Naunyn-Schmiedeberg's Arch Pharmacol (2000) 362 : 453–479
DOI 10.1007/s002100000319

REVIEW ARTICLE

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Structure, function and pharmacology of voltage-gated sodium channels

Received: 11 April 2000 / Accepted: 4 July 2000 / Published online: 24 August 2000

Abstract Voltage-gated sodium channels (VGSCs) are responsible for the initial inwards current during the depolarisation phase of action potential in excitable cells. Therefore, VGSCs are crucial for cardiac and nerve function, since the action potential of nerves and muscle cannot occur without them. Their importance in generation and transmission of signals has been known for more than 40 years but the more recent introduction of new electrophysiological methods and application of molecular biology techniques has led to an explosion of research on many different ion channels, including VGSCs. Their extraordinary biological importance makes them logical and obvious targets for toxins produced by animals and plants for attack or defence. The action of these and similar substances modulating the function of the VGSCs is interesting with respect to their possible use in medicine or use as tools in the study of these molecules. This review summarises recent progress in this research field and, in particular, considers what is known about the relationship of the structure to function, including a current understanding of the pharmacological modulation of VGSCs.

Key words Voltage-gated sodium channels · Channel gating · Molecular mechanisms · Pharmacological modulation · Binding sites

Abbreviations *αI–III* rat brain α-subunit isoforms I–III · *ATX* sea anemone toxin · *BTX* batrachotoxin · *CTX* ciguatoxin · *DI-DIV* domains 1–4 · *DDT* 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane · *GTX* grayanotoxin · *HH1* human heart Na^+ channel isoform-1 · *NMR* nuclear magnetic resonance · *PbTX* brevetoxin · *PKA, PKC* protein kinase A, C · *RH1* rat heart Na^+ channel isoform-1 · *S1–S6* transmembrane segments 1–6 · *SkM1* (*μ1*) skeletal muscle Na^+ channel isoform-1 · *SkM2* skeletal muscle Na^+ channel isoform-2 · *STX* saxitoxin · *TTX* tetrodotoxin · *UTR* untranslated region · *VGSC* voltage-gated sodium channel

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Introduction

Ion channels are crucial components for the activity of living cells. They are integral membrane proteins and allow particular ions to pass through them from one side of the membrane to the other. The plasma membrane acts as a barrier separating the cell contents from the outside, so that the ionic concentration inside the cell can be maintained at levels considerably different from those in extracellular fluids. This difference in ion concentration results in electrical potential difference between the cytoplasm and the external medium resulting in an electrochemical gradient across the plasma membrane for each ion species. Cells make use of these electrochemical gradients in their signalling and control process.

An important role in this process belongs to ion channels. Up to present there is a large number of different ion channels identified and this number is increasing constantly. This diversity leads to problems in their classification and nomenclature. If described in terms of their ionic selectivity and their gating properties, it is possible to define three major groups of ion channels. Channels belonging to the first group are activated by a change in membrane potential and they are described as voltage-gated or voltage-dependent channels. The sodium channels which are being reviewed in this article are belonging to this group. Another group includes channels which are extracellularly-gated by GABA, acetylcholine, ATP or glycine. A third group includes all channels which are activated by intracellular ligands such as calcium ions, ATP, cAMP or cGMP. In addition, there are some channels which cannot be placed in any of this groups.

Among many known voltage-gated channels, sodium channels have been of special importance in the history of physiology. Elucidation of their fundamental properties in

the squid axon by Hodgkin and Huxley (1952) launched modern channel theory. More recently, sodium channels were the first voltage-gated ion channels to be cloned (Noda et al. 1984). This approach using modern molecular biology techniques together with the patch-clamp technique led to better understanding of structure and function of these critical signalling proteins.

In heart, nerve and muscle cells the action potential is initiated by activation of the fast sodium channels. The activation of sodium channels, which are located in the membrane, results in increased permeability for sodium ions followed by an inward movement of sodium ions through these channels. After a period of between 0.5 and several hundreds of milliseconds, the conductivity for sodium ions decreases rapidly due to the inactivation of the channels. Nevertheless, this short yet abundant influx of sodium ions creates a cascade of events resulting in contraction and/or conduction of electrical impulses. As a consequence of an inward sodium current, other voltage-gated channels like potassium and calcium channels are being activated and contributing to the course of the action potential. Calcium channels are responsible for the plateau phase of the action potential and inflowing calcium ions cause the release of relatively large amounts of calcium from the intracellular storage which implements the contraction. Apart from the activated and the inactivated state, sodium channels can be in the resting state. Both resting and inactivated states are non-conducting, but channels that have been inactivated by prolonged depolarisation are refractory until the cell becomes repolarised. The repolarisation converts the channel from the inactivated into the resting state. In other words, the voltage-gated sodium channels (VGSCs) undergo cyclic changes in the form of three functionally distinct states starting with the resting state over the activated to the inactivated state.

This article reviews the current understanding of the function, structure and pharmacological modulation of VGSCs. In the past decade the development of molecular biology techniques enabled a much more accurate investigation of these aspects and resulted in a large increase of available information. Particular topics like activation, inactivation or pharmacology of the VGSCs are reviewed more in detail elsewhere (Catterall 1992; Fozzard and Hanck 1996; Marban et al. 1998).

Molecular organisation

Primary structure and general topology of the VGSC

Early biochemical studies utilising radio-labelled toxins as probes for sodium channels, identified a 260–280 kDa glycoprotein (referred to as α-subunit) as the major component of sodium channels from eel electroplax (Agnew et al. 1980), rat brain (Noda et al. 1986a) and chicken heart (Lombet and Lazdunski 1984). Later on, cloning and sequencing of other sodium channels enabled the prediction of topological arrangements within the α-subunit (Fig. 1A and B). In addition to the coding sequence, the sodium channel mRNA contains a relatively short 5′-UTR and a longer 3′-UTR followed by a poly(A)$^+$ tail.

Apart from the α-subunit, some sodium channels contain one or two smaller subunits called β1 and β2. It has been shown that the sodium channel from rat brain is a heterotrimeric protein containing subunits α, β1 and β2, whereas heart and skeletal muscle contain just α- and β1-subunits (Roberts and Barchi 1987; Satin et al. 1992a). It seems that these tissue-specific patterns are consistent within sodium channels of various species. Exceptions are eel electroplax (Grant 1991; Krafte et al. 1994) and chicken heart (Catterall 1988), containing only α-subunits.

Thanks to a rapid development of molecular biology techniques a large number of sodium channel α-subunits have been cloned and sequenced (Akopian et al. 1996; Chen et al. 1997; Dib-Hajj et al. 1998; Gellens et al. 1992; Kallen et al. 1990; Kayano et al. 1988; Noda et al. 1986b; Rogart et al. 1989; Schaller et al. 1995; Toledo Aral et al. 1997; Trimmer et al. 1989). Table 1 summarises known VGSC α-subunits. Considered in the light of structural models, each isoform represents a kind of naturally occurring mutation, providing clues about which part of the primary structure carries a particular function. To study a functional diversity, most of cloned VGSCs have been expressed in heterologous systems. However, the expression of some cloned VGSCs failed (chicken heart, eel electroplax).

Although the structure of the inactivation gates of the potassium channel (Antz et al. 1997) as well as the one of VGSC (Rohl et al. 1999) have been resolved by NMR lately, this indirect approach remains a major tool for the prediction of the structure of these large molecules. Since it is unlikely that the three-dimensional structure of the whole protein will be completely resolved by x-ray crystallography or NMR in the near future (because of the size and hydrophobic nature of the protein) we are obliged to deduce the secondary structure and some features of tertiary structure from the primary amino acid sequence.

The α-subunit

The α-subunit is the major subunit of the channel. When expressed in a heterologous system, it possesses all of the channel's major properties including voltage-dependent gating and selectivity for sodium. Cloning of the α-subunit of the eel electric organ gave the initial insight into the primary structure of a voltage-gated ion channel. Using oligonucleotides encoding short segments of the electric eel electroplax sodium channel and antibodies directed against it, a cDNA encoding the entire polypeptide was successfully isolated (Noda et al. 1986a). The deduced amino acid sequence revealed a structure which is common to all known VGSCs (Fig. 1A). It is a large protein with four internally homologous domains (DI-DIV), each containing multiple potential α-helical transmembrane segments (S1–S6) of 19–27 residues. These transmembrane segments are connected by non-conserved, hy-

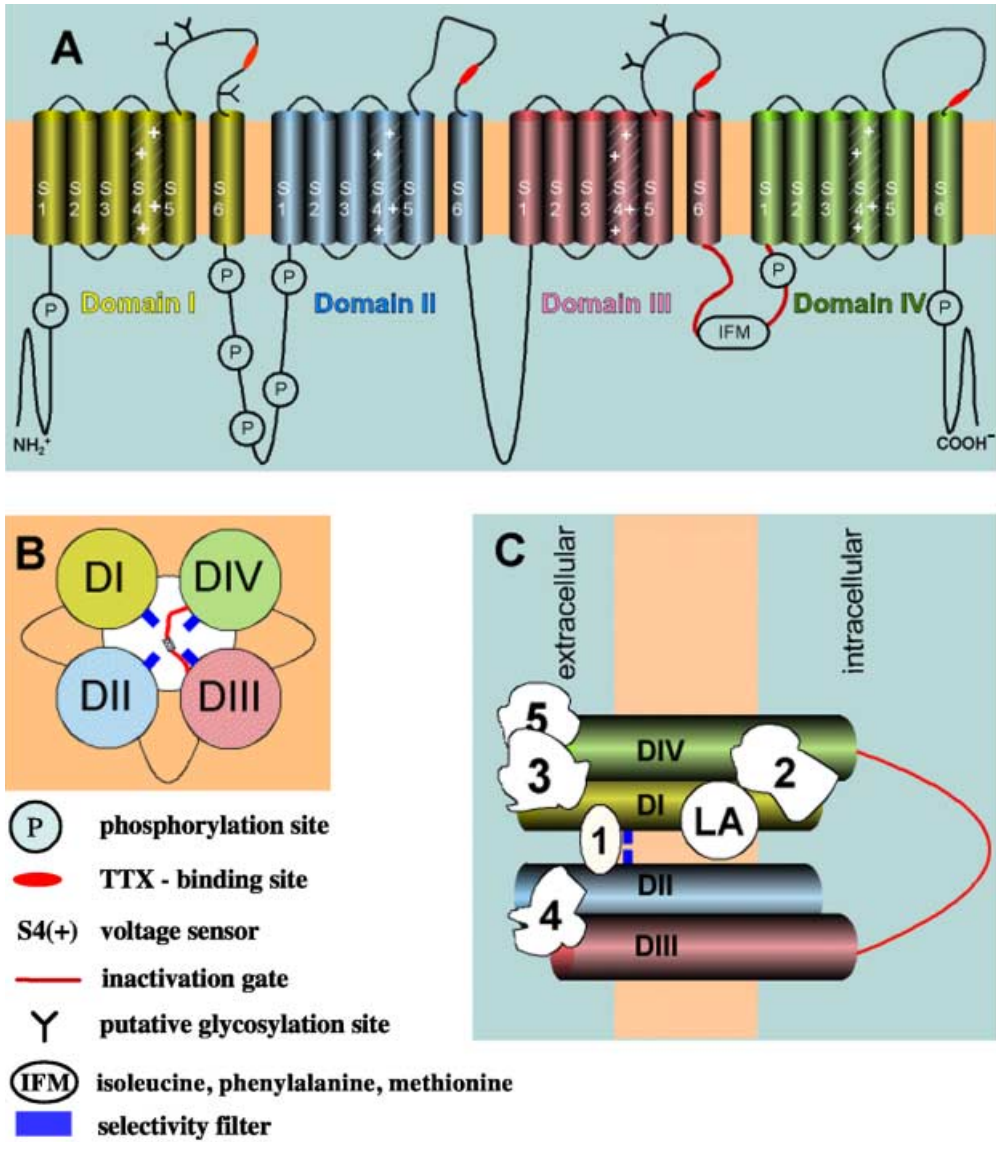

Fig. 1 A–C Topology of the voltage-gated sodium channel (VGSC). **A** The α-subunit of the VGSC consists of four homologous domains (*DI–DIV*), each containing six transmembrane segments *S1–S6*. Single segments and domains are connected by intracellular and extracellular loops. The isoleucine, phenylalanine and methionine (*IFM*) motif has a crucial role in the VGSC inactivation. **B** Transmembrane arrangement of DI–DIV domains of the α-subunit around the channel pore. **C** Binding sites (1–5) of the VGSC ligands (*LA*: putative local anaesthetic binding site, *TTX* tetrodotoxin)

Table 1 Cloned voltage-gated sodium channel (*VGSC*) α-subunits

VGSC α-subunit	Tissue expression	Genbank/EMBL. Accession No	Reference
αI	Rat brain	X03638	Noda et al. (1986a)
αII	Rat brain	X03639	Noda et al. (1986a)
αIII	Rat brain	Y00766	Kayano et al. (1988)
Na6	Rat brain, neurons and glia	L39018	Schaller et al. (1995)
PN1	Peripheral neurons	U79568	Toledo Aral et al. (1997)
μ1 (SkM1)	Rat skeletal muscle	M26643	Trimmer et al. (1989)
SNS/PN3	Sensory neurons	X92184	Akopian et al. (1996)
NaNG	Dog nodose ganglion neurons	U60590	Chen et al. (1997)
NaN	Peripheral sensory neurons within dorsal root ganglia and trigeminal ganglia	AF059030	Dib-Hajj et al. (1998)
SCL-11	Broadly expressed atypical	Y09164	Wood JN, unpublished data, direct submission
HH1	Human heart	M77235	Gellens et al. (1992)
RH1	Rat heart	M27902	Rogart et al. (1989)
SkM2[a]	Rat denervated and immature skeletal muscle		Kallen et al. (1990)

[a]sequences of RHI and SkM2 differ only in a negligible number of nucleotides, none of them resulting in amino acid change (probably cloning artefacts)

drophilic intervening sequences. The fourth transmembrane segment (S4) of each domain is highly positively charged, with a number of conserved arginines or lysines located in each third position. That part of the channel is believed to be a voltage sensor. The amino- and carboxy-termini are on the intracellular side as strongly suggested by antibody binding study with the eel electroplax (Nakayama et al. 1993). The interdomain connecting loops have been localised to the cytoplasmic surface. As shown in Fig. 1A, the connecting loops between DI and DII and between DII and DIII are long, whereas the loop between DIII and DIV is much shorter.

The α-subunit is glycosylated and phosphorylated. The importance of these channel modifications is discussed in a separate chapter. In general, the similarity in amino acid sequence of the α-subunit is greatest in the homologous domains from transmembrane segment S1 through S6, while the intracellular connecting loops as well as the amino- and carboxy-termini are not highly conserved. An exception is the highly conserved interdomain IDIII-IV.

The β-subunits

In addition to the major α-subunit, most VGSCs have one or two small auxiliary β-subunits. The deduced primary structure of the rat brain sodium channel β1-subunit indicates that it is a 23 kDa protein containing a small cytoplasmic domain, a single putative transmembrane segment and a large extracellular domain with four potential N-linked glycosylation sites (Isom et al. 1992). Northern blot analysis revealed that human β1-mRNA is abundantly expressed in skeletal muscle, heart and brain and is encoded by a single gene (Makita et al. 1994).

The sodium channel β2-subunit contains also a single transmembrane segment, a small intracellular carboxyl terminal domain and a large glycosylated extracellular amino-terminal domain (Catterall 1995). The β1-subunit is bound non-covalently whereas the β2 is bound covalently to the α-subunit to form a heterotrimer.

Various biochemical methods have been used to study the role of the β1- and β2-subunits in the functional properties of sodium channels. It has been found that the β2-subunit can be removed from purified rat brain sodium channels without any apparent effect, whereas removal of β1-subunits causes loss of all functional properties (Messner et al. 1986). The loss of activity can be partially prevented by intramolecular cross-linking with the α-subunit, suggesting that the interaction with the β1-subunit may serve, at least in part, to stabilise the structure of the α-subunit.

The β1-subunits have multiple effects on sodium channel function: increased peak current, accelerated activation and inactivation as well as altered voltage-dependence of inactivation. Although α-subunits alone are sufficient to encode functional channels, β1-subunits appear to modulate the kinetics of inactivation. Recombinant brain and skeletal muscle sodium channel α-subunits expressed in *Xenopus* oocytes, exhibit anomalously slow inactivation as compared to sodium currents present in native tissue or with those expressed in oocytes injected with unfractionated rat brain poly(A)$^+$ RNA (Trimmer et al. 1989; Zhou et al. 1991). It has been shown that co-expression of the human β1-subunit with the recombinant human skeletal muscle α-subunit in *Xenopus* oocytes results in sodium currents that inactivate rapidly (Makita et al. 1994). In contrast, the human β1-subunit apparently has no effect on the function of the human heart sodium channel α-subunit.

Interestingly, both subunits contain immunoglobulin-like motifs, similar to those found in many cell-adhesion molecules (Isom et al. 1995). Because nearly all of the immunoglobulin-like motifs identified so far interact with extracellular protein ligands (Williams and Barclay 1988), it is suggested that β1- and β2-subunits probably have similar function (Isom and Catterall 1996) and may act like neural cell-adhesion molecules.

Molecular determinants of the channel gating

Most of the data dealing with functional aspects of gating are obtained from mutagenesis experiments. Currently very little is known about the activation and structure involved in this process. More is known about the voltage sensing. The inactivation is by far the best studied gating process.

The outer vestibule and selectivity filter

The model of the outer mouth of the pore predicts two distinct structures: a selectivity filter and a so-called toxin binding site 1. Valuable tool in the investigation of the shape and structure of the outer vestibule have been the guanidinium toxins TTX and STX. Combination of the chrystallographical determination of their structures and the various mutation experiments improved considerably the understanding of the channel's permeation path. Although structures determining the selectivity of the sodium channel and isoform diversity concerning the toxin binding are functionally and topologically inseparable, we discuss the later topic in details in the chapter dealing with the binding site 1. Here we focus only on the structures involved in the channel selectivity.

By examining permeation of a series of small cationic ions and molecules, Hille was first to estimate the cross-sectional size of the sodium channel (Hille 1971). Using molecular modelling, it was possible to confirm these results (Fozzard and Lipkind 1996). Looking from the extracellular side into the pore, a wide mouth of 1.2 nm is followed by a narrowing of the channel of 0.3–0.5 nm (Fozzard and Lipkind 1996). This narrowing corresponds to a putative ion selectivity filter (Fig. 1B and C). One set of four amino acid residues situated in this region, namely aspartic acid, glycine, lysine and alanine (so-called DEKA locus) forms the narrowest part of the pore acting as the selectivity filter. Together with more extracellularly situated residues glutamic acid, glutamic acid, methionine

and aspartic acid, they are probably involved in channel conductance, selectivity and toxin binding (Backx et al. 1992; Chen et al. 1992; Heinemann et al. 1992b; Noda et al. 1989; Terlau et al. 1991). All of the above mentioned amino acids from both sets are equivalently positioned in the four repeats and forming a three-dimensional cluster which is situated within the segment defined as SS2 (Terlau et al. 1991) or P loop or P segment (Lipkind and Fozzard 1994).

Succeeding results indicate that the lysine residue in DIII of the sodium channel plays a central role in discrimination between monovalent ions Na^+ and K^+ and exclusion of divalent Ca^{2+} ions (Favre et al. 1996). The exact mechanism of discrimination between different cations remained unclear.

The idea of a transmembrane ion path lined by an infolding of the extracellular loops between segments S5 and S6 of each domain existed already for years (Guy and Conti 1990). Based on experimental studies of channel block by toxins and available data on point mutations, Lipkind and Fozzard proposed a model of the outer vestibule (Lipkind and Fozzard 1994). Shortly, the model proposes the existence of a binding pocket for TTX and STX composed of antiparallel β-hairpins which are situated in extracellular connecting loops between S5 and S6 of all four domains. One section of each loop, a highly conserved P segment (SS2), has been implicated as part of the external mouth of the pore and the selectivity filter. Some aspects of this model were revised recently (Penzotti et al. 1998).

The voltage sensors and activation

Gating, a change between the non-conducting and conducting state of a channel, is a response to voltage alteration. This action takes place in all excitable cells. For example, in a heart cell membrane potential changes are controlled by pacemaker cells. Voltage-gated channels which sit in the cell membrane, respond to such voltage shifts by conformational changes. Already with the cloning of the first sodium channel (Noda et al. 1984) a possible voltage sensor was identified. The general criterion for a voltage sensor are charged residues located within an electric field. This feature was recognised by Noda and colleagues in a putative α-helical transmembrane segment of the cloned sodium channel. The S4 segment (Fig. 1A) in each of the four domains of the α-subunit contained an arginine or lysine, both of which are positively charged, at every third position. In the rat brain sodium channel there are five such residues in the S4 segments of the first and second domain (DI and DII), six in S4 of DIII and eight in S4 of DIV. This number is more or less constant in different types of sodium channels. Since S4 are transmembrane segments, they are therefore lying within the electric field and as such good candidates for voltage sensing role. This hypothesis was also supported by the fact that similar patterns are found in other voltage-gated channels. Site-directed mutagenesis experiments (Stühmer et al. 1989) confirmed, at least partially, this attribute of S4. Similar results were obtained by site-directed mutations in S4 of voltage-gated potassium channel (Papazian et al. 1991; Terlau et al. 1997). Several arginine and lysine residues from S4 of domains DI and DII were replaced by neutral or negatively charged residues. This produced a reduction in steepness of the relation between channel opening and the membrane potential when S4 of DI was mutated, but much less when changing S4 of DII. The effect of S4 positive charge mutations was also studied in correlation to fast inactivation kinetics (Chen et al. 1996). It can be concluded that all S4 segments are involved in both activation and inactivation, however, individual S4 segments have different roles in both processes (Kühn and Greeff 1999).

The exact understanding of the conformational changes associated with activation is troublesome because of limited data and the difficulty in determining allosteric versus direct involvement of different residues.

The inner vestibule and inactivation gate

After the activation of the sodium channel, the permeability for sodium rises rapidly and then decays during the depolarisation step. The inactivation of VGSCs is responsible for this decay of sodium current. Compared to the resting state of the channel, which is also a non-conducting state of the channel, inactivation is a process by which the sodium channel becomes unavailable for reopening until after membrane repolarisation.

Presently, we know about at least two modes of sodium channel inactivation: a slow inactivation which develops over seconds to minutes and a fast inactivation with a time scale of milliseconds. Relatively little is known about the slow inactivation. In contrast, the mechanism involved in fast inactivation has been studied intensively.

For quite a long time it has been known that a cytoplasmic loop of the channel might be involved in channel inactivation, according to the “ball and chain” model (Armstrong and Benzanilla 1977). The intracellular loop between domains DIII and DIV is suspected to be this inactivation gate (Noda et al. 1986a). This loop is depicted by the red line in Fig. 1A–C. Already the fact that this large connecting loop is extremely well conserved between different sodium channels, emphasises its physiological importance. The first clear experimental evidence of the role of the DIII-IV linker region as an inactivation gate was delivered through the experiments of Stühmer and colleagues (1989). They have constructed a number of mutant clones and two of them, which have a cut and addition or a cut, respectively, between repeats III and IV (DIII and DIV), were characterised by a dramatic decrease in the rate of inactivation. Just a few months later these results were confirmed by another group which used the inhibition of inactivation by antibodies directed against this region (Vassilev et al. 1989). After identification of this intracellular region as inactivation gate, a cluster of hydrophobic amino acids was found to play a crucial role.

This cluster, containing isoleucine 1488, phenylalanine 1489 and methionine 1490 (IFM) has been identified as a fragment required for sodium channel inactivation (West et al. 1992). More recently it has been shown that small peptides containing this IFM sequence are sufficient to restore fast inactivation of sodium channels with mutations in IDIII-IV connecting loop, leading to the hypothesis that the IFM motif binds within the pore of the sodium channel and blocks it during the inactivation (Eaholtz et al. 1998). This hypothesis implies the presence of amino acid residues in the intracellular mouth of the pore of the sodium channel that are involved in conformational changes which couple activation to inactivation and bind the IFM motif to result in an inactivated state. This part of the channel is described as inactivation linker or inactivation receptor or docking site of the inactivation gate. Using scanning mutagenesis it has been found that several residues located in a short connecting segment between S4 and S5 of the domains DIII (Smith and Goldin 1997) and DIV (McPhee et al. 1998; Tang et al. 1996), situated on the inner vestibule of the channel, play a critical role in gate closure and binding. It has been suggested that the IFM motif of the inactivation gate interacts with F1651 and/or L1660 (in the rat brain αII sodium channel) in the S4-S5DIV loop during inactivation of closed channels. In addition, the S6 transmembrane segment of domain DIV have been shown to be important for inactivation (McPhee et al. 1994) but the peptide containing the IFM motif from the inactivation gate could still restore inactivation in channels carrying mutations in this region (F1764A and V1774A) (McPhee et al. 1995). The authors concluded that these residues do not directly interact with the inactivation gate meaning that they do not function as a docking site for the IFM motif. First demonstration of direct interaction consistent with a docking site model has been demonstrated by mutation experiments within the short connecting loop between segments S4 and S5 (S4-S5) of the domain DIII (Smith and Goldin 1997). The results showed that a highly conserved alanine at position 1329 belonging to S4-S5 of the domain DIII is an important residue for normal inactivation (Smith and Goldin 1997). More detailed investigation of the S4-5 from the domain DIV using the scanning mutagenesis suggested that F1651, L1660 and N1662 play a crucial role in the inactivation process (McPhee et al. 1998). The residues F1651 and/or L1660 are supposed to interact with IFM motif from the inactivation gate.

Recently, the first high-resolution data for VGSC were published. Rohl et al. (1999) cloned and expressed the part of the putative inactivation gate and resolved the structure by NMR. They were able to show that the inactivation gate is an α-helical structure capped by an N-terminal turn. These data represent the first high-resolution data for voltage-gated sodium channels.

At the same time, the molecular mechanism of sodium channel slow inactivation remains mainly unresolved. This physiologically important but poorly understood process is common to all sodium channels. Slow inactivation is thought to play an important role in membrane excitability and firing properties (Sawczuk et al. 1995). Slow inactivation participates in different physiological and pathophysiological events. This process may play only a minor role in normal cardiac function, while playing a more prominent and physiologically important role in brain and muscle (Sawczuk et al. 1995). Several hereditary diseases which might involve defects in slow inactivation are known, such as periodic paralysis (Cummins and Sigworth 1996; Ruff 1994).

It is known that the slow inactivation of cardiac sodium channels is very limited compared to slow inactivation in nerve and skeletal muscle and this represents an important physiological difference between cardiac sodium channels and other sodium channel subtypes (Richmond et al. 1998). Similarly as in the case of fast inactivation, slow inactivation of the skeletal muscle isoform (SkM1) but not cardiac isoform (HH1) is modulated by the β1-subunit (Vilin et al. 1999). The mechanism of this modulation is still not known. It seems that P-loops might be structural determinants of this process through the interaction with the β1-subunit.

It is believed that slow and fast inactivation are independent events. This is supported by the fact that cytoplasmic application of proteolytic enzymes abolishes fast inactivation without disrupting slow inactivation (Valenzuela and Bennett 1994). It is not known if there is a slow inactivation gate as in the case of the fast inactivation. However, the IFM motif which plays an important role in fast inactivation produces also a slow use-dependent inactivation when applied to the cytoplasmic side (Eaholtz et al. 1999). It has been proposed that fast block represents binding of the inactivation gate to the inactivation gate receptor in the short S4-S5 connecting loop of the DIV, while slow block represents binding of the IFM peptide deeper in the pore. At the same time, there is a relationship between the outer mouth of the pore and the slow inactivation process. Mutation of a external pore-lining residue had an effect on slow inactivation (Balser et al. 1996). Mutation of a single arginine residue in the transmembrane segment S6 of the DI (S6DI) also altered drastically slow inactivation of the channel (Wang and Wang 1997). For these reasons the "C-type" inactivation of the Shaker K channels has been proposed to apply for VGSCs as well. Another point mutation in the transmembrane segment S2 of the domain DIV altered both activation and inactivation of the sodium channel (Fleig et al. 1994). The slow inactivation was also impaired by mutations localised on the cytoplasmic side of the channel (Cummins and Sigworth 1996).

Slow and fast inactivation also differ in that the kinetics and steady-state levels of slow inactivation are influenced by the concentration of extracellular alkali and organic cations (Townsend and Horn 1997). The decay kinetics are faster for the organic cations than for the alkali metal cations. In contrast to the fast inactivation which is rather insensitive to changes of $[Na^+]_o$ (Oxford and Yeh 1985; Tang et al. 1996), raising extracellular cation concentration inhibits slow inactivation. These findings, together with mutation experiments within the outer channel pore indicate that the conformational change during

slow inactivation might depend on the occupancy of the putative "Na^+ binding site" at the outer mouth of the channel. Using chimeras between human heart and rat skeletal muscle it could be demonstrated that all four domains can modulate the sodium channel slow inactivation, but the domains DIII and DIV are shown to be less important than DI or DII (O'Reilly et al. 1999). Future experiments of this kind may allow identification of structures involved in slow activation as well as structures mediating interaction between fast and slow inactivation.

Role of post-translational modifications of VGSCs

Gating properties of VGSCs can be modulated by glycosylation, phosphorylation, fatty acylation and sulphation. Here we concentrate on glycosylation and phosphorylation which contribute mostly to the final structure of the channel in vivo. Fatty acylation and sulphation are described elsewhere (Schmidt and Catterall 1987).

Glycosylation of VGSCs

All of the VGSC subunits are post-translationally modified by glycosylation. Fifteen to 30% of the mass of Na^+ α-subunits and approximately 25% of the mass of β1 and β2-subunits are carbohydrates (Catterall 1988). The carbohydrate moieties include chains containing large amounts of sialic acid (James and Agnew 1987), an unusual post-translational modification of vertebrate proteins. The function of these carbohydrates has not yet been well examined. However, the α1-subunit of the Ca^{2+} channel lacks extensive glycosylation (Catterall et al. 1988; Henning et al. 1996), which suggests that the critical functions of the channel can be fulfilled without direct participation of carbohydrates. It is known that glycosylation is an essential process in the maintenance of the normal steady state of biosynthesis and degradation of sodium channels (Waechter et al. 1983). It is also considered to be important for the functional expression of voltage-activated channels in embryonic neurons during early stages of cell growth in culture. The channels become less dependent of glycosylation in mature neurons. The hydrophilic extracellular loops are the consensus sites for N-linked glycosylation lying between S5 and S6 of domain DI and DIII (Fig. 1A). There are 14 potential sites for N-linked glycosylation in the regions of HH1 (Gellens et al. 1992). β1-subunit contains four potential sites for N-linked glycosylation in the extracellular domain which is not required for function of the β1-subunit (McCormick et al. 1998). Experiments on whole-cell patch-clamped rat neocortical neurons show that the Na^+-current decreased progressively in the presence of an inhibitor of protein N-glycosylation (Zona et al. 1990). Moreover, recent experiments with transiently expressed cardiac and skeletal muscle channels have shown that glycosylation differentially regulates Na^+ channel function in these two isoforms (Zhang et al. 1999). Glycosylation can influence the toxin binding as shown for STX binding in the neuroblastoma cells (Waechter et al. 1983).

Phosphorylation of VGSCs

Phosphorylation consensus sequences are found on the IDI-II and IDIII-IV linkers and on both intracellular termini (Fig. 1A). Although still controversial, the role of the phosphorylation in the channel function has been studied by many research groups and is very complex. Generally, channel phosphorylation results from a complex balance between activities of protein kinases, different kinase isoforms, and phosphatases. Since different cells express different protein kinases and phosphatases the phosphorylation of the same sodium channel may vary between different cells. Even variations within the same cell may occur if conditions change.

It has been shown that cytosolic segments such as the interdomain loop between domains DI and DII (IDI-II) or domains DIII and DIV (IDIII-IV) represent potential targets for protein phosphorylation (Chen et al. 1995; Dascal and Lotan 1991; Sigel and Baur 1988) by cAMP-dependent protein kinase (PKA) or protein kinase C (PKC). The loop between domains DI and DII contains four serines which are phosphorylated in vitro and in vivo by PKA (Murphy et al. 1993). The serine residue occurring in the highly conserved IDIII-IV (S1506) is required for the PKA effect on the rat brain isoform αII (Li et al. 1993; West et al. 1991), while it is not necessary for the cardiac isoform (Frohnwieser et al. 1995). Phosphorylation of this site is required for slowing of Na^+ channel inactivation and reduction of the peak current by PKC.

PKA has been shown to modulate VGSC individually in different isoforms: PKA-mediated phosphorylation has been shown biochemically for neuronal (Costa et al. 1982; Murphy et al. 1993), skeletal muscle (Ukomadu et al. 1992) and cardiac (Cohen and Levitt 1993) isoforms of VGSCs. The cardiac channel has eight candidate consensus PKA phosphorylation sites in the loop between domains DI and DII, all of which are distinct from the neuronal channel. In vitro studies of the expressed cardiac sodium channel demonstrate cAMP-dependent phosphorylation on only two of these serines (Murphy et al. 1996).

The functional consequences of PKA activation have also been studied for different heterologously expressed isoforms (Frohnwieser et al. 1997; Gershon et al. 1992; Schreibmayer et al. 1994). The activity of the neuronal isoform (αII) becomes attenuated upon PKA stimulation due to forskolin or isoproterenol stimulation (Gershon et al. 1992; Li et al. 1993; Smith and Goldin 1996). In contrast to the attenuation in neuronal isoforms, the cardiac isoform RH1 is stimulated by PKA (Schreibmayer et al. 1994) and skeletal muscle channel (SkM1) currents remain unchanged (Frohnwieser et al. 1997). In other words, PKA and PKC have similar effects in αII: so-called convergent modulation as described by Li et al. (1993) while cardiac sodium channels are modulated oppositely by PKA and PKC: so-called divergent modulation.

Many Ca^{2+} and K^+ channels are also regulated by pathways involving protein phosphorylation, suggesting that long-term modulation of ion channel function by phosphorylation is a wide-spread regulatory mechanism. It is also very probable that different phosphorylation patterns account for, at least partially, functional differences between channel isoforms.

Pharmacology of the VGSCs: binding sites and ligands

A large number of biological toxins exert their toxic effects by modifying the properties of sodium channels. These include the water-soluble heterocyclic guanidines: TTX and STX, the lipid-soluble polycyclic compounds: veratridine, aconitine and BTX and the low-molecular weight polypeptide venoms isolated from scorpion venoms and sea anemone as well as many others.

According to their binding properties, Catterall (1980) defined five groups of toxins (Fig. 1C and Table 2). Briefly, site 1 binds TTX, STX and μ-conotoxin which block ion conductance. Site 2 binds BTX, veratridine and aconitine, resulting in persistent activation of the sodium channel. Site 3 binds the scorpion α-toxins and sea anemone toxins which slow or block inactivation. Agents which bind on this site also enhance the persistent activation of the sodium channel caused by toxins acting at the receptor site 2. Receptor site 4 binds scorpion β-toxins that shift the voltage-dependence of activation to more negative membrane potentials without modifying the sodium channel inactivation. Finally, receptor site 5 binds brevetoxin and CTX, agents that cause repetitive neuronal firing, shift the voltage dependence and block sodium channel inactivation. Except substances with defined binding sites, changes of extracellular divalent ion concentration, pH and ionic strength influence the channel gating. No direct chemical modifications of activation are known (Hille 1992). More recently, another distinct binding site 6 was proposed in addition to the classically defined five sites: the insecticide binding site.

As defined by Hille (1992) and summarised in Table 2, there are several major classes of VGSC modifiers: those removing inactivation, others slowing inactivation, toxins shifting activation and agents affecting voltage dependence of gating. Some of lipid-soluble toxins, like aconitine or veratridine exert combined effects by shifting activation and slowing inactivation. Another set of pharmacological agents, typified by DPI 201-106, has been used to slow or remove inactivation of cardiac sodium channels (Scholtysik et al. 1985). These agents prolong the action potential duration and increase cardiac contractility, making them potentially useful for inotropic and antiarrhythmic therapy in patients with depressed cardiac contractility (Hille 1992). Below we review the most important sodium channel modifiers which have greatly improved our understanding of the VGSC function and structure.

TTX, STX and conotoxins (binding site 1)

The first two naturally occurring toxins are probably the best investigated sodium channel modifiers. Tetrodotoxin (TTX) is a potent poison (LD_{50} 0.1 mg/kg) found in ovaries and liver with lesser amounts in intestines and skin of the puffer (fugu) fish and its relatives from the family *Tetraodontidae*. The fish do not synthesise TTX, but instead concentrate it from ingested bacteria. Restaurants in Japan and Korea serve the fugu fish as delicacy

Table 2 Toxin binding sites associated with the VGSC

Binding Site	Neurotoxin	Physiological effect
1	Tetrodotoxin (TTX) Saxitoxin (STX) μ-Conotoxins	Ion channel block ⇒ Inhibition of ion transport
2	Veratridine Batrachotoxin (BTX) Aconitine Grayanotoxin (GTX)	Persistent activation
3	North African scorpion α-toxin Sea-anemone toxins (ATX) δ-Atracotoxins	Slow inactivation; enhancement of persistent activation
4	American scorpion β-toxins	Transient repetitive activity and block ⇒ shift voltage-dependent activation to more negative potentials
5	Brevetoxins (PbTx) Ciguatoxins (CTX)	Repetitive activity; persistent activation ⇒ shift voltage-dependent activation to more negative potentials
6?	Pyrethroids DDT	Repetitive activity and/or block; Slowing of activation, inactivation and deactivation
Unidentified sites	DPI 201-106 Local anaesthetics	Prolongation of action potential Ion channel block

after removing poisonous organs. Nevertheless, many fatalities are reported every year ranging from Papua New Guinea, over Japan to Thailand and Vietnam. The total number of deaths per year is estimated to be 120 (Klaassen 1996). Furthermore, there is an interesting ethnopharmacological link between TTX poisoning and Voodoo folklore in Haiti. TTX has been found to be the major active pharmacological ingredient responsible for the introduction of zombie state catalepsy (Benedek and Rivier 1989). It has been isolated from a powder used in voodoo rituals, which has been prepared from various animals (fish, frog) and plants by special extraction methods.

STX, like TTX, is a blocker of sodium channel activation known also as paralytic shellfish poison (PSP). It is a toxin found in certain molluscs, arthropods, echinoderms and some other marine animals that have ingested toxic protists mostly of the order *Dinoflagellata*, unicellular organisms in the marine plankton. In addition, STX is a product of certain freshwater cyanobacteria. In this way filter-feeding shell, fish or clams become poisonous themselves and can cause paralytic shellfish poisoning through the food chain in both domestic animals and humans.

TTX and STX are perhaps the most specific VGSC toxins. In most cases binding is rapidly reversible (seconds and minutes) and of high affinity. Their equilibrium dissociation constant K_d is 1–10 nM for skeletal muscle or nerve isoforms and for less sensitive cardiac VGSC a few micromolar. The two guanidinium toxins and their structural analogues were valuable tools in the investigation of the shape and structure of the outer vestibule as well as for modelling of TTX and STX binding site (Lipkind and Fozzard 1994). Binding studies with tritiated STX suggested that also the β1-subunit participates in forming the TTX/STX binding site (Messner and Catterall 1986). In combination with site-directed mutation experiments it was possible to make a quite refined picture of this part of the channel. For better understanding the amino acid sequence of this region from three VGSC isoforms is shown in the Fig. 2. A single point mutation of glutamic acid 387 in SS2 segment (or P segment; see also the chapter about the outer vestibule and the selectivity filter) of DI from rat brain II isoform (AC X03639) to glutamine (E387Q) results in complete loss of TTX and STX block (Noda et al. 1989). Subsequently, systematic mutations of mainly charged residues in SS2 segments of all four repeats were performed (Terlau et al. 1991). Different mutations affected TTX and STX sensitivity to different grades, however neutralisation of any of the six conserved residues of glutamic acid or aspartic acid reduced the sensitivity by at least three orders of magnitude. TTX interacted primarily with the residues in the segments of the first and second repeat. Concerning STX, neutralisation of aspartic acid at position 1717 of the repeat IV (D1717N9) abolished the block by this toxin, while just partially reducing the block by TTX.

It was generally accepted that TTX and STX bind to the same site on the channel. This idea was based upon different facts like that two toxins completely block current, competitively block each other in binding assays, are

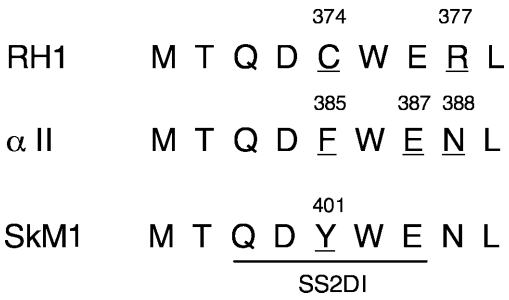

Fig. 2 The positions of amino acid residues (one-letter code) that have been identified as major determinants for tetrodotoxin/saxitoxin (TTX/STX) and cation (Cd^{2+}, Zn^{2+}) sensitivity differences among VGSC isoforms are underlined and numbered as in Terlau et al. 1991. SS2DI is a segment belonging to the connecting loop between S5 and S6 of the domain DI and contributing together with corresponding segments from other repeats to the TTX/STX binding site and selectivity filter (*RH1* rat heart Na^+ channel isoform-1, α*II* rat brain Na^+ channel isoform 2, *SkM1* skeletal muscle Na^+ channel isoform-1)

of similar size, possess similar functional groups and mutations in the outer vestibule seem to have qualitatively similar effects on the affinity of both toxins (Noda et al. 1989; Terlau et al. 1991) All these similarities led Lipkind and Fozzard (1994) to propose a molecular model of the TTX and STX binding pocket composed of antiparallel β-hairpins formed from peptide segments of the four S5-S6 extracellular loops of the VGSC. On the other side, some more recent experimental results suggested that there might be some differences in this respect (Kirsch et al. 1994). To elucidate those disagreements in the specific interactions between the channel vestibule and the two toxins, a quantitative comparison of the effects of the mutations at eight important vestibule sites was performed (Penzotti et al. 1998). For this study the skeletal muscle VGSC isoform (μ1 or SkM1) expressed in *X. laevis* oocytes was used. Results showed that the most dramatic differences between TTX and STX block were introduced by the mutation of a tyrosine residue at the position 401 (Fig. 2). The effects of the mutation were more pronounced through aspartic acid than through cysteine substitution. At the same time, mutations in that position affected STX blocking efficacy much less than that of TTX. For the STX block, two outer ring residues glutamic acid 758 and aspartic acid 1532 were more important. All three mentioned residues (Y401, E758 and D1532) are situated closer to the extracellular part of the outer mouth of the pore. More intracellularly located charged residues from the inner selectivity region (DEKA locus) interact similarly with STX and TTX, supporting the idea that the two toxins block the pore by the same mechanism in spite of differences in interaction with amino acid residues situated around the pore.

In addition to the TTX/STX binding properties, VGSCs from different tissues have distinct properties concerning the sensitivity to TTX itself. The cardiac muscle sodium channels are less sensitive than those of brain and skeletal muscle: they require micromolar (TTX-resistant or TTX-R) instead of nanomolar (TTX-sensitive or TTX-S) concen-

tration of TTX to be blocked. The first hypothesis that explains high/low-affinity TTX and STX binding in the VGSCs has focused on electrostatic attractions between the positively charged guanidinium groups and negatively charged acidic groups in their binding site on the channel (Hille 1992). Cardiac and brain isoforms have only two residues in the P segment which are different (Fig. 2). A first one is cysteine at position 374 in the cardiac isoform (RH1) in place of phenylalanine 385 in brain (αII) or tyrosine 401 in skeletal muscle (SkM1). The second difference concerns an arginine residue in position 377 (in RH1) in place of an asparagine (position 388 in α II or position 404 in μ1). To prove the later hypothesis, mutations N388R in brain (Terlau et al. 1991) and R377N in the heart isoform (Satin et al. 1992b) were performed. These experiments ruled out the simple electrostatic attraction hypothesis, since these mutations showed a minimal effect on toxin binding, which was also shown by Heinemann and colleagues (1992a). In addition they showed that the mutation F385C not only diminishes sensitivity to the guanidinium toxins but also increases sensitivity to Zn^{2+} and Cd^{2+}, thus conferring properties of heart sodium channel on brain sodium channel.

A second characteristic that distinguishes cardiac from brain and skeletal muscle VGSCs and is practically inseparable from the sensitivity for toxins is the increased sensitivity of TTX-R channels to blockage by divalent cations such as Cd^{2+} and Zn^{2+} (Frelin et al. 1986). These cations also competitively inhibit TTX and STX binding, which suggests that they share a common binding site on sodium channels. These divalent ions co-ordinate well with cysteines and histidines, suggesting that a cysteine residue might account for the possession of TTX-S or TTX-R properties. As previously mentioned cysteine at position 374 in the cardiac isoform could be a residue which accounts for these affinity differences. After RH1 (AC M27902) mutation of the respective cysteine residue to tyrosine (C374Y), the TTX sensitivity increased dramatically (730-fold) and in the same time the sensitivity for divalent ions (Cd^{2+}) decreased (28-fold) (Satin et al. 1992b). This means that the RH1 mutant channel showed nerve-like sensitivity to TTX and cation block and that the mutated Cys residue was critical for differences between isoforms. This was also shown by mutation experiments of the corresponding residues in the skeletal muscle isoform (Y401C) which also reduced TTX block and increased Cd^{2+} block (Backx et al. 1992). Some later experiments confirmed these results (Penzotti et al. 1998). Finally, chimeric study with rat skeletal muscle (TTX-S) and heart isoform (TTX-R) clones, confirmed previous mutation experiments and showed that the TTX sensitivity could be switched between the two isoform by exchange of a respective tyrosine 401 in μ1 and cysteine 374 in SkM2 (Chen et al. 1992). It can be said that the respective residue is an important, but not exclusive, contributor to the difference in guanidinium toxin affinity between skeletal and cardiac isoforms.

Conotoxins are biologically active peptides from the venom of the cone snails from the *Conus* family. Each *Conus* venom contains remarkably diverse peptides which are 10–30 amino acids in length. For example small peptides from the *Conus geographus* venom target potassium, calcium and sodium channels, acetylcholine receptors and NMDA receptors (Olivera et al. 1991). A small 22-amino-acid-long peptide designated μ-conotoxin GIII targets VGSCs and causes the block. μ-Conotoxin is a very interesting molecule for scientists working with VGSCs since the structure of this peptide has been determined at very high resolution and can be used therefore as a molecular probe for testing of various models of the VGSC structure (McIntosh et al. 1999). Based on mutagenesis studies of the μ-conotoxin, it has been suggested that the positively charged group of arginine 13 is directly involved in the binding to the sodium channels (Dudley et al. 1995; Li et al. 1997).

There are several similarities between the conotoxins and guanidinium toxins TTX and STX. They block VGSCs, a guanidinium group is required for binding and studies have shown competitive inhibition between STX and μ-conotoxin GIIIA and GIIIB suggesting that binding sites for TTX/STX and conotoxin overlap (Moczydlowski et al. 1986; Ohizumi et al. 1986; Yanagawa et al. 1986). There is also an analogy between the effect of these toxins on VGSCs and differences in sensitivity of various channel isoforms. The skeletal muscle and eel electroplax isoforms exhibit high affinity to the μ-conotoxins, whereas brain and heart isoforms are resistant to the block (Stephan et al. 1994).

In spite of these similarities and in contrast to the results obtained with mutation experiments within TTX/STX binding domain, where the substitution of a single glutamate can abolish TTX and STX sensitivity in the rat brain (αII) isoform (Noda et al. 1989), no single amino acid residue has been found that has an overriding influence on either the potency nor specificity of μ-conotoxin. The mutation in rat SkM1, E403Q (corresponding to the mentioned E387 of αII) prevented current block by TTX and STX, but reduced μ-conotoxin sensitivity by only about 4-fold (Stephan et al. 1994). The Y401C mutation, which reduces the TTX sensitivity of SkM1, diminishes the effect of μ-conotoxin somewhat, but considerable sensitivity is retained (French and Dudley 1999). The mutation which resulted in the largest reduction in μ-conotoxin affinity was the substitution of glutamate at position 758 for glutamine (E758Q) in the outer vestibule region. This mutation decreased the μ-conotoxin binding affinity by 48-fold and caused at the same time the reduction of TTX binding by a factor of 375 (Dudley et al. 1995). These findings supported the hypothesis about overlapping μ-conotoxin and guanidinium toxin binding sites, but still did not deliver an explanation for the difference in the μ-conotoxin binding affinity for different sodium channels. In the situation where molecular clones of isoforms with different affinities for the ligand are available, principally two strategies can be applied: chimera studies and mutagenesis. Chimera studies using μ-conotoxin sensitive rat SkM1 and μ-conotoxin-resistant rat SkM2 channels, demonstrated that μ-conotoxin interacts with all of the four domains

apparently without a dominant interaction with any of them (Chen et al. 1992). However, more recent results showed that DI and especially DII play a more important role in forming the μ-conotoxin receptor than other domains (Chahine et al. 1998). Mutation strategy has been used to dissect the complex interactions between the receptor and ligand already in the case of TTX or STX (Noda et al. 1989; Satin et al. 1992b; Stühmer et al. 1989; Terlau et al. 1991). Efforts to define the μ-conotoxin binding region by cysteine mutagenesis method resulted in identification of seven pore-lining residues that significantly influence binding to SkM1 channels: D400, Y401, W402, E758, W1239, D1241 and W1531. Yet, these residues do not contribute equally to the toxin binding. Charged residues at positions 758 and 1241 seem to be the most important (Li et al. 1997).

The difficulty in determining the exact binding site of the μ-conotoxin might be due to the fact that this toxin is a much larger molecule than TTX or STX and therefore interacting with the larger binding domain on a more complex way. The large contact area of μ-conotoxin increases the likelihood that isoform differences are determined by steric hindrances as well as specific attractions.

Because of their highly selectivity and potency, conopeptides are interesting because of their potential as drugs. With the discovery of a TTX-R neuronal type of the channel (Akopian et al. 1996) and conopeptides which can discriminate among TTX-S VGSC subtypes (Shon et al. 1998), additional possibilities for therapeutic intervention are opened.

Veratridine, aconitine and BTX (binding site 2)

A variety of different neurotoxins modifies the sodium channel gating by keeping the channel open for longer. In contrast to TTX or STX molecules which can affect the channel only from the outside, a group of lipid-soluble compounds can access the sodium channel binding site embedded in the plasma membrane. This group of compounds includes alkaloids like veratridine, BTX, aconitine and GTX. They bind to the binding site 2 of the VGSC (Fig. 1C, Table 2). All these toxins exist in nature: BTX is secreted by the skin of Colombian arrow poison frogs of the genus *Phyllobates* and the other three toxins are of plant origin. Veratridine is isolated from veratrum alkaloids mixture from plants of the family *Liliaceae*. Aconitine is an alkaloid produced by the plant *Aconitum napellus*. GTX is found in rhododendrons and other plants of the heather family.

These compounds exert their neurotoxic action on nerve and muscle membranes by persistent activation of VGSC at the resting membrane potential (Lazdunski and Renaud 1982). This hyperexcitability is explained by two effects: first, a shift of the voltage dependence of activation of VGSC towards more negative effects and second, a block of the fast inactivation of these channels. Because these molecules cause sodium channels to open more easily and to stay open longer than normal, they can be called agonists or activators of sodium channels. BTX can be considered as a full agonist since all aspects of VGCS function are altered upon exposure to BTX: beside the two previously mentioned effects, BTX impairs single channel conductance and ion selectivity as well.

These toxins bind preferentially to an open state of VGSC (Hille and Barnes 1988; Lazdunski and Renaud 1982) and so are their binding and action enhanced by all treatments that promote or prolong opening of VGSC, including modification with scorpion toxins, DPI 201-106 or repetitive depolarisation. Especially BTX binding to the site 2 is allosterically modulated by other neurotoxins. The α-polypeptide toxins from scorpion and sea anemone (Catterall et al. 1981), the α-cyano pyrethroid insecticides (Lombet et al. 1988) and brevetoxin (Trainer et al. 1993) enhance BTX binding. In contrast, local anaesthetics (Postma and Catterall 1984) and anticonvulsants diphenylhydantoin and carbamazepine (Willow and Catterall 1982) act as competitive antagonists of BTX binding. Electrophysiological experiments have provided indirect evidence for competitive interactions between BTX and certain local anaesthetics (Postma and Catterall 1984) meaning that the neurotoxin receptor site 2 is involved in the action of local anaesthetics. It could be shown that local anaesthetics have little effect on sites 1 and 3 (Catterall 1981; Henderson et al. 1973) which is an interesting result considering the fact that binding sites 2 and 3 are allosterically coupled. The effect of veratridine, aconitine and BTX is both concentration- and frequency-dependent and is inhibited by TTX (Zilberter et al. 1994). This and the fact that binding of the radio-labelled BTX is inhibited by TTX (Brown 1986) imply the functional coupling of binding sites 1 and 2. However, this result is partially in contradiction to the finding that BTX does not influence the binding of ligands to the site 1 (Catterall et al. 1979).

In heart muscle, veratridine, BTX and GTX all produce a concentration-dependent reversible positive inotropic effect (Honerjäger 1982). This effect is not reported for aconitine. The positive inotropic effect is the consequence of the activation of VGSC and prolongation of their opening time. This results in increased intracellular sodium concentration which reduces the driving force for the Na^+/Ca^{2+} exchanger. Higher intracellular Ca^{2+} concentration means stronger force of contraction. In this way an increased concentration of intracellular Na^+ results in positive inotropy. There are also evidence that beside the alteration of gating kinetics, BTX increases the relative permeability of the VGSC toward larger ions, including Ca^{2+} ions (Khodorov and Revenko 1979). This direct effect might contribute to the positive inotropic action of BTX.

More recently, several attempts to characterise the binding site for these toxins have been made. Trainer et al. (1996) identified, using photoaffinity labelling and peptide mapping with sequence-specific antibodies, the transmembrane segment S6 of the domain DI (S6DI) as an important constituent of BTX receptor site. This finding was supported by point mutation experiments in this region which showed that a substitution of a single residue yields a BTX insensitive channel (Wang and Wang 1998). The

critical residue in the S6DI was asparagine 434, which in addition plays a critical role in the slow inactivation process (Wang and Wang 1997). Most recently, another S6 segment in the domain DIV was identified as a part of the BTX receptor so that together with the S6DI they are probably situated adjacent to the local anaesthetic receptor. Moreover, mutation experiments of the local anaesthetic receptor site in transmembrane segment S6DIV supported this hypothesis and indicated that binding sites for BTX and local anaesthetics in this region are not identical but overlapping (Linford et al. 1998).

Comparable to the binding of TTX and STX to the binding site 1, veratridine and BTX bind to the same putative binding site 2 although their binding domains are not completely identical. This is also suggested by the fact that *Phyllobates* frogs which produce BTX have BTX-resistant VGSC but these channels are sensitive to veratridine (Warnick et al. 1975).

One approach to examine the binding properties of veratridine arose from the fact that this toxin influences the gating of the VGSCs by slowing their inactivation. As mentioned before the inactivation process is mediated by an inactivation gate formed by the intracellular loop connecting domains DIII and DIV (West et al. 1992). The IFM motif present in inactivation gate is crucial for this process (Eaholtz et al. 1994). The peptide containing IFM motif and two positive charges (in form of the KIFMK peptide) are sufficient to mimic the inactivation gate and can produce the open channel block (Eaholtz et al. 1999). The investigation of the influence of this peptide on the binding of the veratridine revealed that the peptide and veratridine compete for the binding on the channel (Ghatpande and Sikdar 1997). This does not mean necessarily that veratridine shares a common receptor with the KIFMK peptide although this possibility is suggested.

Sea anemone toxins, scorpion α-toxins and funnel-web spider toxins (binding site 3)

The binding site 3 is the site of action for polypeptide scorpion α-toxins and sea anemone toxins (ATX) which were isolated and identified some 20 years ago (Norton 1991). They were first isolated as cardiac stimulants and neurotoxins. The mechanism of their action is the binding to the VGSCs of excitable tissues and selective inhibition of their inactivation, principally by inhibiting transitions of the channel from the open to the inactivated state.

Pharmacological properties of sea anemone toxins are excellently reviewed by Norton (1991). Shortly, they are polypeptides of 3–300 kDa isolated from sea anemone tentacles. The toxins isolated from genera *Anthopleura* (anthopleurin: Ap-A or Ap-B) and *Anemonia* (ATX I, II or V), are relatively large proteins classified as type-I sea anemone toxins (Norton 1991). To exert their action on sodium channels, they have to be applied from the extracellular side. As discussed previously, according to the current understanding of VGSC structure, domains which are known to affect inactivation are primarily located intracellularly. The fact that sea anemone toxins modify the inactivation although they act from the extracellular side, makes them interesting for use as probes in studies of VGSCs.

Scorpion α-toxins are produced by north African genera *Androctonus*, *Buthus* and *Leiurus*. Scorpion α-toxin V isolated from the venom of *Leiurus quinquestriatus* (LqTx) binds to neurotoxin receptor site 3 on the sodium channel in a voltage dependent manner and slows or block sodium channel inactivation (Meves et al. 1986). Scorpion α-toxins also allosterically enhance persistent activation by neurotoxins that bind to the site 2 such as BTX (Sharkey et al. 1987; Thomsen et al. 1995). The structure of several toxins from the venom of the respective scorpion has been resolved by NMR (Buisine et al. 1997; Martins et al. 1995).

Although little is known about the structure of the extracellular part of the sodium channel outside of the immediate region of the pore vestibule, several regions have been proposed to participate in sea anemone and scorpion α-toxin binding. Studies involving binding of scorpion toxin derivatives by photoaffinity labelling of the extracellular loop S5-S6DI (Tejedor and Catterall 1988) and protection against such binding by site-specific antibodies directed against S5-S6DI (Thomsen and Catterall 1989) have implicated involvement of the domain DI. In addition, it seems that parts of DIV also play a role in the toxin binding as shown with chimeric studies (Benzinger et al. 1997) and binding of the site-directed antipeptide antibodies against S5-S6 loop in domain DIV (Thomsen and Catterall 1989). The effort to characterise the residues which constitute the binding site 3 has been made more recently using site directed mutagenesis (Rogers et al. 1996). It has been shown that mutation of the glutamic acid 1613 of the rat brain sodium channel from the transmembrane segment S3DIV reduces the binding of the sea anemone toxin ATX-II by 80-fold. This mutation did not have any effect on STX binding. Beside E1613, seven additional residues from the short extracellular loop connecting S3 and S4 of the domain DIV (S3-S4DIV) had significant effects on ATX-II an scorpion α-toxin binding. These experiments singled out E1613 and S3-S4DIV as critical determinants for the binding of respective toxins. According to this model these parts of the channel are also involved in the coupling of channel activation to fast inactivation. Analogous mutation experiments were also performed with rat cardiac channel isoform (RH1). The D1612 which is corresponding to E1613 in rat brain sodium channel, was identified as a crucial residue for interaction with Ap-B toxin (Benzinger et al. 1998).

A relatively new class of toxins interacting with neurotoxin receptor site 3 have been isolated from Australian funnel-web spiders from the family *Atracinae*. δ-Atracotoxin-Ar1 and δ-atracotoxin-Hv1 are peptide neurotoxins consisting of a single chain of 42 amino acids (Little et al. 1998b). These toxins show no significant sequence homology to any other known neurotoxins. After determination of their structure by NMR (Fletcher et al. 1997; Pellaghy et al. 1997) it could be seen that the folding of these

peptides is completely different to the previously determined structures of scorpion α-toxin or Ap-B, despite similar actions on sodium current inactivation. These toxins slow sodium current inactivation and shift voltage dependence of activation in a similar manner to scorpion α-toxins (Little et al. 1998a). The demonstration that δ-Atracotoxins bind to the site 3, has been delivered through equilibrium competition assays with scorpion α-toxin, BTX and STX (Little et al. 1998b). δ-Atracotoxin and scorpion α-toxins appear to bind to partially overlapping sites on the sodium channel within the area described as site 3. They display also positive allosteric interactions with neurotoxin binding site 3, which is comparable with allosteric coupling of sites 2 and 3 as shown already for some other toxins (Sharkey et al. 1987)

Scorpion β-toxins (binding site 4)

Scorpion toxins isolated from the venom of North and South American scorpions from the genus *Centuroides* bind to neurotoxin receptor site 4 (Fig. 1C, Table 2) and are classified as scorpion β-toxins. The scorpion α- and β-toxins do not share the common neurotoxin binding site, since binding of one type does not interfere with the binding of the other (Wheeler et al. 1983). A scorpion toxin with high affinity to VGSCs was isolated as well from the venom of Brazilian scorpion *Tityus serrulatus* and named Tityus γ toxin (TiTxγ) (Lazdunski et al. 1986). The three dimensional structure of several scorpion β-toxins has been resolved recently by NMR (Jablonsky et al. 1999; Pintar et al. 1999).

In contrast to the results with scorpion α-toxins, the venom from American scorpions have no effect on inactivation but shift the voltage dependence of activation to more negative membrane potentials (Cahalan 1975; Couraud et al. 1982; Meves et al. 1982). As a result of this effect, sodium currents are created at membrane potentials as low as –70 mV at which the sodium channel is normally in the resting (closed) state. More recently it was proposed that "voltage sensor trapping" presents the fundamental mechanism of action of these polypeptide toxins (Cestele et al. 1998). This mechanism suggests the binding of scorpion β-toxin to the extracellular end of the S4DII segment or to the residues in the S3-S4DII loop during the translocation of positive charges of the voltage sensor region (S4) at the transition from a resting to an open state of the channel. By binding to the extracellular end of the S4DII segment, the toxin can stabilise and trap it in the outward (activated) position and thereby enhance channel activation in response to subsequent depolarising pulses. The mutation G845 N in the S3-S4DII may abolish the effect of scorpion β-toxin on the voltage-dependence of activation by preventing binding of the toxin to the extracellular end of the activated conformation of the S4DII segment, because it introduces unfavourable steric or polar/non-polar interactions.

In recent years scorpion toxins gained on importance mostly because of the fact that some of them show high selectivity to insect sodium channels (Gordon et al. 1996), meaning that they are not toxic for warm blooded animals. This and the fact that they are biodegradable make them potential biopesticides of the future (Gurevitz et al. 1998). Previously described scorpion α- and β-toxins are mainly active against both insects and mammals, whereas so-called excitatory and depressant scorpion toxins show absolute selectivity to sodium channels. This topic is reviewed much more in details elsewhere (Gurevitz et al. 1998).

Brevetoxins and CTX (binding site 5)

Brevetoxins (PbTx) and CTX are lipid-soluble polyether marine toxins. PbTX is produced by the marine dinoflagellate *Ptychodiscus brevis*, an organism linked to red tide outbreaks, and the accompanying toxicity of marine animals as well as neurotoxic shellfish poisoning in humans. CTX originate from the marine dinoflagellate *Gambierdiscus toxicus* and poisoning in humans occurs by the consumption of ciguatoxic reef fish (Swift and Swift 1993). A total of eight toxins (PbTx-1 to -8) have been isolated and purified from *Ptychodiscus brevis* (Wu and Narahashi 1988).

Both toxins bind to a unique site on the channel protein α-subunit known as the binding site 5 (Poli et al. 1986) and modify both, the activation and inactivation processes of VGSCs (Dechraoui et al. 1999; Gawley et al. 1995) Electrophysiological experiments have shown that the two groups of marine toxins depolarise excitable membranes by both causing a shift in VGSC activation towards more negative potentials and by inhibiting normal inactivation (Baden 1989; Wu and Narahashi 1988).

PbTxs are complex polyether neurotoxins containing a so-called ring A that is essential for their activity (Baden et al. 1994). The one of brevetoxins, a sodium channel activator PbTx-3, acts through a prolongation of open times as well as an increased frequency in channel openings of the cardiac VGSC (Schreibmayer and Jeglitsch 1992). In addition PbTx-3 induces a shift in activation to more negative membrane potentials (Jeglitsch et al. 1998). As consequence, channel openings are observed even at the resting membrane potential (Gawley et al. 1995). Besides, an inhibition of Na^+ channel inactivation at different membrane potentials has been perceived in cardiomyocytes and rat nodose neurons (Schreibmayer and Jeglitsch 1992) but not in neuroblastoma cells (Sheridan and Adler 1989). The above mentioned experiments reveal that PbTx-3 is a gating modifier with unique properties. In contrast to other gating modifiers which bind to site 2 or site 3 and act primarily via stabilisation of already open channels or reactivation of inactivated channels (Schreibmayer and Jeglitsch 1992), PbTx-3 stabilises pre-open states as well as a whole set of different open states. Voltage-clamp experiments showed several striking characteristics of VGSCs modified by PbTx2 or PbTx-3 including activation of the channel at membrane potentials of –160 to –80 mV with extremely slow kinetics as well as absence of fast inactivation (Huang and Wu 1985).

The receptor site is located on the α-subunit of the sodium channel. During the binding the toxin backbone resides in the vicinity of the S5-S6 extracellular loop of domain DIV of the protein (Catterall et al. 1992; Gawley et al. 1995). Allosteric interactions include the enhanced binding of BTX and veratridine in presence of brevetoxin (Catterall and Gainer 1985; Lombet et al. 1987; Wada et al. 1992). There was no effect of BTX on brevetoxin binding which can be explained by the fact that BTX binds to open channels with much higher affinity than to closed channels (Wu and Narahashi 1988). There was no interaction of brevetoxin with toxins of binding site 1 or 3 (Catterall and Gainer 1985; Lombet et al. 1987; Trainer et al. 1996; Wada et al. 1992). Both CTX and brevetoxins enhance pyrethroid-induced sodium influx into neuroblastoma cells, rat muscle cells or synaptosomes (Bidard et al. 1984; Lombet et al. 1987).

The membrane depolarisation or spontaneous action potentials in neuroblastoma cells or frog nodes of Ranvier caused by CTX could be explained as a result of VGSC opening at the resting potential and a failure of inactivation of open channels (Benoit et al. 1986; Huang and Wu 1985). The increase of neuronal excitability by CTX-1 is suggested to be a consequence of an increased transition rate of the inactivated to the resting state of TTX-R sodium channels during depolarisation (Strachan et al. 1999). In contrast to other neurotoxins such as veratridine, scorpion α-toxin, and brevetoxins (Baden et al. 1994), CTX-1 had no effect on activation or inactivation kinetics of TTX-sensitive and TTX-resistant sodium channel currents (Strachan et al. 1999).

Potential relevance for neurodegenerative diseases should be examined since it has been shown that the activation of VGSC caused by these marine toxins leads to a modification of neurotransmitter release (Meunier et al. 1997; Molgo et al. 1990).

Insecticides (binding site 6)

Pyrethroid insecticides are potent excitatory neurotoxins that disrupt normal nerve function by actions on VGSCs and cause hyperexcitation and paralysis in animals. Pyrethroids are especially interesting because they exhibit a high degree of selective toxicity between mammals and invertebrates. This is due to several features of pyrethroids like temperature-dependent potency, distinct sensitivity of vertebrate/mammalian VGSC or different enzymatic detoxification rate (Narahashi et al. 1998; Song and Narahashi 1996).

Pyrethroids constitute a large group of neurotoxic insecticides, which were originally developed as synthetic analogues of the natural pyrethrins present in the flower heads of certain *Chrysanthemum* species. Biological effects of pyrethroids include effects on other ion channels: calcium (Narahashi 1986), chloride channel of the gamma-aminobutyric acid (GABA)A receptor-ionophore sites (Saleh et al. 1993) and receptors like nicotinic acetylcholine receptor (Abbassy et al. 1983; Eriksson and Nordberg 1990) but the main target are VGSCs. The effect of pyrethroids and dichlorodiphenyltrichlorethane (DDT) on VGSCs in excitable membranes is a prolongation of the sodium current which results in the development of a depolarising after-potential. These effects are responsible for the induction of repetitive activity which is the most characteristic effect of pyrethroid poisoning in the nervous system. The modification of VGSC gating by pyrethroids has been studied in voltage clamp experiments in crayfish (Lund and Narahashi 1981a; Narahashi 1986) squid axon membranes (Lund and Narahashi 1981b), myelinated nerve fibres of frogs (Vijverberg et al. 1982) and mouse neuroblastoma cells (Ruigt et al. 1987). Electrophysiological studies using *Xenopus laevis* oocytes with brain α-subunit (αIIa) indicated that pyrethroid insecticides such as permethrin and cypermethrin prolong sodium currents, increase the amplitude of sodium current, and induce repetitive bursts of action potentials or use-dependent nerve block (Ruigt et al. 1987; Smith et al. 1998; Trainer et al. 1993). Co-expression of the rat brain β-subunit increased the affinity of sodium channel binding site for pyrethroid insecticides (Smith et al. 1998).

In expressed rat brain sodium channel α-subunit (αIIa), cypermethrin induced a slowly decaying tail-current following a depolarisation pulse. In addition, a sustained non-inactivating component of the sodium current, so-called „late current“, was induced after expression of the $\alpha IIa\beta_1$-subunit (Smith et al. 1998). The tail current developed in parallel with the activation of the sodium current and reached its maximum at the peak of the sodium current (Ruigt et al. 1987). It is suggested that the tail current results from stabilisation of the activation gate and is always proportional to the number of sodium channels which are open at the end of the depolarisation (Ruigt et al. 1987). In contrast, the induction of the “late current” implies that the pyrethroid insecticides interact with the closing of the inactivation gate (Smith et al. 1998). The generation of the tail current was also been described in rat dorsal root ganglion neurons, mouse neuroblastoma cells and squid giant axon (De Weille et al. 1990; Ruigt et al. 1987; Song et al. 1996). The incorporation of an α-cyano group as in type-II pyrethroids, enhances the effect of pyrethroids on the tail current compared to pyrethroids devoid of an α-cyano group (type-I pyrethroids like tetramethrin) (Ruigt et al. 1987). Moreover, time of exposure, membrane potential and temperature are important parameters for pyrethroid-induced tail-currents (Ruigt et al. 1987). The pyrethroid insecticide tetramethrin was more potent in modifying TTX-R sodium channel currents than TTX-S sodium channels (Narahashi et al. 1998). Despite of pyrethroid effects on activation and inactivation processes of VGSCs, there is evidence that pyrethroids and DDT preferentially interact with open sodium channels (De Weille et al. 1990; Vijverberg et al. 1982). Modification of VGSCs is enhanced by depolarisation suggesting a rapid and high affinity binding to the activated state of the channel (Narahashi 1992).

As previously described in the chapter about phosphorylation of VGSCs, the brain sodium channel is phosphorylated by protein kinases during the depolarisation but

the exact meaning of phosphorylation is still not well understood (Costa et al. 1982; Costa and Catterall 1984). Yet, it has been shown that pyrethroids, e.g. 1R-deltamethrin and DDT inhibit phosphorylation of the α-subunit (Ishikawa et al. 1989).

Allosteric interactions between pyrethroids and several toxins such as alkaloid activators (site 2), scorpion α-toxins (site 3), veratridine (site 2), and brevetoxin/conotoxin (site 5) have been described (Bloomquist and Soderlund 1988; Linford et al. 1998; Lombet et al. 1988; Trainer et al. 1993; Trainer et al. 1997). For example, there is an allosteric coupling among the receptor sites for BTX (site 2) (Brown et al. 1988; Eells et al. 1993) and pyrethroids in purified sodium channels of the brain. The enhancement of BTX-binding was shown to be voltage-dependent and concentration dependent (Eells et al. 1993). Although allosterically modulated by other neurotoxins, pyrethroids act at a new neurotoxin receptor site on the VGSCs (Lombet et al. 1988).

It is possible that allosteric modulation may provide a new approach to increase selective activity of insecticides on target organisms by simultaneous application of allosterically interacting drugs (Gordon 1997). Considering increasing knock down resistance operated by increased metabolism and/or alteration of the target site, a comparative study of neurotoxin receptor sites on mammalian and insecticide sodium channels may offer new targets and approaches to the development of highly selective insecticides. In addition, possible combinations of two different compounds may be able to increase insecticide activity without apparent increase in mammalian toxicity (Zlotkin 1999).

DPI 201-106: a synthetic VGSC modifier

Many naturally occurring compounds, like veratridine, aconitine, BTX, GTX and CTX, prolong the open state of sodium channels, mainly by causing activation of channel opening. Other toxins isolated from African scorpions (scorpion α-toxins) or sea anemone toxins prolong the open state of the channel through the inhibition of the inactivation. The first synthetic compound to prolong the opening of the VGSC was 4-[3-(4-diphenylmethyl-1-piperazinyl)-2-hydroxypropoxy]-1H-indole-2-carbonitrile (DPI 201-106). DPI 201-106 was shown to produce a cAMP-independent positive inotropic response (Scholtysik et al. 1985). This effect could be abolished by a highly selective VGSC blocker, TTX (Buggisch et al. 1985), meaning that the positive inotropic effect of DPI 201-106 is directly coupled to an increased influx of Na^+ through sodium channels and that DPI-modified Na^+ channels must be expected to be exclusively responsible for the prolongation of the action potential. This property of DPI 201-106 is also very useful in determining the clinical relevance of selective modulation of the VGSCs. Comparable prolongation of the action potential duration can be achieved after application of ATX II toxin. However, the important difference in terms of selectivity for VGSCs is that ATX causes the prolongation of the action potential also by inhibiting outward K^+ currents and not only through VGSC activation (Isenberg and Ravens 1984). Another positive property of this compound is its vasodilatatory effect associated with negative chronotropy (Hof and Hof 1985) which suggested that DPI 201-106 might have an Ca^{2+} channel antagonistic effect. The blocking effect on Ca^{2+} channels was subsequently proven by both voltage clamp experiments using isolated cardiocytes and radioligand binding studies (Holck and Osterrieder 1988; Siegl et al. 1988). The same effect has been proven for vascular preparations (Hof and Hof 1985).

Further studies demonstrated that DPI 201-106 increases the mean open time of VGSCs by inhibiting channel inactivation, thus allowing Na^+ influx to continue during the action potential in rat, guinea-pig and human cardiac muscles (Hoey et al. 1993, 1994; Holck and Osterrieder 1988; Kohlhardt et al. 1986).

The compound piperazinyl-indole DPI 201-106 is a racemic mixture. *S*-DPI (DPI 205-430) tends to act as a VGSC agonist, whereas *R*-DPI (205-429) tends to act as a sodium channel antagonist. The binding site for both enantiomers remained unknown. Although modification by DPI 201-106 can be achieved after treating the external side of the membrane (Kohlhardt et al. 1986), the drug does not necessarily find a target in the outer part of the sodium channel. Due to its lipophilic properties, DPI 201-106 may be able to pass through the lipid phase so that in analogy to local anaesthetics the molecule could gain access to its site of action either laterally during membrane permeation, or after reaching the cytoplasmic side, from the inner channel mouth. Because of its hydrophobicity, DPI 201-106 is most likely to interact with hydrophobic pockets i.e. sodium channel domains formed predominantly by apolar residues such as alanine, valine, leucine or isoleucine.

Although DPI 201-106 showed promising properties as a positive inotropic agent in vivo, both proarrhythmic (Novosel et al. 1993) as well as antiarrhythmic effects were reported. The most pronounced antiarrhythmic effect was prolongation of the Q-T interval of the electrocardiogram (Kostis et al. 1987; Rüegg and Nüesch 1987). Prolongation of the Q-T interval may increase incidence of torsade de pointes, a type of malignant arrhythmia which is intermediate between tachycardia and ventricular fibrillation (Fontaine 1992). The subsequently developed positive inotropic compound with much lower antiarrhythmic effect was the DPI congener 4-[3′-(1″-benzhydrylazetidine-3″-oxy)-2′-hydroxypropoxy]-1H-indole-2-carbonitrile (BDF 9148) (Baumgart et al. 1994; Hoey et al. 1993; Ravens et al. 1995). They are similar in structure, except that BDF 9148 contains an azetidine-oxy where DPI 201-106 has a piperazinyl moiety. Also some other purine-based analogues of DPI 201-106 were synthesised and shown to be much more potent (Estep et al. 1995).

The exact mechanism of action potential prolongation as well as structures involved in DPI 201-106 binding to VGSC remained unknown. The fact that bovine heart cardiocytes are completely resistant to DPI 201-106 and its purine derivative SDZ 211-939 might help to elucidate the mechanism of their action (Scholtysik and Schaad 1992).

Local anaesthetic binding site

Local anaesthetics (LA) such as lidocaine and procaine produce their effect by blocking the sodium channels (Butterworth and Strichartz 1990). Beside sodium channels, LA have been shown to affect several ion channels like potassium (Kinoshita et al. 1999) and calcium (Xiong et al. 1999) or neurotransmitter receptors (Ye et al. 1999). They are all relatively small lipid-soluble molecules, usually with amine groups that become positively charged under acidic conditions. More recently, useful information as to how local anaesthetics work came from experiments with QX-314, a quarternary analogue of lidocaine that is positively charged at all times and hence is not lipid soluble. In cardiac VGSCs the blocking action of QX-314 is present when the drug is applied from either side of the membrane but inhibition of the brain sodium channel occurs only when the drug is applied from intracellular side (Qu et al. 1995). Differences in the action of permanently charged local anaesthetics like QX-314 have been shown for heart and brain sodium channels, and for dorsal root ganglion neurons (Bräu and Elliott 1998).

In vitro studies on a wide variety of cells using whole-cell voltage clamp recordings or expression of α-subunit of the VGSC in *Xenopus laevis* oocytes or mammalian cells have demonstrated an inhibition of VGSCs in a use (-state)-dependent action (Arlock 1988). The action of local anaesthetics was increased at more depolarised holding potentials, whereas at more hyperpolarised potentials the block was diminished (Arlock 1988; Li et al. 1999) suggesting a more pronounced inhibition of sodium currents due to an increase in the proportion of high-affinity-inactivated channels.

Many attempts to characterise the binding site of LA have been reported. Several studies revealed an interaction of veratridine (Deffois et al. 1996) and BTX (Linford et al. 1998; Postma and Catterall 1984) binding with LA in a concentration-dependent manner suggesting an allosteric interaction of these two binding sites. Apart from the binding site 2, there are no significant effects of LA binding on neurotoxin binding at other receptor sites reported. For example, binding of the local anaesthetic PD85,639 did not compete for the anticonvulsant drugs phenytoin and carbamazepine binding in sodium channels of synaptosomes and synaptosomal membranes (Thomsen et al. 1993). Recently published results show that a hydrophobic binding site exists that is different from site 2 but allosterically coupled to binding site 2 (Ratnakumari and Hemmings 1997). Several studies showed a preferential interaction with the inactivated state of the sodium channel (Li et al. 1999; Ratnakumari and Hemmings 1997). In contrast to those findings are recently published data that indicate that the LA lidocaine does not compete with fast inactivation (Vedantham and Cannon 1999). These experiments give evidence that the blocking effect of local anaesthetics does not result from a slower recovery from fast inactivation, and does not involve an accumulation of fast-inactivated channels as described in the other studies mentioned before (Vedantham and Cannon 1999). This represents a new model which focus on activation processes as more relevant for local anaesthetic action.

To define the location of the local anaesthetic receptor site more precisely, site directed mutations have been used (Li et al. 1999; Linford et al. 1998; Qu et al. 1995; Ragsdale et al. 1994; Ragsdale et al. 1996; Wang et al. 1998). Most prominent changes were attained by mutations of the cytosolic side of S6DIV. Mutation F1764A and Y1771A from rat brain type IIa (αII) abolished the block of the channel by local anaesthetics (Ragsdale et al. 1994; Ragsdale et al. 1996). In experiments conducted with the slow-inactivating isoform III (αIII) from the rat brain which is primarily expressed during embryogenesis, residues phenylalanine 1710 and tyrosine 1717 from DIVS6 were defined as residues responsible for local anaesthetic binding (Li et al. 1999). These residues were proposed to face toward the channel pore during binding of LA. This is implying that the local anaesthetic receptor site is located in the pore of the channel. According to this model the local anaesthetic binding site should be physically close to the selectivity filter of the VGSCs and to the binding site of the inactivation gate. In addition, mutation of the threonine 1755 in rat heart into valine as in IIa isoform have been identified as a residue which controls the passage through the pore to the receptor responsible for the action of QX-314 when applied at the extracellular site. Mutation of the analogous amino acid in rat brain type IIa (I1760A) produced a cardiac phenotype of the channel (Ragsdale et al. 1994), leading to a higher sensitivity to external block by a quaternary derivative of lidocaine. Furthermore, phenylalanine 1762 of the cardiac isoform was identified as a critical residue for QX-314 binding, independently of whether applied intra- or extracellularly (Qu et al. 1995), supporting a model with a single binding site for QX-314. Similar findings have been reported for skeletal muscle (Wang et al. 1998).

Nevertheless, it has to be mentioned that site-directed mutations of amino acids on the other three S6 in domains DI-DIII – which could interact with other transmembrane segments – have not been studied until now. Since these four domains of the channel are thought to be arranged around the channel pore, it is likely that the other S6 segments may also contribute to the local anaesthetic receptor. Residues in the pore loops that form the selectivity filter are also good candidates for future experiments which should define determinants of the local anaesthetic action.

Therapeutic use of VGSC modulators and future aspects for drugs with VGSC specificity

VGSC modulators in neurology: animal models and mechanisms of action

VGSCs are responsible for the initiation and propagation of action potentials in both nerve and muscle cells (Hille 1992; Ragsdale et al. 1996). Their functional properties influence the threshold for action potential generation and

the frequency of neuronal firing. Therefore, neuromodulation of sodium channel function is likely to have an important influence on generation of the high frequency action potentials that are an essential element of epilepsy. Sodium channels are potential molecular targets of a number of antiepileptic agents. Despite of diverse chemical structures, their pharmacological profiles share several common characteristics (Ragsdale and Avoli 1998). Antiepileptic and neuroprotective drugs have different sites of action such as potentiation of GABAergic mechanisms, blockade of ion channels (sodium, calcium and potassium) and blockade of glutamate/AMPA receptors (Loescher 1998; Meldrum 1996a). A number of clinically important antiepileptic drugs exert their therapeutic effects principally by blocking brain VGSCs at clinically relevant concentrations. VGSCs have become widely accepted as the primary target of antiepileptic drugs such as phenytoin, carbamazepine and lamotrigine. In addition, these drugs may offer significant potential for treatment of neuronal damage caused by ischemia and neurodegenerative diseases. Antiepileptic drugs such as phenytoin and carbamazepine, which are effective against grand mal and partial seizures, are potent blockers of neuronal sodium channels at therapeutically relevant concentrations (Ragsdale et al. 1991).

Functional sodium channels of the nervous system are located in axonal cell membranes, cell bodies and dendrites of neurons of the mammalian brain. Type-II (αII) channels predominate in cortex, hippocampus and cerebellum, whereas type-I (αI) channels are more abundant in the spinal cord than type IIa. Type-IIa (αIIa) channels are found in greater densities in axons and dendrites while type-I channels are found in greater densities in cell bodies and dendrites. Sodium channel type I is expressed predominantly at late postnatal states whereas type-II channel is expressed throughout the developmental stages (Beckh et al 1989). Type-III (αIII) channels occur in significant amounts in the immature brain and is expressed predominantly at foetal and late postnatal states (Beckh et al. 1989). Type-IV (αIV) channels are highly represented in cortex, hippocampus and brain stem [for review see (Ragsdale and Avoli 1998)]. In situ mRNA hybridization experiments indicate that type-IV channels are expressed in neurons and glia of the brain and peripheral nerves (Taylor and Narasimhan 1997). About 80% of the sodium channels of the neocortex are type IIa and 14% type I.

Sodium channel blocking antiepileptic drugs are effective in different animal models that are used for identification and development of new anticonvulsants. It has been shown that sodium channel blockers are effective in MES (maximal electroshock seizure test) which is thought to be predictive for anticonvulsant efficacy against generalised tonic-clonic seizures (Benes et al. 1999; Loescher 1998). In addition antiepileptic drugs that inhibit sodium current of VGSCs are effective in the kindling model which predicts clinical effect against partial seizures whereas chemically induced clonic seizures in rodents (PTZ, strychnine or excitatory amino acids) cannot be suppressed by sodium channel blocking drugs (Loescher 1998; Ragsdale and Avoli 1998). In addition, they are effective in partial and generalised tonic-clonic seizures in humans (Ragsdale and Avoli 1998). It is well documented that these drugs inhibit sustained repetitive firing of action potentials in current clamp recordings of cultured neurons. Moreover, an attenuation of ionic currents through VGSCs has been shown at therapeutically relevant concentrations for phenytoin, carbamazepine, lamotrigine, topiramate, felbamate and riluzole that will be described in detail below. Sodium channel blocking effects have also found for antidepressants, neuroleptics and anti-hyperalgesic drugs (Trezise et al. 1998).

Phenytoin-like anticonvulsant drugs reduce the severity of seizures and raise the threshold for after-discharges in the kindling model. Furthermore, antiepileptics like phenytoin have little or no activity against generalised absence and myoclonic seizures in rodents.

There is evidence for over-expression of sodium channels in neuronal membranes in a mutant mice strain (El mice) that shows stimulus-sensitive limbic seizures (Sashihara et al. 1994). Kindled seizures in rodents and complex partial seizures in man seem to share common characteristics such as changes contributing to the occurrence of seizures including changes in ion channels and in the function of excitatory and inhibitory transmitter systems.

First generation anticonvulsant drugs such as phenytoin, carbamazepine and valproate have been demonstrated to suppress the abnormal neuronal excitability associated with seizures by means of complex voltage- and frequency-dependent inhibition of ionic currents through VGSCs which is concentration-dependent (Burack et al. 1995; Chao and Alzheimer 1995; Kuo et al. 1997; Taylor and Narasimhan 1997; Tomaselli et al. 1989). Carbamazepine and phenytoin act as competitive inhibitors of sodium channel activation by the full agonist BTX and veratridine (binding site 2) indicating that both drugs act as allosteric inhibitors of neurotoxin-activated sodium channels (Willow et al. 1984; Worley and Baraban 1987).

New drugs (second generation drugs) such as lamotrigine, topiramate, felbamate and oxcarbamazepine share the same mechanisms (Cheung et al. 1992; Coulter 1997; Kuo and Lu 1997; MacDonald and Greenfield 1997; Ragsdale and Avoli 1998; Taglialatela et al. 1996; Taverna et al. 1999; Van den Berg et al. 1993; Xie et al. 1995; Xie and Hagan 1998; Zona et al. 1997). These anticonvulsant drugs also exert effects on receptors other than sodium channels, and therefore these drugs are no selective sodium channel blockers. The neuroprotective and anticonvulsant agent riluzole was reported to reduce inward sodium currents (EC_{50} 51 μM) in primary cultures of rat cortical neurons (Zona et al. 1998) and in oocytes expressing α-subunit of brain VGSC (Hebert et al. 1994). It is important that therapeutic concentrations of the above mentioned antiepileptic drugs did exert sodium blocking effect in whole cell electrophysiological experiments (Chao and Alzheimer 1995; Francis and Burnham 1992; Zona et al. 1998). Direct support for VGSC blocking effects due to phenytoin-like anticonvulsants derives from experiments with rat hippocampal slices in vitro. Low concentrations of TTX

cause anticonvulsant effects that are similar to those of phenytoin or carbamazepine.

Gabapentin, which is known to potentiate GABAergic inhibitory action, has been shown to decrease sustained firing of sodium-dependent action potentials but the data are inconsistent (Rock et al. 1993; Taylor et al. 1998).

A common effect of these sodium channel blockers is a shift of the steady-state inactivation curve of the sodium current towards more negative values (Kuo 1998; Ragsdale et al. 1991; Zona et al. 1997, 1998). In addition, antiepileptic drugs like lamotrigine are also effective as neuroprotective agents in rodent models of stroke or focal ischemia. This effect correlates with the suppression of glutamate release which is probably due to the action on sodium channels (Baumgold 1987).

A retardation of the recovery from inactivation of VGSC has been reported for phenytoin-like anticonvulsants (e.g. propofol) suggesting an interaction with the inactivated state of the channel which has also been described for local anaesthetics (Ragsdale et al. 1996; Ratnakumari and Hemmings 1997; Van den Berg et al. 1993; Zimanyi et al. 1989). Prolonged depolarizations are effective in increasing drug binding and block of VGSCs, which is consistent with the idea that these drugs bind preferentially to inactivated VGSCs (Quandt 1988). Based on the mutagenesis experiments done by Ragsdale et al. (1996), local anaesthetics and phenytoin may share a common or overlapping receptor site because both substances cause similar effects on binding and block of the channel due to mutations of the local anaesthetic binding site. Toxin probes have been used as selective high-affinity probes of sodium channel function and they compete with the action of sodium channel blockers such as phenytoin. VGSC blockers inhibit the binding of BTX (site 2) in brain synaptosomes (Benes et al. 1999) and mouse spinal cord neurons suggesting a modulation of neurotoxin binding to receptor site 2 (Cheung et al. 1992). In addition, the veratridine evoked amino acid release has been shown to be inhibited by action of the antiepileptic lamotrigine in vitro in rat hippocampal slices and in whole cell voltage clamp recordings (Cheung et al. 1992; Xie and Hagan 1998).

It is currently not known if sodium channel blockers exert their action on fast inactivation (Kuo and Lu 1997) or slow inactivation (Trezise et al. 1998). The actions of sodium channel blockers are more pronounced at more depolarised potentials implicating that the inactivated state of the channel has a much higher affinity for the drug than the resting state, as postulated in “modulated receptor hypothesis” (Hille 1992). Chao and Alzheimer (1995) have demonstrated that phenytoin inhibited non-activating (persistent) sodium current which enhances neuronal excitability near firing threshold in isolated rat neurons from neocortex and neostriatum. Persistent sodium current may influence the behaviour of neurons during situations of abnormal excitability which occurs in epilepsy. The persistent sodium current may be an important component of the sodium current involved in seizures. Such persistent currents appear to account for the pacemaker potentials associated with repetitive firing. Segal and Douglas (1997) demonstrated an effect of phenytoin on the late sodium channel openings in hippocampal neurons in vitro. These effects were demonstrated at the concentration where fast (peak) sodium current of resting neurons remains intact (Ragsdale et al. 1991). Because non-activating sodium currents occur at more negative potentials than fast sodium currents, their inhibition may prevent membrane depolarisation before a neuron starts to fire (Taverna et al. 1999). Similar results were reported by Zona et al. (1997) and Taverna et al. (1999). This might present a novel mechanism which contribute to the anticonvulsant profile of phenytoin (Chao and Alzheimer 1995) which has also been demonstrated for valproate (Taverna et al. 1998).

Minimal effects were seen in a variety of neurons which show a normal pattern of sodium channel activity (Xie and Hagan 1998). Microelectrode recordings of mouse spinal cord neurons indicate that phenytoin inhibited high frequency repetitive firing of action potentials evoked by prolonged depolarising current pulses, whereas spontaneous neuronal activity was not affected (McLean and Macdonald 1983). The inhibitory action of sodium channel blockers is strongly dependent on the membrane potential as well as on the opening frequency of the channels (Kuo and Lu 1997; Xie and Hagan 1998). The use-dependent block of brain αIIa VGSCs by TTX has been studied in *Xenopus* oocytes. TTX-binding affinity has been shown to increased due to repetitive stimulation (Boccaccio et al. 1999, Conti et al. 1996). Concentration-response relationships with phenytoin supported the hypothesis that the voltage dependence of tonic block resulted from higher affinity of the VGSC blockers for inactivated states and not for resting channels (Kuo 1998; Ragsdale et al. 1991). Experiments on rat hippocampal neurons in vitro indicate that phenytoin binds tightly to fast inactivated states of VGSCs (Kuo and Bean 1994). Phenytoin binding to resting channels is about 100 times weaker. Kuo (1998) has suggested that the anticonvulsant receptor may not exist in the resting state of the sodium channels. Thus, there may be correlative conformational changes of the receptor during the gating process (Kuo et al. 1997; Kuo 1998) that may be controlled by the membrane potential. Binding rates of anticonvulsants to the open state of the receptor are too slow to be involved in the blocking activity of these sodium channel blockers (Kuo et al. 1997).

As already mentioned, there is apparent dissimilarity in the chemical structure of phenytoin, carbamazepine and lamotrigine. Therefore, it is interesting that these anticonvulsants seem to bind to a common receptor in VGSCs. The only common structural motive shared by these drugs are two phenyl groups separated by one to two C-C or C-N single bounds, and it has been suggested that the common receptor for antiepileptic drugs in the inactivated state of the channel also contains two phenyl groups, which are probably part of the side chain groups of some aromatic amino acids constituting the channel (Kuo 1998). In this respect it is interesting that mutations of the aromatic amino acid (phenylalanine) disrupt the binding to inactivated states of the channel. These findings support the hy-

pothesis that part of the receptor of the VGSCs is formed by amino acid residues phenylalanine or tyrosine (position 1764 and 1771) in domain DIV transmembrane segment S6 of the α-subunit (Ragsdale et al. 1996).

Cellular excitability from CA1 neurons of the hippocampal focus in kindled rats has been demonstrated to be persistently increased. The amplitude of the sodium current amplitude was increased by about 20% as shown in whole cell voltage-clamp experiments (Vreugdenhil et al. 1998a). These findings are interesting considering that the kindling model involves many processes important for the pathogenesis of epilepsy (Loescher 1998). Whole-cell voltage clamp measurements in dentate granule cells isolated from resected hippocampus of patients with therapy resistant temporal lobe epilepsy indicate a large current density and the presence of a component showing a slow recovery from inactivation (Reckziegel et al. 1998). Experiments on CA1 hippocampal neurons and neurons that belong to the temporal lobe neocortex of patients with pharmacoresistant temporal lobe epilepsy using whole-cell voltage clamp conditions, did not indicate a changed modulation of the sodium current by valproate (Vreugdenhil et al. 1998b). Valproate-induced modulation of sodium current inactivation was not affected by kindling epileptogenesis in rats (Vreugdenhil et al. 1998a). In contrast to these findings, a study on patients with temporal lobe epilepsy demonstrated that modulation of sodium current inactivation was only about 50% in CA1 neurons of patients with mesial temporal sclerosis compared to neocortical neurons of the same patients, and half of that encountered in CA1 neurons from patients without mesial temporal sclerosis (Vreugdenhil et al. 1998a). The same effect was found in CA1 neurons isolated from the epileptic focus of kindled rats (Vreugdenhil et al. 1998a). The rapid component of recovery from inactivation was not affected by carbamazepine in a similar study on hippocampal cells isolated from patients with temporal lobe epilepsy (Reckziegel et al. 1999). An alteration of the expression of VGSCs may be responsible for these effects. These findings indicate that the modulation of the sodium current inactivation is selective for antiepileptic drugs.

Therapeutic potential of VGSC blockers

Epilepsy

The above mentioned antiepileptic drugs apart from lamotrigine and topiramate are effective against both partial and generalised seizures but cannot be used for pharmacotherapy of absence seizures (Coulter 1997). The primary anticonvulsant mechanism of phenytoin-like antiepileptics seems to be the voltage-dependent blockade of sodium channels. The anticonvulsant spectrum of VGSC blockers is not identical. There are basically three reasons for different action: a different selectivity on different neuronal VGSCs (types I, II, IIa, III, IV), differences in their kinetic action, or differences in their additional actions beyond those on VGSCs (Meldrum 1996b).

There is a need for the development of new antiepileptic drugs because seizures in one of three patients with epilepsy are resistant to treatment with currently available anticonvulsant drugs (Loescher 1998). Further understanding of mapping the receptor site of antiepileptic drugs acting through the VGCS will improve the knowledge of the molecular basis of state-dependent channel block. Due to a better understanding of the mechanisms of different sodium channel blockers which includes knowledge about the binding site of these anticonvulsant drugs, a rational design of more effective therapeutic drugs may be possible (Ragsdale et al. 1996).

Changes in the ratio of sodium channel expression between different subtypes in epileptic human brain tissue may show that the development of subtype specific sodium channel blockers may be a promising tool. The recent cloning and functional expression of various sodium channel subtypes provides useful systems for the potential discovery of subtype-selective blocking agents (Taylor and Narasimhan 1997).

Ralitoline, a new sodium channel blocker did not demonstrate any further advantage over standard drugs such as phenytoin or carbamazepine (Loescher and Schmidt 1994). It has been demonstrated that epileptic seizures are multifactorial, even for the same type of seizure, and therefore anticonvulsant drugs with several mechanisms such as valproate have advantages compared to drugs with a selective effect (Loescher 1998). Nevertheless, a better understanding of pathophysiology of epilepsy and binding site of the VGSC blockers may improve the development of selective antiepileptic drugs with decreased adverse effects (Meldrum 1996b). The mutagenesis approach may represent a promising tool for characterisation of the binding site of antiepileptic drugs that may facilitate the rational design of more effective therapeutic anticonvulsants (Ragsdale et al. 1996). Moreover, the carbamazepine insensitivity of the removal of inactivation could be an interesting concept to explain the medical intractable temporal lobe epilepsy (Reckziegel et al. 1999).

Neuropathic pain

Sodium channel blockers such as carbamazepine and low doses of lidocaine are used to treat neuropathic pain (trigeminal neuralgia or diabetic neuralgia etc.). The pathophysiology of these diseases may include increased numbers of VGSCs and increased spontaneous action potentials in peripheral nerves. Several drugs that are used in chronic pain have been demonstrated to inhibit veratridine-induced sodium influx (Deffois et al. 1996). Studies in animal models demonstrated a reduction of abnormal tonic firing of nociceptor neurons.

Neuroprotection

There is evidence from animal models that sodium channel blockers are neuroprotective in global and focal ische-

mia (Baumgold 1987; Meldrum 1994). Several VGSC-blockers reduce brain damage from ischemia at doses that exert little side effects which stands in contrast to antagonists of excitatory amino acids or L-type calcium channel blockers (Taylor and Narasimhan 1997). Moreover, drugs such as riluzole have been shown to decrease ischaemic glutamate release (Baumgold 1987; Meldrum 1994). Furthermore, prevention from hypoxic damage to white matter tracts in stroke or neural trauma have been shown for sodium channel blockers. Therefore, modulators of VGSCs may offer advantages for treating certain types of stroke where damage of white matter tracts is prominent.

VGSC modulators in cardiology

A simple rule for the treatment of all patients with heart failure cannot be formulated because of the various aetiologies, haemodynamic features, clinical manifestations and severity of heart failures. Current therapy is based principally on the combination of a diuretic, a vasodilator, preferably an angiotensin-converting enzyme inhibitor, and usually a digitalis glycoside. The main objective in the use of diuretics and vasodilators is to reduce the pre- and afterload which leads to the reduction of the cardiac work. The cardiac glycosides are used principally to increase the force of the contraction which is reduced in heart failure due to a systolic dysfunction. Digitalis, introduced by William Withering more than 200 years ago and still used as a major positive inotropic drug, is far from being an ideal compound. The major problem with digitalis and related substances is the low therapeutic index. They can easily produce life-threatening cardiac arrhythmias, nausea as well as anorexia and vision disturbances.

In the past decade, several promising compounds like phosphodiesterase inhibitors (milrinone, vesnarinone, pimobendan etc.) have been synthesised and studied. The disappointment followed after it has been found that although showing a good short-term inotropic support of a heart failure, phosphodiesterase inhibitors significantly increase mortality when used for a longer period of time (Packer 1989). Another type of agents, β-adrenoceptor agonists, have various disadvantages like β-adrenoceptor down-regulation in progressed heart failures (Doggrell et al. 1994) or drug toxicity. Therefore, the development of positive inotropic drugs remains an important goal in cardiopharmacology.

One possible approach to positive inotropy is through the modulation of ion channels resulting in an increase of intracellular calcium. This can be achieved through the modulation of calcium, sodium and/or potassium ion channels. The main clinical disadvantage of Ca^{2+} channel activators is their lack of selectivity for the myocardium over the vasculature, producing profound vasoconstriction and damaging effects primarily within the central nervous system. The most promising channel modulators are drugs which increase Na^+ influx or decrease K^+ efflux (Doggrell et al. 1994; Varro and Papp 1995). The potential of these compounds in the treatment of patients with heart failure is still not fully evaluated.

An increase in Na^+ influx can be accomplished either by channel activation or by inhibition of channel inactivation or both. Compounds which activate sodium channels like alkaloids veratridine, BTX or aconitine or sea anemone toxins show strong pro-arrhythmogenic and vasoconstrictory activity limiting their usefulness. In contrast, compounds which prolong the opening of the sodium channel, like DPI 201-106 or BDF 9148 (Dumont 1972) have much more potential for treatment of patients with congestive heart failure. A major advantage of these compounds over other ion channel modulators is that they are in principle not arrhythmogenic (Doggrell et al. 1995) and not vasoconstrictive since the probability of blood vessel Na^+ channel being in the closed state is higher than in cardiac channels. Moreover, DPI 201-106 and BDF9148 were shown to have a vasodilatatory effect (Doggrell and Liang 1998; Hof and Hof 1985) which is desirable in patients suffering from heart failure. Another possible combination of ion channel modulator actions that may be useful in the clinic would be a compound that prolongs the opening of cardiac Na^+ channels to induce positive inotropy and vascular K^+ channel opening to produce vasodilatation.

Although (or perhaps we can say because) an ideal positive inotropic drug is still not available, there is a certain pessimism in the search for more suitable positive inotropes. This is partially a result of disappointment by numerous compounds (more than 100!) which have emerged in the past 20 years and which were not able to fulfil all requirements like selectivity or low toxicity. These arguments have been supported by the belief that the increase in intracellular calcium produced by these agents compromises the viability of myocardial cells. Nevertheless, there is an enormous potential for therapeutic advances in the treatment of heart failure with ion channel modulators, especially VGSC modulators. This potential should be better exploited in the future although the experience of decades suggests that progress will come slowly. Certain is that a better understanding of the VGSC structure and function is one of prerequisites for further development in this field. In particular, events such as activation and inactivation of the channels are interesting since they can serve as targets for VGSC modulators.

Acknowledgements The authors thank Prof. W. Löscher, Dept. Pharmacology, Toxicology and Pharmacy, School of Veterinary Medicine, Hannover and Dr. Tatjana Heinrich, Murdoch University, Western Australia for critically reading the manuscript. We acknowledge gratefully the help of J. Lis and M. Schneider.

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