VSDs are physically connected to the pore domains by S4–S5 linkers. Structural observations (Long et al. 2005b) together with site-directed mutagenesis studies (Slesinger et al. 1993, Lu et al. 2002, Labro et al. 2005) support the idea of electromechanical coupling where the S4–S5 linker communicates the conformational change in the voltage sensor to the pore domain through interactions with the bottom part of S6. This bottom part of S6 is separated from the rest of S6 by a highly conserved-PXP-motif (del Camino et al. 2000, Labro et al. 2003) that creates a flexible gating hinge which allows rotational motions required for intracellular gate opening. In the closed conformation the intracellular S6 gate seals off the permeation pathway by forming a hydrophobic constriction. Upon VSD movements to the activated state, it appears that the S6 segments already rearrange, however without opening the intracellular gate (del Camino et al. 2005). Once all four VSDs are in the activated state, the intracellular gate will open resulting in the ionic K^+ current. The S4–S5 linker and bottom S6 form

together the first interface region required for electrochemical coupling. A second interface region is located between the S1 of the VSD and the pore helix near the extracellular surface, which acts as a second anchor point for the electrochemical coupling (Lee et al. 2009, Bocksteins et al. 2011).

In summary, the electromechanical coupling between the VSDs and the pore domain results in three different K_v channel conformations: i) a resting state where the VSDs are “down” and the intracellular gate is closed, ii) an activated-not-open state where the VSDs are “up” and the intracellular gate is not yet open, and iii) a open state where the intracellular gate is open.

Saxitoxins and Tetrodotoxin

Saxitoxin

Saxitoxin (STX) was the first paralytic shellfish toxin to be isolated and is still considered as one of the most potent neurotoxins known today. Saxitoxin adopts its name from the bivalve in which it was firstly discovered in 1957, the Alaskan butter clam, *Saxidomus giganteus* (Whitefleet-Smith et al. 1985). Nevertheless, STX and its analogs are produced by several marine organisms such as dinoflagellates and cyanobacteria, while only one terrestrial organism, the Panamanian golden frog *Atelopus zeteki*, is reported to produce STX. The biosynthesis of saxitoxins will not be discussed here as it is the subject of another chapter in volume 1 of this book (Diagostino et al. 2014).

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STX is an alkaloid of 299 Da. It contains a rigid tricyclic core with two guanidino groups and a flexible carbamoyl side chain, allowing STX to exert a high conformational heterogeneity in solution (Schantz et al. 1975, Berlinck and Kossuga 2005). At physiological pH, the 1,2,3-guanidinium group is deprotonated, while the 7,8,9-guanidinium group is partial positively charged (Strichartz 1984, Llewellyn 2009, Wiese et al. 2010). In 1975, the crystal structure of STX has been elucidated, characterizing the 3,4-propinoperhydropurine tricyclic system. More than 57 naturally occurring derivatives have been reported. These STX analogs all possess the same tricyclic core but differ in moieties and nature of the side groups (Wiese et al. 2010). An example hereof is that while STX is not sulfated, mono-sulfated (gonyautoxins or GTXs 1–6), and di-sulfated (C1–4 toxins) analogs have been reported (van Apeldoorn et al. 2007, Llewellyn 2009). Furthermore, decarbamoyl variants of each of these analogs are known. A more drastic example of structural differing STX analogs can be found in zetekitoxin AB. This STX derivative, isolated from the Panamanian golden frog *Atelopus zeteki*, possesses a unique 1,2-oxazolidine ring-fused lactam, a sulfate ester, and a N-hydroxycarbamate side group moiety. Zetekitoxin AB

showed to be an extreme potent neurotoxin with a toxicity 500-fold higher as compared to STX (Yotsu-Yamashita et al. 2004, Lee and Ruben 2008).

Tetrodotoxin

Tetrodotoxin (TTX) is a neurotoxic marine compound produced by bacteria including those belonging to the genera *Plesiomonas shigelloides*, *Pseudomonas* sp., *Vibrio* sp. *Aeromonas* and *Alteromonas*. The 319 Da alkaloid TTX was first characterized as a highly toxic compound, abundantly present in the ovary and liver of puffer fish belonging to the family of Tetraodontidae, hence the name tetrodotoxin. It is remarkable to note that since its discovery in puffer fish, TTX has been isolated in a wide range of organisms belonging to marine as well terrestrial life. Besides fish, the occurrence of TTX has been reported in organisms such as amphibians, arthropods, mollusks, nematodes, bacteria, dinoflagellates and echinoderms (Lee and Ruben 2008). This wide distribution can be explained by the concept of endosymbiosis where TTX-producing microorganisms provide TTX, or a precursor thereof, and herewith an evolutionary advantage for their symbionts or hosts; the latter can use the TTX as a defense mechanism against predators or to increase the efficiency of prey capturing. For instance, the venom of the Australian blue-ringed octopus contains TTX produced by endosymbiotic bacteria (Hwang et al. 1989).

The crystal structure of TTX was elucidated in 1964, on the basis of which Robert Woodward received the noble prize in 1965 (Tsuda et al. 1964, Goto et al. 1965). TTX is an orthoester composed of a pyrimidine ring with six hydroxyl groups and a guanidinium group that is positively charged at physiological pH (Noguchi and Watanabe 2005, Pires et al. 2005). Similar to STX, several TTX analogs have been reported. However, the TTX analogs found in marine organisms display less chemical diversity while analogs found in terrestrial organisms such as newts and anurans display side group diversity comparable to the STX analogs (Pires et al. 2005, Llewellyn 2009).

STX and TTX are the most studied and well characterized members of the large family of guanidinium-containing marine compounds. Even though STX and TTX and their respective analogs belong to structural divergent families, they do bind with high affinity to the same target, the neurotoxin receptor site 1 of Na_v channels (Fig. 1). Consequently, both toxins exert the same pharmacological effect. Upon binding at a narrow part within the channel pore, they obstruct the ion conductance through the channels by physically blocking the pathway of the Na⁺ ions (Fig. 3 left panel). Both toxins do not significantly alter the kinetics of gating (Fig. 3 right panel).

Fig. 3. Illustration of the effects of guanidinium toxins on Na_v channels: pore block without changes in the kinetics.

STX and TTX inhibit Na_v channel conductance through the neurotoxin site 1

More than eight putative binding sites on Na_v channels have been determined (Catterall et al. 2007, Stevens et al. 2011). Among these, the neurotoxin receptor site 1 is probably the best characterized and delineated. Originally, site 1 was functionally defined by its ability to interact with the guanidinium toxins TTX and STX. Afterwards, μ -conotoxins (peptide toxins produced by marine snails of the genus *Conus*) were also found to bind at a micro-site within the neurotoxin site 1 (Stephan et al. 1994). The μ -conotoxin site was reported to be not identical to the TTX/STX binding site but overlapping, allowing both synergistic and antagonistic interactions between μ -conotoxins and guanidinium toxins (French et al. 2010).

As early as 1965 a first structural hypothesis on the interaction between guanidinium toxins and Na_v channels proposed a simple mechanical model based on the observation that free guanidinium ions can pass through the Na_v channel. In this model it was seen that the guanidinium group forms the blocking complex by entering the pore, and subsequently the toxin gets stuck because the rest of the molecule is too big to pass through the channel (Kao and Nishiyama 1965). This hypothesis was later extended with the proposal that the binding site of these toxins was the ionic selectivity filter (Hille 1971). The determination of the TTX and STX structures allowed a further extension of this hypothesis with a concept of the toxin orientation when entering the pore and the possible molecular interactions between the guanidinium toxin and the channel (Hille 1975). Even though these early hypotheses were formed without the availability of crystal structures of Na_v channels nor K_v channels, it is interesting to note that the recent advances in channel structure determinations do seem to confirm these early structural hypotheses.

The first molecular determinants of the guanidinium binding site resulted from mutagenesis experiments in which one of the Glu residues of the outer selectivity ring of $\text{Na}_v1.2$ was neutralized (E378Q). This point mutation had minor influence on the magnitude of the sodium peak

current and did not alter the kinetics of channel gating. Remarkably, this single charge neutralization rendered Na_v1.2 channels completely resistant for guanidinium toxin induced inhibition (Noda et al. 1989). Further identification of the TTX/STX binding site was obtained by mutating amino acids residues within the P-loops among which also those contributing to the inner and outer selectivity ring. Mutations of the D384, E942, K1422, A1714 (DEKA) and of E387, E945, M1425, D1717 (EEMD) significantly affected TTX and STX binding (Fig. 1). Furthermore, these mutations strongly influenced channel conductance. Mutating neighboring residues did not result in significant altered TTX/STX sensitivity nor did it alter channel conductance (Terlau et al. 1991). This indicated that channel residues interacting with guanidinium toxins are also involved in ion conductance and channel permeation. Therefore, it was concluded that TTX and STX bind within the outer vestibule and physically occlude the pore upon interaction with residues critical for ion conductance and selectivity (Terlau et al. 1991, Chiamvimonvat et al. 1996, Favre et al. 1996, Fozzard and Lipkind 2010). The recent advances in the crystal structure of Na_v channels corroborate the previous observations and provide a broader foundation to interpret guanidinium toxin induced inhibition of channels (Payandeh et al. 2011).

The elucidation of the TTX/STX binding site also sheds light on the scientific puzzle regarding the difference in TTX sensitivity observed for Na_v channel isoforms. It has been well established that Na_v1.1–Na_v1.4 and Na_v1.6–Na_v1.7 are more sensitive towards TTX compared with Na_v1.5, Na_v1.8–Na_v1.9. Although all isoforms can be blocked by TTX, the latter ones require 250-fold higher concentrations. These differences in sensitivity can be accounted for by the residues constituting the TTX/STX binding site. For instance, in Na_v1.1–Na_v1.3 this site is composed of the sequence DFWEN while in Na_v1.4 it is DYWEN. When comparing with the sequence in Na_v1.5, DCWED, two differences can be observed. The aromatic residue at position 2 is replaced with a Cys while the amino acid at position 5 is an Asp. Mutagenesis experiments to investigate the relative energetic contributions of these amino acids within the TTX/STX binding site to the binding of guanidinium toxins and divalent cations have shown that neutralizing the C-terminal Asp in Na_v1.5 does not render these channels TTX sensitive (Satin et al. 1992). However, providing an aromatic nature at position 2 by replacing the Cys for a Tyr did increase the TTX sensitivity of these cardiac Na_v channel isoforms by 1,000-fold (Satin et al. 1992, Fozzard and Lipkind 2010). Similar studies in which the Tyr at position 2 (Tyr-401) of Na_v1.4 channels was mutated to a Cys indicated a 200-fold decrease of STX sensitivity (Favre et al. 1995).

Delineation of the guanidinium toxin binding site, together with mutagenesis data, allowed the construction of homology models of the pore structure enabling docking studies to further investigate the interaction of

TTX and STX with the Na_v channels. It should be noted that, at present, all models used to study the TTX/STX interaction are based on the crystal structure of prokaryotic K⁺ channels as a starting template rather than the recently available structures of bacterial Na_v channels (Tikhonov and Zhorov 2012). Several arguments disfavor the use of bacterial Na_v channel structures as template for docking studies. For example, sequence alignment analysis between the prokaryotic Na_vAb and the eukaryotic Na_v1.4 indicated low homology in the pore loop region (Tikhonov and Zhorov 2012). Similar results were obtained when sequence homology of the outer vestibule region of Na_vAb and Na_v1.7 was examined (Walker et al. 2012). Furthermore, Na_vAb is a TTX insensitive channel. The X-ray structure of Na_vAb reveals that within the outer vestibule the amino acids composing the outer selectivity ring (EE(D/M)D) do not face the inner lining of the pore and as such they are not available for toxin interaction (Walker et al. 2012). As a consequence, attempts to dock TTX or STX in the straightforward Na_vAb structure model of eukaryotic Na_v channels were unsuccessful, mainly due to inconsistencies regarding the interactions of TTX or STX with the carboxylate side groups of the outer selectivity ring residues (Payandeh et al. 2011, Tikhonov and Zhorov 2012). All together, it can be concluded that the structure of Na_vAb, at least regarding the guanidinium toxin-channel interaction, is no more accurate starting template for construction of a eukaryotic Na_v channel homology model than prokaryotic K⁺ channels such as KcsA or KvAP (Walker et al. 2012).

KcsA is a bacterial potassium channel of which the structure has been determined at a 3.2 Å resolution (Doyle et al. 1998). This potassium selective channel, isolated from *Streptomyces lividans*, is highly homologous to invertebrate K⁺ channels and is composed of 2 transmembrane α -helical segments connected by a loop which represent the S5-P loop-S6 of invertebrate K_v channels. A functional channel is formed upon tetramerization of four such subunits. The amino acid composition in the pore region is nearly identical to that found in the vertebrate K_v channels (MacKinnon 1991). A model of the S5-P loop-S6 pore forming region of Na_v1.4 was built based on the backbone coordinates of the closed KcsA channel structure together with an α -helix-turn- β -strand motif for the P-loops in order to mimic an appropriate outer vestibule for binding of site 1 ligands such as the guanidinium toxins (Lipkind and Fozzard 2000, Fozzard and Lipkind 2010). In this model, the arrangement of the four P-loops results in the configuration of the TTX/STX binding surface (Lipkind and Fozzard 2000). Several characteristics of the interaction between the guanidinium toxins and residues within the P-loops of the channel were revealed in docking experiments using this model (Fig. 2). For TTX, a strong non-covalent binding between the molecule's non-polar surface and the Tyr-401 of DI was observed. Furthermore, the 1,2,3 guanidinium group of TTX enters deeply

in the pore and interacts directly with the Glu-755 of DII and the Asp-400 of DI. Identical reactions with the Glu-755 and Asp-400 were performed with the 7,8,9 guanidinium group of STX while its 1,2,3 guanidinium group interacts specifically with Asp-1532 of DIV. Furthermore, the Glu-758 of DII interacted with the C-12 *gem*-diol of STX (Fozzard and Lipkind 2010).

Functional groups on TTX and STX involved in the molecular mechanism of action

To have a better understanding of the molecular mechanism by which guanidinium toxins interact with their targets, several structure-function studies have been conducted. Investigating the binding interaction of both natural and synthetic analogs of STX and TTX allowed to determine the functional groups important for their potent activity. The results of competitive binding experiments between STX and its analog neosaxitoxin (NEO) have excluded the 1,2,3 guanidinium group as a functional important group. NEO differs from its reference molecule with an oxime group at the C2 (Shimizu et al. 1976). Since neo-STX, under physiological conditions, possesses twice as many negative charges compared to STX, it could be concluded that if the 1,2,3 guanidinium group has any role in the molecular interaction with the Na_v channel, a significant difference in potency between both molecules should occur. Therefore, the equipotency of STX and its analog neo-STX excludes the 1,2,3 guanidinium group and identifies the 7,8,9 guanidinium group as the functional active group involved in binding (Kao and Walker 1982, Kao et al. 1983). Decarbamoyl-saxitoxin (dcSTX) is a synthetic STX derivate in which the carbamoyl group at the C6 is removed. The slightly lower potency of dcSTX suggests that this side group is not essential for the activity but most likely is involved in stabilizing interactions between STX and the receptor binding site (Kao and Walker 1982). In voltage clamp studies on squid axons it was shown that reduced-STX could inhibit Na_v channels, but interestingly, with a 100-fold reduction in affinity (Kao and Walker 1982). This is a remarkable observation since reduced-STX only structurally differs from STX in a dehydroxylation of one of the alcohol side groups of the C12 (Ghazarossian et al. 1976). It thus seems that the formation of hydrogen bonds via the hydroxyl groups of C12 is an important requirement for the interaction of STX with the neurotoxin receptor site 1.

Altogether, it might be concluded that STX exhibits its Na_v channel toxicity by binding within the outer vestibule with the 7,8,9 guanidinium group directed into the narrow part of the pore, forming an ionic bond with an anionic site on the receptor. More specific, the 7,8,9 guanidinium group is in direct contact with the Asp-400 and Glu-755 residues constituting the inner selectivity ring or DEKA. The hydroxyl groups of STX's C12 form

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stabilizing hydrogen bonds at other sites. These interactions will allow STX to position itself to obstruct the Na^+ ion pathway and, as such, inhibit the sodium conductance through the channel.

When comparing the structures of STX and TTX by aligning the 7,8,3 guanidinium group of STX with the 1,2,3 guanidinium group of TTX, it is interesting to note that the two hydroxyls at the C12 of STX are very well aligned with the hydroxyls of C9 and C10 of TTX (Kao and Walker 1982). Following this alignment one would deduce that the functional important groups of TTX are composed of the 1,2,3 guanidinium group and the C9 and C10 hydroxyls. Indeed, TTX derivatives in which the alcohol groups on C9 and C10 were modified, showed a complete abolishment of binding on Na_v channels (Narahashi et al. 1967). Furthermore, the reduced potency of 4-epiTTX where the C9 and C10 side groups were dehydroxylated and the low affinity of 4,9 anhydroTTX, a TTX analog in which the C9 OH group is unavailable for interaction due to formation of a inner oxygen bridge with C4, further support the involvement of the C9 and C10 side groups in TTX potency and binding (Narahashi et al. 1967, Kao and Yasumoto 1985, Rosker et al. 2007). From a pharmacological point of view, it is interesting to note that, even though 4,9 anhydroTTX displays a lower affinity than TTX, this analog is a selective inhibitor of a particular Na_v channel isoform, $\text{Na}_v1.6$ (Rosker et al. 2007). Several lines of evidence suggest a causative role for $\text{Na}_v1.6$ channels in neurodegenerative diseases such as multiple sclerosis (Caldwell et al. 2000). A selective inhibitor like 4,9 anhydroTTX might thus represent an interesting lead compound in the development of novel therapeutics.

Two TTX analogs, isolated from an Okinawan newt, *Cynops ensicauda*, were tested for their Na_v channel blocking effects on the voltage-clamped frog skeletal muscle fiber. Both these analogs have certain hydroxyl groups unavailable for binding interactions since in 6-epiTTX, the C-6-OH is in an epimeric position while in 11-deoxyTTX, the C11 has a methyl in place of a hydroxymethyl group. The low binding affinity of both analogs as observed in these experiments, suggests an important role for the C11 hydroxymethyl group (Yang et al. 1992).

It is believed that the molecular mechanism through which TTX interacts with Na_v channels is initiated by the entering of the molecule in an orientation strongly guided by the negatively charged residues of the vestibule (McNulty et al. 2007). In fact, the lower TTX affinity, when charged vestibule residues are neutralized, can be accounted for by a reduced association rate constant of binding rather than an increased dissociation rate constant. This implies that the initial orienting electrostatic interaction is a notable contribution of the carboxylates to the TTX binding (Penzotti et al. 1998, Boccaccio et al. 1999, Fozzard and Lipkind 2010). Entering the vestibule in this specific orientation positions the C9 and C10 hydroxyl and

the C11 hydroxymethyl groups in close proximity with, respectively, the Glu-758 and Glu-403 residues of the outer selectivity ring. Interestingly, in STX the two hydrogen side groups of C12 form hydrogen bonds with the Glu-758. In contrast, for TTX only one hydroxyl group could form a bond with Glu-758, structurally explaining the weaker interaction of TTX with this residue (Fozzard and Lipkind 2010). More recently, the discovery of two natural occurring point mutations in human Na_v1.7 channels displayed a marked difference in STX and TTX affinity for these channels (Walker et al. 2012).

Altogether, it can be concluded that even though TTX and STX do bind at the same neurotoxin receptor site of Na_v channels, small but significant differences do exist in the specific channel interactions of both TTX and STX, which can be explained by the differences in structure of these guanidinium toxins.

Brevetoxins

Marine dinoflagellates produce highly lipophilic, cyclic polyether compounds which target Na_v channels through the neurotoxin receptor site 5. Brevetoxins (*Karenia brevis*) and ciguatoxins (*Gambierdiscus toxicus*) are such multi-ring ladder-shaped polyether toxins. Brevetoxins (PbTXs) consist of 11 transfused rings, 23 stereocenters and an overall linear low-energy conformation (Jeglitsch et al. 1998). Since the discovery of the first brevetoxin, PbTX-2 in 1981, at least 14 brevetoxins have been described. The two most potent brevetoxins are PbTX-1 and PbTX-2 that slightly differ from each other in their backbone structure. PbTX-1 and PbTX-2, also known as type A PbTXs and type B PbTXs, respectively, are considered to be the parent toxins since all other brevetoxins can be seen as derivatives from one of these two structural backbones. All PbTXs possess a lactone in the A-ring and a strictly rigid region or H-K rings system that forms a ladder structure and which is separated from the A-ring by a spacer region with limited flexibility (Gawley et al. 1995).

PbTXs are believed to interact with Na_v channels by intercalating in the membrane between DI and DIV in a head-down orientation. Several studies have indicated that these toxins position themselves across the plasma membrane, parallel with the transmembrane segments, with the A-ring toward the intracellular side and the tail end of the molecule facing the extracellular side (Trainer et al. 1994, Jeglitsch et al. 1998). It is believed that the ladder toxins interact with the channels by adopting an orientation which is complementary to the protein structure of the binding site, most likely an α -helix structure. The molecular interaction between toxin and channel probably occurs through a network of hydrogen bonds. This model is supported by the observation that the average distance between

neighboring ether oxygen atoms of brevetoxins corresponds well with the pitch of an α -helix (Murata et al. 2008). At least 13 derivatives of these two back bone structures have been the subject of molecular pharmacological studies and it was found that these derivatives possess a different toxicity that can be correlated with their respective affinities for the binding sites on the Na_v channels (Baden et al. 2005).

Experiments in which a photoreactive PbTX-3 derivative was used as probe implicated S6 of DI and S5 of DIV in the formation of the neurotoxin receptor site 5 (Trainer et al. 1991, 1994). However, the key residues involved in brevetoxin activity still remain unknown. Although ion selectivity of the Na_v channels was not influenced, PbTX binding at site 5 does lead to distinct alteration in channel gating: (i) the voltage dependence of activation is shifted toward hyperpolarized potentials; (ii) channels remain longer in the open configuration which results in a longer mean open time; (iii) the inactivation is slowed down or inhibited; and (iv) brevetoxins have, among all known Na_v channel modifying toxins, the unique capability to stabilize more than one conductance level. As such brevetoxin binding induces distinct subconductance states in addition to the normal 21 pS single channel conductance (Schreibmayer and Jeglitsch 1992, Jeglitsch et al. 1998, Baden et al. 2005). It is believed that the terminal, rigid four ring system is involved in channel binding while the functional lactone A-ring is responsible for the alterations in channel inactivation and prolongation of the mean open time. The nature of the A-ring and its proximity to the inactivation loop of the Na_v channel might result in the observed induction of subconductance states. The exact mechanism of these PbTX induced subconductance states remains to be elucidated. However, one theory, partially supported by molecular modeling, proposes an interaction of the A-ring with an inactivation particle within the intracellular loop connecting DIII and DIV, resulting in a different interaction with the receptor and therefore leading to partial closed or partial open channels (Baden et al. 1994, Jeglitsch et al. 1998, Purkerson-Parker et al. 2000).

Noteworthy, from the same dinoflagellate that produces brevetoxins, another polyether molecule, brevenal, could be isolated. Brevenal is an essentially nontoxic short-chain trans-syn polyether molecule (Bourdelaïs et al. 2004). It was found that the nontoxic brevenal competes with the brevetoxins for their binding site at the Na_v channels. Moreover, brevenal could competitively displace brevetoxin in synaptosome receptor binding experiments. Concentrations of brevenal required for competition with brevetoxins was two orders of magnitude higher than the brevetoxins itself. Nevertheless, by competing for the same specific binding site and rendering this site unavailable for brevetoxins to exert their lethal toxicity, brevenal acts as a molecular antagonist with potential therapeutic value (Bourdelaïs et al. 2004).

Ciguatoxins

Ciguatoxins are a family of heat-stable, lipid-soluble cyclic polyether toxins that are derived from the epiphytic marine dinoflagellate *Gambierdiscus toxicus*. These organisms live on common, larger reef-colonizing species of red, green, and brown algae (Lehane and Lewis 2000). Ciguatoxins accumulate in tropical and sub-tropical fishes through the aquatic food chain and cause ciguatera, a food-borne disease mostly observed in regions of the Pacific Ocean, the West Indian Ocean and the Caribbean Sea (Dickey and Plakas 2010, Skinner et al. 2011). This can lead to fish kills with severe impact on the fishing industries (Lehane and Lewis 2000). Consumption of ciguatoxin-contaminated fish by humans results in gastrointestinal, neurological and cardiac symptoms that can last up to several months (Lewis 2006, Dickey and Plakas 2010). However, contaminated fish rarely accumulate sufficient levels of ciguatoxins to be lethal for humans. The first link between ciguatoxins and ciguatera was established in 1977, when a Japanese/French team found large numbers of the species *Gambierdiscus toxicus* in a toxic sample of detritus (dead organic material such as coral rubble) collected in the Gambier Islands in French Polynesia during a ciguatera outbreak (Miyahara et al. 1979). However, despite the fact that these toxins have been investigated for a long time, progress on their mechanisms of action has been slow due to their scarcity and the complexity of their molecular interactions. At least 12 Caribbean (C-CTX) and 29 Pacific ciguatoxin congeners (P-CTX) have been reported thus far (Dickey and Plakas 2010), but most studies on the molecular pharmacology of ciguatoxins have been done with P-CTX-1 (also known as P-CTX-1B) and in lesser extent with P-CTX-4B (also known as gambiertoxin), C-CTX-1 and P-CTX-3C.

Ciguatoxins enhance Na_v channel activity through the neurotoxin site 5

Na_v channels are considered to be the primary molecular targets of ciguatoxins, which are classified (together with the similar brevetoxins) as receptor site 5 toxins (Catterall et al. 2007, Stevens et al. 2011) (Fig. 1). However, more recently ciguatoxins have been shown to modulate (in this case inhibit) K_v channels as well. The first link between the action of ciguatoxins and Na_v channels was reported in 1984. Purified P-CTX-1 appeared to enhance the excitability in nerve and muscle cells and at synaptic terminals. This effect could be inhibited by TTX (a Na_v channel pore blocker), suggesting that P-CTX-1 is a novel type of Na_v channel toxin (Bidard et al. 1984). Similarly, experiments on frog myelinated nerve fibres showed that at low nM concentrations P-CTX-1 induced spontaneous action potentials that were due to a specific modulation of Na^+ channels

(Benoit et al. 1986). In the presence of P-CTX-1, the channels were activated at resting potentials and a sub-population of channels failed to inactivate during prolonged depolarizations. Further studies with P-CTX-1 showed a similar enhancement of the cellular excitability, linking the effects to modulation of TTX-sensitive Na_v channels (Birinyi-Strachan et al. 2005). P-CTX-1 caused a concentration-dependent reduction in peak Na^+ current amplitude in rat myotubes (Hidalgo et al. 2002). In rat dorsal root ganglion (DRG) neurons, P-CTX-1 caused a reduction of the peak current amplitude of both TTX-sensitive and TTX-resistant Na_v channels, however through a different mechanism (Strachan et al. 1999) (Fig. 4). P-CTX-1 modulated TTX-sensitive Na_v channels through a hyperpolarized shift in the voltage dependence of activation and inactivation. P-CTX-1 also caused a rapid rise in the membrane leakage current, mediated through a spontaneous opening of TTX-sensitive Na_v channels at resting potentials. In contrast, P-CTX-1 modulated TTX-resistant Na_v channels by increasing the rate of recovery from inactivation, without altering the voltage dependence

Nav channels

Kv channels

Fig. 4. Illustration of the various effects of ciguatoxins on Na_v and K_v channels with largely effects on the activation and/or the inactivation gating.

of activation and inactivation. However, in amphibian neurons P-CTX-1 partially eliminated the inactivation and shifted the voltage dependence of activation towards more negative potentials without reducing the peak current amplitude (Benoit et al. 1986, Molgo et al. 1990, Benoit and Legrand 1992, Benoit et al. 1996). In isolated rat parasympathetic neurons P-CTX-1 caused an increased neuronal excitability by shifting the voltage dependence of activation towards more negative potentials and by creating a persistent, non-inactivating Na^+ current (Hogg et al. 1998).

Besides the extensively studied P-CTX-1, other ciguatoxin congeners modulate Na_v currents in a similar fashion. Caribbean ciguatoxin-1 (C-CTX-1) caused spontaneous and repetitive action potential discharges and marked swelling of the nodes of Ranvier in single frog myelinated axons (Mattei et al. 1999). This C-CTX-1-induced nodal swelling could be prevented by TTX. These observations suggested that a fraction of Na^+ channels open at the resting potential, resulting in spontaneous and repetitive action potential discharges and a steady Na^+ entry that disturbs the osmotic equilibrium. In rat parasympathic neurons, C-CTX-1 (and P-CTX-1) induced oscillations in the membrane potential and action potential firing (Hogg et al. 2002). In contrast, brevetoxin-3 (PbTx-3) caused membrane depolarizations that were not associated with the oscillation of the membrane potentials, suggesting that ciguatoxins induce membrane potential oscillations via different (TTX-sensitive) Na_v channels or different mechanisms of action compared to brevetoxins. C-CTX-1 also evoked neuromuscular effects in frog myelinated axons and at the neuromuscular junction (Mattei et al. 2010). Synthetic P-CTX-3C (100 nM) caused a 30-mV negative shift in the voltage dependence of activation and inactivation of Na_v channels in mouse taste cells (Ghiaroni et al. 2006). Investigation of the action of synthetic P-CTX-3C on several Na_v channel isoforms revealed that P-CTX-3C modulated r Na_v 1.2, 1.4, 1.5 and 1.8 isoforms by a hyperpolarizing shift of the activation and inactivation process and by inducing a sustained leak Na^+ current, where Na_v 1.8 was the most sensitive isoform (Yamaoka et al. 2004, 2009).

Neurotoxins can bind to at least eight identified binding sites on Na_v channels and modulate their function, resulting in either a loss or gain of Na_v channel function (Catterall et al. 2007, Stevens et al. 2011). Competition binding assays between radiolabelled derivatives of PbTx-3 and purified ciguatoxin fractions (Lombet et al. 1987) or isolated ciguatoxin-isoforms (Lewis et al. 1991, Dechraoui et al. 1999, Dechraoui and Ramsdell 2003) showed that these two classes of toxins share a common receptor site on Na_v channels distinct from the other receptor sites. As mentioned before, this neurotoxin site 5 is formed by the membrane-embedded regions S6 from DI and S5 from DIV (Trainer et al. 1991, 1994). More recently, chimeric constructs between Na_v 1.8 and Na_v 1.4 channels implicated DI and DII in

the high affinity of Nav1.8 for synthetic ciguatoxin P-CTX-3C (Yamaoka et al. 2009). Subsequently, the same group suggested a functional relationship between this ciguatoxin and a charged residue in DII voltage sensor of Na_v1.4 (Yamaoka et al. 2011). Hence, the receptor site 5 on Na_v channels has not yet been fully characterized and further studies are required to elucidate the binding site and the molecular mechanism of ciguatoxins on Na_v channels. Structure-activity relationship studies with 51-hydroxy-P-CTX-3C (an analog of P-CTX-3C) showed a critical importance of the nine-membered F-ring for potent activity (Inoue et al. 2008, Ishihara et al. 2010). Modifications of the F-ring decreased the affinity or potency in different biological assays (e.g., competitive ligand-receptor interactions, *in vitro* effect on mouse neuroblastoma cells and *in vivo* toxicity to mice). Furthermore, a fragment containing the AB-ring structure of P-CTX-1 was shown to evoke effects in the guinea pig atrial and neuroblastoma cell assays, thought to be mediated by activation of Na⁺ channels (Hokama et al. 2006).

Ciguatoxins display a dual action by also inhibiting K_v channels

Although Na_v channels may serve as their primary molecular targets, in the last decade several ciguatoxins have been shown to target K_v channels with high affinity as well. The first study linking the effect of ciguatoxins on K_v channels was reported in 2002, showing the inhibitory effect of 5 nM P-CTX-1 on K⁺ currents in rat myotubes (Hidalgo et al. 2002). Another study showed similar effects with P-CTX-1 on neuronal excitability in DRG neurons (Birinyi-Strachan et al. 2005). Under voltage-clamp conditions, nM concentrations of P-CTX-1 inhibited both delayed-rectifier and A-type K⁺ currents in a concentration-dependent manner. These results appeared to underlie the prolongation of the action potential duration and contribute to tonic firing of action potentials. In neuromuscular junctions C-CTX-1 both up-regulated Na⁺ currents and inhibited K⁺ currents, which appeared to be responsible for the muscle symptoms observed in ciguatera (Mattei et al. 2010). A comparative study of the effect of P-CTX-1, P-CTX-3C and 51-OH P-CTX-3C on Na_v and K_v channels in cerebellar neurons showed that these toxins, in addition to modulating Na_v currents, also inhibited K_v currents in the nM range (Perez et al. 2011). A similar dual action was observed with P-CTX-4B where nM concentrations modulated Na_v channels as well inhibited K_v channels in single myelinated axons (Schlumberger et al. 2010a).

Ciguatoxins seem to target two separate classes of voltage-gated channels (e.g., Na_v and K_v channels) with nanomolar affinity, but through a different mechanism. Specifically, ciguatoxins cause a gain of function of Na_v channels by shifting the activation to more hyperpolarized potentials and by destabilizing the inactivation process, whereas the inhibition of K_v

channels results in a loss of K_v function. Hence, both effects can contribute to the increased neuronal excitability observed clinically with ciguatera poisoning. Indeed, most studied ciguatoxin congeners (P-CTX-1, P-CTX-4B, C-CTX-1 and P-CTX-3C) seem to display this (high-affinity) dual action on both Na_v and K_v channels. However, almost all of these studies to date have been performed in various native cell types and cultures which express multiple channel isoforms that contribute to the Na^+ or K^+ current. Therefore, future studies on individual Na_v and K_v isoforms are required to shed light on the selectivity and mechanism of action of ciguatoxins on Na_v and K_v channels.

Gambierol

Gambierol is a ciguatoxin-like toxin that has been isolated initially from the dinoflagellate *Gambierdiscus toxicus* in 1993 (Satake et al. 1993). Gambierol has a ladder-shaped polyether structure similar to other ciguatoxins, however it contains only 8 trans/syn-fused ether rings compared to 9–13 in most ciguatoxins. Scarcity from natural sources encouraged different laboratories to successfully synthesize gambierol by different chemical strategies, despite its complex configuration (Fuwa et al. 2002, Kadota et al. 2003, Johnson et al. 2006, Furuta et al. 2009). The availability of the synthetic product has greatly advanced the investigation of gambierol on Na_v and K_v channels. In particular, studies on individual K_v channel isoforms expressed in heterologous expression systems have identified the molecular determinants and mechanism of action of gambierol on $K_v3.1$ channels (Kopljär et al. 2009, Kopljär et al. 2013).

Gambierol selectively targets K_v1 and K_v3 channels

Initially, gambierol was assumed to be a Na_v channel modulator, like most ciguatoxins. Studies in rat brain synaptosomes and cerebellar granule neurons suggested that gambierol binds (in the micromolar range) at the neurotoxin site 5 in Na_v channels and acts as an antagonist for brevetoxin (PbTX-3) binding, however without modulating the channels itself (Inoue et al. 2003, Lepage et al. 2007). Furthermore, at μM concentrations gambierol evoked membrane depolarizations, decreased ciguatoxin-induced depolarizations and increased cytosolic Ca^{2+} in neuroblastoma cells, suggesting that the toxin modulates ion fluxes by acting as a partial agonist of sodium channels (Louzao et al. 2006). However, the methods used to investigate the effect of gambierol on Na^+ channels were indirect. In contrast, patch-clamp studies on mouse taste cells showed that at nM concentrations gambierol inhibited K^+ currents, while no effect was seen

on Na⁺ and Ca²⁺ currents (Ghiaroni et al. 2005). This revealed new insights into gambierol pharmacology, suggesting that K_v channels might serve as primary targets. An extensive screening on different K_v (K_v1.1–1.6, *Shaker*, and hERG) and Na_v (Na_v1.1–1.8 and insect Para) channel isoforms in *Xenopus laevis* oocytes (Cuypers et al. 2008) confirmed this hypothesis of gambierol being selective towards K_v channels. Gambierol inhibited most of the K_v1 channels in the nM range where K_v1.2 was the most sensitive target (IC₅₀ of 35 nM) while the Na_v channels were unaffected, even in the μM range. Further examination of gambierol on other K_v channels (K_v2, K_v3 and K_v4 subfamily members) showed that K_v1 and K_v3 members were sensitive to nM concentrations, while no effect was observed on K_v2 and K_v4 members. K_v3.1 was the most sensitive channel with an apparent IC₅₀ of 1 nM and, to the best of our knowledge, is the most potent K_v3.1 inhibitor reported up to date.

Gambierol inhibits K_v channels via a novel binding site that shares similarity with the neurotoxin site 5 in Na_v channels

The outcome of the above studies raised several interesting questions regarding gambierol pharmacology on K_v channels. Why does gambierol have a specific affinity for K_v1 and K_v3 subfamilies? Where does gambierol bind and what is the mechanism of inhibition? As mentioned above, ciguatoxins and brevetoxins modulate Na_v channels by interacting with the neurotoxin site 5 (Catterall et al. 2007). This site was determined using radiolabelled brevetoxin (PbTX-3), indicating that it is located outside the permeation pore and consists of residues in S5 of DIV and S6 of DI (Trainer et al. 1991, 1994). In case of K_v channels, no toxins or other (pharmacological) compounds had been described to bind in this region of the channel. However, exploiting the high affinity for K_v3.1 channels allowed the identification of critical molecular determinants for the binding of gambierol in K_v channels (Kopljär et al. 2009).

The rationale of the study was that gambierol could inhibit K_v channels via one of the established binding sites (external pore block, internal cavity block and VSD gating modification) or via a novel site that could be the equivalent of the neurotoxin site 5 in Na_v channels. Chimeras between K_v3.1 (sensitive) and K_v2.1 (insensitive) channels showed the importance of the S6 segment for the sensitivity of different K_v isoforms, whereas S5 and pore region swaps did not significantly alter the potency of gambierol. Single amino acid substitutions in S6 (between K_v3.1 and K_v2.1) showed that the T427V mutation drastically reduced the affinity for K_v3.1. This T427 residue appears to underlie the selectivity of gambierol since it is conserved in K_v1 and K_v3 subfamilies, whereas K_v2 and K_v4 channels possess a valine at this position, associated with a >1,000-fold decrease in affinity. Additional

mutations in S5 (F349, F351) and S6 (M430) where shown to affect, in a lesser extent, the affinity as well. These results are in line with the studies that identified the interaction regions for neurotoxin site 5 toxins in Na_v channels.

In general, many compounds are attracted to the dominantly hydrophobic nature of the internal cavity which is delineated by S6 helices, where S6 interaction residues point towards the permeation pathway so that binding of compounds results in an occlusion of the ion pore (Doyle et al. 1998, Sanguinetti and Tristani-Firouzi 2006). In contrast, both the crystal structure of the open K_v1.2 channel (Long et al. 2005a) as the closed state model of the same crystal structure (Pathak et al. 2007) show that the threonine side chain (T427 in K_v3.1) points away from the conduction pathway (central pore) towards the lipid environment. Since K_v subfamilies 1–4 share a structurally conserved pore region, one might expect that T427 in K_v3.1 also points towards the lipid environment, suggesting that gambierol does not bind within the cavity of the channel. Competition experiments showed that gambierol did not compete with the internal cavity blocker ~~flecainide~~ (Herrera et al. 2005), nor with 4-AP that binds near the gating hinge at the bottom of the permeation pathway (Caballero et al. 2007), indicating that gambierol binds to a site distinct from the internal cavity. Furthermore, the main molecular determinant for gambierol binding, T427, does not belong to the set of residues implicated in internal cavity block by various drugs (Herrera et al. 2005, Decher et al. 2006, Eldstrom et al. 2007).

flecainide

The possibility that gambierol acts at one of the other two well-described binding sites, namely as an external pore blocker or gating modifier (cfr. hanatoxin) was not supported by the experimental data. Swapping the S5–S6 linker, containing the pore region, from K_v2.1 into the K_v3.1 background did not alter the affinity for gambierol, excluding the possibility of gambierol being an external pore blocker. Furthermore, peptide gating modifier toxins such as hanatoxin, BDS-II and HpTx2 (Swartz and MacKinnon 1995, Yeung et al. 2005, DeSimone et al. 2009) modify the gating of K_v channels by binding to the paddle-motif of the VSD (Alabi et al. 2007) and hereby stabilizing the closed conformation by acting as a cargo (Swartz and MacKinnon 1997, Phillips et al. 2005). As result the voltage dependency of activation is shifted towards more depolarizing potentials. However, the results with the S6 chimera and the T427 mutant indicate that the binding site of this poyether toxin differs from that of classical gating modifier peptides.

Taking in account the gambierol's size (~22 Å) and its amphipathic nature, one might expect that the binding site is situated within the plasma membrane. This hypothesis was supported by inside-out experiments showing that the onset of inhibition is independent of the site of application (intra- or extracellular). Furthermore, gambierol inhibited closed channels

with high-affinity implicating that the binding site is accessible in the closed state where the pore gate is closed. In contrast, gambierol could not inhibit open $K_v3.1$ channels indicating that the open state has a low affinity for gambierol (Kopljar et al. 2013). Hence, it seems that gambierol inhibits K_v channels via a novel binding site which may be the equivalent of the neurotoxin site 5 in Na_v channels.

In the closed state model of the r $K_v1.2$ crystal structure, gambierol could be fitted in a space between the VSD and the pore domain roughly parallel to the S5 and S6 helices (of a single subunit). However, it is quite possible that the binding site involves regions of neighbouring subunits as seems to be the case for brevetoxins and ciguatoxins on Na_v channels, where the binding site is formed by S5 of DIV and S6 of DI (Trainer et al. 1991, 1994). Ladder-shaped polyether toxins have been proposed to target transmembrane proteins within the lipid bilayer (Murata et al. 2008). Their unique structure in which the spacing of the ether-oxygens closely matches the pitch of α -helices of transmembrane proteins enables multiple interactions involving hydrogen bonding and/or hydrophobic interactions. (Ujihara et al. 2008, 2010, Torikai et al. 2008, Murata et al. 2008). Hence, its amphipathic nature might allow gambierol to modulate K_v channels through the lipid bilayer, where the closed channel conformation represents the high-affinity binding site. In $K_v3.1$, the interaction residues F349, F351 in S5 and M430 in S6 are indeed non-polar, whereas the key residue T427 has a polar character. Additional mutations of the T427 residue to an Ala, Val, Cys, Ser and Lys showed that the affinity toward $K_v3.1$ decreased proportional to a decreased capacity for hydrogen bonding of the mutant amino acid. This suggests that the hydrogen-bonding ability of residue T427 in $K_v3.1$ is a major determinant of gambierol inhibition. Structure-activity relationship (SAR) studies with gambierol on mice identified that the C28=C29 double bond and the unsaturated side chain were required for a high toxicity (Fuwa et al. 2004). Recently a study with fragments of gambierol showed that the EFGH-rings were sufficient for the potent inhibitory effect towards K_v channels (Alonso et al. 2012). These SAR studies suggest that the key determinants for K_v channel inhibition reside in that section of the gambierol structure which also contains a hydroxyl group and could therefore be a possible candidate to interact with the T427 residue in $K_v3.1$.

Gambierol displays a novel mechanism of gating modification

Ciguatoxins mostly modulate Na_v channels by shifting the activation and inactivation to more hyperpolarized potentials or by altering the inactivation process (Strachan et al. 1999). This results in a persistent activation leading to an increased neuronal excitability. Conversely, gambierol reduces K_v channel activity by binding to an analog of site 5 in K_v channels (Kopljar

et al. 2009). Hence, despite that ciguatoxins bind to a similar binding site in Na_v and K_v channels, the modulation of channel function is opposite. This suggests that the high-affinity binding stabilizes different conformations in K_v (resting-closed) and Na_v channels (open).

How can gambierol inhibit ion permeation if it does not occlude the permeation pathway as external pore blockers or internal cavity blockers do? A possible mechanism of action could be modulation of the gating machinery that results in a “loss of function” of K_v channels. However, short depolarizing steps up to +140 mV did not show a significant activation of the ionic current (Kopljär et al. 2009) as can be seen with classical gating modifier peptides (Yeung et al. 2005, Swartz 2007). Moreover, longer depolarizations might result in gambierol unbinding which would mask the effect of a gating modification. To overcome this problem the effect of gambierol on VSD movements was investigated (Kopljär et al. 2013). These positively charged domains are capable of detecting changes in the membrane potential and respond by changing their conformation. The result is a transient charge displacement detectable as a gating current (Swartz 2008, Bezanilla 2008). Gambierol shifted the voltage dependence of gating charge movement (Q/V curve) in $\text{K}_v3.1$ channels by 120 mV towards more depolarized potentials (Kopljär et al. 2013). Such a strong shift is quite remarkable since most gating modifier peptides give rise to 50 mV shifts (Swartz 2007). Hence, with such a huge shift, no gating current movements are observed in the physiological range. Gambierol apparently immobilizes the VSDs in their resting state. Consequently, the channels cannot pass the concerted step of activation (requiring all VSDs to be in their activated state) and no channel opening will occur. Interestingly, the gating current data showed that during strong depolarizations the VSD movements in presence of gambierol were limited to those between the resting and the activated state, where still no ion permeation occurs. Hence, two interactions between gambierol and K_v channels are possible: a high-affinity interaction with the resting (closed) state and a low-affinity interaction with the activated state while the open state is not sensitive (Kopljär et al. 2013). This is different from the mechanism of gating modification by toxin peptides (e.g., hanatoxin and BDS-II), where the toxin sits as a cargo on the voltage sensor (Phillips et al. 2005, Wang et al. 2007). Hence, at more positive potentials, the channels can reach the open conformation with hanatoxin still bound. This implicates that the mechanism of gating modification for gambierol is quite different because of a different binding site and a different effect on VSD movement compared to classical gating modifier peptides.

The authors propose two possible mechanisms for channel inhibition by gambierol, both resulting in anchoring the gating machinery in the high-affinity resting state (Kopljär et al. 2013) (Fig. 5B). A first mechanism is based on anchoring the S6 activation gate in its resting (closed) state. Since

Fig. 5. A. Gating scheme for Kv channels. The four VSDs move independently from the resting state (R) to the activated-not-open (A) state. When all four VSDs have reached the A state, a concerted step results in channel opening (i.e., ionic current). Immobilization of a single VSD by gambierol precludes the movement of all four VSDs to the A state, resulting in no ionic permeation. B. Two possible mechanistic models for Kv channel inhibition by gambierol. Residue T427 (Kv3.1) in S6 is shown in red. ©Kopljár et al. 2013 originally published in *J. Gen. Physiol.* doi: 10.1085/jgp.201210890.

Color image of this figure appears in the color plate section at the end of the book.

VSD transitions from the resting to the activated-not-open state already induce rearrangements of the intracellular S6 gate region (del Camino et al. 2005), immobilization of this gate would prohibit channel opening. A second mechanism would be that gambierol interacts directly with the VSD domain and anchors it to the pore domain, causing a stabilization of the VSD in the resting state. Hence, further studies are required to completely understand the molecular basis of gambierol-induced gating modification of $K_v3.1$ channels.

Generally, external pore and internal cavity blockers interact in a one to one stoichiometry despite that K_v channels possess four possible binding sites due to the symmetry of the pore (MacKinnon 1991). However, toxins that bind outside the permeation pathway could bind to up to four binding sites because of the fourfold symmetry in K_v channels. This is true for gating modifier peptides of K_v channels which bind to the four identical voltage sensor paddles (Swartz 2007). In contrast, a Na_v channel has four distinct VSDs and it has been shown that gating modifier peptides bind preferentially to one or two VSDs to exhibit its effect on Na_v channels (Bosmans et al. 2008). Assuming that gambierol interacts outside the permeation pathway at a space between the pore domain and the VSD, one would expect four binding sites. Experiments with single high-affinity binding site concatemers showed that the effective single toxin-binding site interaction has an IC_{50} of 18–20 nM, which corresponds to an IC_{50} of approximately 3 nM in WT $K_v3.1$ channels with four independent binding

sites (Kopljár et al. 2013). Most likely, gambierol can bind up to four binding sites but binding to a single site is already sufficient for gambierol to inhibit ionic permeation. This was supported by gating currents analysis where the decrease in the slow component of the deactivating gating current (representative for channels that have passed the concerted step of opening) during gambierol onset had a similar time course as the inhibition of the ionic current (Kopljár et al. 2013).

While the presence of gambierol in ciguateric fish has not yet been reported, *in vivo* studies in mice show that gambierol can cause pathological effects (Ito et al. 2003). Gambierol exhibits potent toxicity against mice with a lethal dose of 80 $\mu\text{g.kg}^{-1}$ by intraperitoneal or intravenous administration and 150 $\mu\text{g.kg}^{-1}$ by oral administration. The main injury is observed in the lungs and the heart resulting in respiratory and cardiac failure. More recently, the effect of gambierol has been investigated on mouse and frog skeletal neuromuscular preparations and *Xenopus* skeletal myocytes where it has been shown that gambierol enhances muscle contraction, prolongs action potential duration and inhibits a transient outward K^+ current in skeletal muscle cells (Schlumberger et al. 2010b).

While the ciguatoxins display the dual action of potentiating Na_v channels and inhibiting K_v channels, the smaller gambierol apparently only inhibits K_v channels. The putative binding site in a cleft between S5 and S6 would be the equivalent of the neurotoxin site 5 in Na_v channels. Mechanistically, gambierol acts as a gating modifier that strongly stabilizes the rested, closed state.

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