Sodium Channel Inactivation: Molecular Determinants and Modulation

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I. Introduction

A. Scope

The subject of this article is the voltage-gated sodium channel that plays a key role in membrane excitation and is dealt with in an enormous number of papers. Inactivation (defined in sect. IIA) of this channel appears to be its most vulnerable kinetic feature as it is influenced, mostly slowed or abolished, by all kinds of chemical agents such as drugs, toxins, or mutations, often of only a single amino acid residue of the channel molecule. The amino

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acid sequence of the sodium channel molecule and its transmembrane topology have been known for quite some time. Nevertheless, relating function to structural details is still unsatisfactory, but site-directed mutations are increasingly employed to solve such questions. The molecular exploration of hereditary diseases has helped much to identify relevant regions.

Papers dealing with sodium channels, even those that are limited to inactivation, are so numerous that often only summaries, in particular of older work, can be quoted. Reviews of more recent papers on structure and function of sodium channels have been written by Catterall (67, 68), Denac et al. (106), Fozzard and Hanck (130), Marban et al. (272), and Ogata and Ohishi (331); extensive reviews of kinetic aspects of inactivation by Patlak (341) and of mechanisms by Goldin (146) have appeared. Highly readable accounts are found in books on ion channels in general (5, 181). Other reviews will be quoted in several other sections.

In this paper inactivation is first described as observed in electrophysiological experiments including patch-clamp studies. These are followed by interpretations by kinetic models and gating current experiments. Then the structure will be dealt with on a molecular level as derived from experimental point mutations. Next chemical modulation will be described and finally genetic modulation, observed in hereditary diseases (channelopathies).

B. Terminology

There exist many isoforms of the sodium channel (144) that have been differently termed. To eliminate the confusion a panel of leading researchers has agreed on a uniform nomenclature following that for potassium channels (147). These terms will be given, at least once, in parentheses to those used in the original papers.

II. PHENOMENA

A. Time Course of (Fast) Inactivation

The typical voltage-gated sodium channel opens on depolarization and closes rapidly on repolarization or, more slowly, on sustained depolarization. The latter process is termed inactivation and leaves the channel refractory for some time after repolarization. In the classical study of the squid giant axon, Hodgkin and Huxley (184, 185) described the sodium conductance (channel gating) being proportional to \(m^3 \cdot h\), where \(m\) and \(h\) are variables of time and potential that can assume values between 0 and 1: \(m\) increases and \(h\) decreases on depolarization. This leads to a nonmonotonic time course with \(1 - h\) expressing inactivation whereby \(h\) develops exponentially as \(h(t) = h_\infty - (h_\infty - h_0) \exp (-t/\tau_h)\) with \(h_0\) and \(h_\infty\) the starting and final value of \(h\), respectively, and \(\tau_h\) the inactivation time constant. For large depolarizations \(h_\infty\) becomes 0, meaning that inactivation reaches completion.

Changes in methods led to results that necessitated different formalisms. Thus in squid axons internally perfused with NaF solution, Chandler and Meves (75) found inactivation to be incomplete \((h_\infty = -0.1)\) and at very positive membrane potentials \(h_\infty\) increasing to ~0.3 or more. These authors explained their results by assuming that \(h\) is the sum of two components, \(h = h_1 + h_2\), with the components connected through an inactive (closed) state. Only during very long depolarizing pulses lasting for several seconds did the “maintained” current inactivate, which Chandler and Meves (76) described by an additional variable \(s\) so that \(h = (h_1 + h_2)s\). Such slow inactivation of varying time constants is observed in many preparations as will be shown below; in KF-perfused squid axons, it gives rise to action potentials lasting for seconds. On blocking \(I_K\), a noninactivating fraction of \(I_{Na}\) was later found also in nonperfused squid axons (412). It was further studied at very positive membrane potentials and with an inverse \(Na^+\) gradient (92). The voltage dependence of this fraction has again been examined in more detail recently (359), but the conclusions have been questioned (88).

In amphibian myelinated nerve fibers, inactivation was originally studied with short impulses and could be described by a monoeponential process (131). When in this preparation the potassium current was eliminated with tetraethylammonium ions (TEA), or by other means, the sodium current could be followed for any length of time. Inactivation then turned out to be diphasic (see Fig. 4, trace “toxin free”) and so was recovery from inactivation (84) which was interpreted by a second inactivating state in series. Several authors observed diphasic inactivation (see Ref. 230), but some interpreted it differently, recognizing that recovery from inactivation posed the more difficult part of description. Ochs et al. (330) tested several three-state models and found their results to be best fit with two open states connected through a closed one. To account for biphasic tail currents on repolarization, Elinder and Århem (119) suggested another model with two open states leading to two different inactivated states. Schmidtmaier (394) formulated a cyclic three-state model with one open and two closed inactivated states. Whereas these models were based on the idea of a single population of sodium channels, Benoit et al. (35) assumed that there are two, however interconvertible, types of sodium channels that differ not only in their inactivation kinetics but also in their susceptibility to blocking agents.

In the papers mentioned so far, it was tacitly assumed, as implied by the \(m \cdot h\) formalism, that inactiva-
tion proceeds independently from activation. This was questioned on the grounds of experiments on *Myxicola* axons. If in this preparation steady-state inactivation was determined with the classical protocol of a conditioning pulse of varying amplitude followed by a test pulse of constant amplitude (184), the position of the observed \( h_m(V) \) curve was clearly shifted to more negative potentials for weaker test pulses (151). Such shift had been predicted by Hoyt (197) for activation-inactivation coupling. Another point in favor of coupling was seen in a delayed onset of inactivation as determined with a constant test pulse following a conditioning pulse of constant amplitude but varying duration. Such delay was reported for *Myxicola* axons (151) and squid axons (45), but in the latter preparation, the delay could be minimized by leaving a gap between conditioning and test pulse to allow activation to subside (140). This does not seem to apply to *Myxicola* axons in which introduction of a gap did not eliminate the delay (148, 149, 150) nor did it in neuronal and cardiac channels (42). Kniffki et al. (230) showed that in toad nodes of Ranvier the initial delay is due to the activation during the conditioning pulse and implicit in the classical \( m \cdot h \) description. Thus the situation is complicated, moreover since the kinetic experiments are prone to flaws in method, some of which were reviewed by Meves (282).

The question of whether or not inactivation is coupled to activation is of course essential for understanding the mechanism of inactivation. A great number of kinetic models have been proposed that attempt to reconcile results on bulk sodium currents with so-called gating currents (see sect. uD) or single-channel recordings (see sect. uD).

### B. Differences Between Channel Isoforms

Activation and inactivation characteristics like \( V_{m} \), the potential at which activation reaches half-maximal values, and \( V_{h} \), the potential at which \( h_m = 0.5 \), differ in different isoforms, but also among species and, with cloned channels, depend on the cells in which they are expressed (269; see below). Thus, in \( \text{Na}^+ \) channels of human heart (hH1 = Na\(_{1.5}\)), skeletal muscle (hSkM1 = Na\(_{1.4}\)), and rat brain (rIIA = Na\(_{1.2}\)), expressed in mammalian tsa201 cells, \( V_{m} = -48, -28, \) and \( -22 \) mV and \( V_{h} = -92, -72, \) and \( -61 \) mV, respectively. The kinetics of activation and inactivation are also quite different. Thus \( I_{1/2} \), the time to reach half of their maximal current amplitude, values are 0.82, 0.48, and 0.40 ms for the three isoforms at 20°C (Ref. 333, containing ample references). The time constants of the rapid component of inactivation decrease with depolarization and reach asymptotic values at \( V > 20 \) mV of 0.41 ms (hH1), 0.26 ms (hSkM1), and 0.27 ms (rIIA). Clearly at positive potentials, where channels inactivate mostly from the open state, heart channels do so more slowly. Recovery from inactivation is also quite different: \( \tau_{\text{rec}} \), measured at \( V = -100 \) mV, was 44.7 ms (hH1), 4.7 ms (hSkM1), and 7.6 ms (rIIA). Extensive descriptions of these isoforms (70) and their evolution (142) have recently been compiled.

### C. Slow Inactivation

After prolonged depolarization, often achieved by changes of holding potential in the voltage clamp, recovery from inactivation may proceed very slowly with time constants in the second to minute range and in several phases. Such changes have been termed “slow” or “ultra-slow” inactivation and have been reported for amphibian myelinated nerve fibers (52, 129, 238, 318, 458), frog muscle (12), frog ventricular myocytes (132), and rat muscle (382, 415). Slow changes in \( I_{\text{Na}} \), following changes in membrane potential have also been observed in lobster, crayfish (378), *Myxicola* (392), and squid (1, 75, 76, 287) axons, in the latter preparation even after block of fast inactivation by intra-axonal application of pronase (379).

Slow inactivation was also observed in mammalian neuronal cells, such as rat hippocampal neurons (288), tetrodotoxin-resistant cells of dorsal root ganglia of rat (332), or cultivated neuroblastoma cells (353). In human cardiac muscle (471), slow inactivation is present but is only 40% complete compared with 80% observed in channels of human skeletal muscle (367).

Fast inactivation is important for action potential repolarization, and in mammalian nodes of Ranvier, which almost lack phasic potassium channels, it is the only repolarizing force besides the leakage current (85). Slow inactivation, on the other hand, may play a role in regulating excitability (383), such as by modulating burst discharges as can be demonstrated by computations (120). In reality, however, this modulation appears to be complicated since slow inactivation not only depends on resting potential but also on previous history of action potential firing (288, 448). Also, persistent \( I_{\text{Na}} \) of tetrodotoxin (TTX)-resistant channels (100) may affect the resting potential as suggested by computer simulations (175).

### D. Single-Channel Results

Single-channel records of normal sodium channels were preferentially obtained from cell-attached neuroblastoma and myocardial cells. They show one or a few short openings at the first 10–20 ms (at room temperature) after the start of a depolarizing impulse which, if kept on, leads the channels in an inactivated state. An early thorough analysis of single neuroblastoma channels revealed a fast “microscopic” inactivation from the open state even in the case of slow “macroscopic” inactivation as expressed by \( \tau_{\text{f}} \) at smaller depolarizations where a slower activation is rate limiting (6, 7). The underlying scheme predicts that removal
or inactivation would unmask the slow activation reaching its peak much later than $I_{Na}$ of untreated cells. This was indeed observed in neuroblastoma cells intracellularly treated with papain (153) but seems to be the exception rather than the rule, since other channel subtypes show a fast activation combined with relatively slow inactivation.

In some heart cells, openings lasting clearly longer than in neuronal cells were observed that correspond to a slow inactivating state as illustrated by ensemble-averaged records; even a transition from fast to slowly inactivating state was observed as well as a persistent state (48, 49). In rat skeletal muscle, slow inactivation manifests itself as a decreasing number of channels of unchanged open time and conductance (380).

An extensive single-channel study has also been done on cells of the rat entorhinal cortex which generate a persistent current caused by prolonged and often delayed bursts (267). Open times within these bursts (but not interburst closed times) were strongly voltage dependent. In neuroblastoma cells, multiple, longer, and late openings were seen only in channels modified by batrachotoxin (354), sea anemone toxin, scorpion toxin, or chloramine-T (226, 303, 308, 323). Sea anemone toxin prolonged the open state (including repeated openings) in cardiomyocytes (121, 402) as well as in cloned myocardial channels (hH1 = Na, 1.5; Ref. 74). In a comparative study on single cardiac and neuronal channels, modified by sea anemone toxin, initial clusters of multiple openings and periodic subsequent reopenings were observed in either preparation. However, toxin induced a larger persistent current in neuronal than in cardiac channels (42).

Similar single-channel phenomena have been observed in mutant channels with slowed inactivation (280) whereby particularly delayed inactivation is accompanied with very long mean open times (244, 417). Mutant channels completely deficient of inactivation (IFM/QQQ; see sect. IV) showed repeated openings and closings throughout long depolarizing pulses (159). In almost all these experiments, single-channel conductance was unaffected by modifications of kinetics. Analysis, however, may be complicated by the existence of conductance substrates and changes following excisions of membrane patches (see Ref. 309).

E. Temperature Effects

In their classical description of ion channel kinetics in squid axons, Hodgkin and Huxley (185) assumed a temperature coefficient, $Q_{10}$, of inactivation of ~3. Similar results have since been obtained with many other preparations (181). Hence, in comparing time constants it is necessary to consider the temperature at which they were measured. Usually the $Q_{10}$ values of the time constant(s) increase at lower temperatures as one would expect if kinetics are described by a simple Arrhenius plot of log $\tau_n$ vs. $T^{-1}$. However, in some preparations such as skeletal muscle, a break in this plot is observed which may point to a transition in the lipid membrane phase (85, 405), complicating the interpretation in terms of activation enthalpy. In rat nerve, the steady-state inactivation $h_n(V)$ was found to be shifted in the hyperpolarizing direction on cooling (404), so was $h_n(V)$ in *Xenopus* nerve (207), whereas in rat muscle only the steady-state curve of slow inactivation $s_n(V)$ was similarly shifted; this may explain the reduced availability of mammalian sodium channels if tested at room rather than at body temperature (381).

III. INTERPRETATIONS

A. Kinetic Models

As already mentioned in section II A, the classical description of sodium channel gating by Hodgkin and Huxley (184, 185) implied activation and inactivation to be independent processes, linked only by their dependence on membrane potential. However, later results such as the existence of more than one inactivation component, single-channel results, and the discovery of four nonidentical domains to form the sodium channel $\alpha$-subunit required extended models, competently reviewed by Hille (181). Most of these models propose that inactivation derives its voltage dependence from that of activation. However, often amino acid substitutions in the voltage sensors shift $h_n(V)$ in the opposite direction compared with shifts in $m_n(V)$, arguing against a strict activation-inactivation coupling (236).

The general concept is that on depolarization a channel moves from (several) closed (C) resting state(s) through an open (O) state to one (or several) inactivated (I) state(s). Gating is assumed to be a Markov process meaning that the rate constants of transitions between these states are “oblivious” of how a given state is reached. A critical feature to model is the recovery from inactivation that does not seem to pass through the open state as no ionic current is usually seen during this period. An exception is observed in cerebellar neurons that produce a “resurgent” current on repolarization (2, 360). This current is assumed to reflect unblocking of an open-channel block in a portion of channels by a hypothetical particle during strong depolarization, constituting an additional mechanism of inactivation. It thus enhances recovery from inactivation, which enables these neurons to fire at high frequency (360). In most other channels the details of recovery require C$\rightarrow$I and I$\rightarrow$C steps leading to cyclic connections of states. Such cycles underlie the restraint of microscopic reversibility so that the product of rate constants going clockwise must equal the product going counterclockwise. In the following scheme 1 this would mean $k_{3C}k_{qC}k_{1C} = k_{1C}k_{qC}k_{3C}$.
In a study of various five-state models, that of the kind of scheme 1 yielded the best fit of single-channel results (see sect. IV) as tested with the maximum likelihood method (193), allowing inactivation both from closed and open states. Block by a synthetic likelihood method (193), allowing inactivation both schemes 2 with two inactivated states or even more complicated models. Details are found in the excellent review of Patlak (342).

Another model based on single-channel results has been proposed by Correa and Bezanilla (93) and allows for a second open state that is reached through an inactivated state; this scheme may underlie the classical description of Chandler and Meves (75) mentioned in section IIA.

It should be noted that the classical Hodgkin-Huxley formulation \( m^3 \cdot h \) can be restated as a consecutive movement through eight states as in scheme 3 with \( k_{12} = 3 \alpha_m k_{21} = \beta_m k_{23} = 2 \alpha_m k_{32} = 2 \beta_m k_{34} = \alpha_m \) and \( k_{43} = 3 \beta_m \) (180); to describe a number of single-channel results, it appears not to be inferior to a model of the type of scheme 1 (78).

A similar model involving five closed states has been proposed for hippocampal neurons (241) and was later supplemented with another open state to describe inactivation in heart channels and its temperature dependence (200).

While the kinetic models described so far are based on voltage-clamp experiments that employ square voltage pulses, a different method called “nonequilibrium response spectroscopy” uses rapidly fluctuating (up to 14 kHz) potentials to extract more kinetic details (290).

**B. Gating Currents and Inactivation**

The potential-dependent gating of channels requires a voltage sensor bearing charges that move within the membrane as its potential changes, generating a tiny “gating current.” This became measurable on channel opening and closing after suppression of the much larger ionic current (16, 221), whereas movement of the inactivation gate did not seem to contribute a specific fraction of gating current. Instead, inactivation causes a substantial fraction (–2/3) to become immobilized, and immobilization proceeds and recovers with the same time course as does inactivation (17, 286, 287; for reviews, see Refs. 11, 15). This indicates that inactivation is coupled to activation from which it derives most of its voltage dependence (181). Nevertheless, some genuine voltage dependence of inactivation exists (194, 219, 220). The advantage of gating current measurements is to reveal kinetic steps that are not accompanied by changes of ionic currents.

A great number of papers have been published dealing, one way or the other, with the relationship between inactivation and gating current, many of them in K⁺ channels. As for Na⁺ channels, more recent studies tried to reconcile gating currents with details of channel structure, in particular the existence of four domains, D1–D4, each with six segments of which S4 acts as voltage sensor (see sect. \( \nu \)A). Although segments S4 of all domains seem to be involved in activation (237), D4S4 clearly has the largest effect on inactivation (236), and mutation of central arginines has specific effects on inactivation and gating charge immobilization in rNav1.2 (239). S4 immobilization in D3 and D4 (but not in D1 or D2) has also been observed by site-directed fluorescence labeling (73).

Another approach is to compare effects of modulating agents on ionic currents with those on gating currents (223). As one would expect, irreversibly removing inactivation in squid axons by chloramine-T (CT) or pronase also stops charge immobilization but with other similarly acting agents like batrachotoxin (BTX) immobilization remains (439). In amphibian nodes of Ranvier, comparable results were obtained with CT (111) but not with BTX, which eliminated immobilization (114) as did internally applied iodate (113). Comparative studies on nodes of Ranvier, also with site 3 toxins, have been summarized by Meves (284). In mammalian muscle channels (rNav1.4) and cardiac channels (hNav1.5), site 3 toxins of Anthopleura, ApA and ApB, reduce the maximal gating charge by about one-third, although muscle channels inactivate clearly faster than heart channels (407). Studies of ApA effects on gating charge in mutant hNav1.5 revealed the importance of the outermost arginine in D4S4 for charge immobilization.
immobilization (409) Charge immobilization has also been achieved in squid axons by intracellular treatment with the positively charged sulphydryl reagents MTSET (2-trimethylammonioethylmethane thiosulfonate) and MTS-PTrEA ([3(triethylammonium)propyl)methanesulfonate]. These reagents irreversibly modify native cysteine(s) in a potential-dependent manner and promote inactivation from closed states, whereas they do not affect activation; MTS-PTrEA shifts the steady-state inactivation curve to more negative potential values and renders it much shallower (222).

IV. MOLECULAR MECHANISMS

A. Channel Structure and How to Relate It to Function

1. Structure

During the last 20 years the molecular analysis of the sodium channel has made enormous progress through experiments most recently described by Catterall (68). Here it may suffice to summarize our present knowledge. The channel purified from mammalian brain consists of the large \( \alpha \)-subunit (260 kDa) with the pore, the \( \beta_1 \) (36 kDa) and \( \beta_2 \)-subunits (33 kDa) that contain extracellular immunoglobulin-like folds as illustrated by Figure 1 in section IV B. The \( \alpha \)-subunit consists of four domains (D1-D4) each with six transmembrane \( \alpha \)-helical segments (S1-S6) of which S4 bears several positive charges originating from arginine or lysine residues. The proteins of the domains wrap around a central pore such that the P-loops (SS1-SS2) between S5 and S6 form part of the pore lining.

The positively charged residues of S4 are interspaced by two hydrophobic residues forming a spiral ribbon of positive charges on the \( \alpha \)-helix. The “classical” view has it that negatively charged residues in adjacent segments form ion pairs that stabilize S4 against the pull exerted at rest by the (inside) negative membrane potential. Depolarization releases S4, the voltage sensor, to move outward initiating the structural changes that open the pore (66, 166). This general concept has been varied and adapted to comply with new evidence, in particular gained from the three-dimensional structure of the bacterial potassium channel KcsA as reviewed by Bezanilla (43).

Comparable results of sodium channels are soon to be expected as a recently described bacterial channel, NaChBac, may provide the protein in quantities needed for structural studies (365). A mutation, G219P, in S6 of this channel, dramatically reduces the rate of the already very slow deactivation and inactivation and shifts membrane potential (\( V_m \)) by \(-51 \text{ mV} \) (521). It seems that this mutation strongly favors bending of the S6 helix and that G219 serves as a gating hinge. A low-resolution three-dimensional structure study of a sodium channel (employing cryo-electron microscopy and image reconstruction) revealed, in addition to the ion-conducting central pore, four peripherally located transmembrane “gating pores” in which the S4 movement is thought to take place (69, 391).

More recently, X-ray crystallography of another bacterial channel, KvAP, yielded results that led to a quite
different interpretation of voltage sensor movement. S4 and part of S3 are supposed to form a “paddle” at the periphery of the channel which, on depolarization, moves like a lever through the surrounding membrane lipid from the intracellular to the extracellular side, thereby opening the pore (205, 206). This unconventional view, summarized by MacKinnon (265), is at present controversially discussed on the basis of new experimental results (4, 29, 53, 133, 247; see also comment in Ref. 190). Whatever the outcome, it certainly will affect our interpretation of sodium channel gating.

2. Methods

To derive function from structure, many methods have been employed. Early experiments revealed the sideness of certain modulating agents or procedures giving valuable information as to where certain functions may be located. Thus, for instance, scorpion toxins act only from outside, pronase treatment only from inside the excitable cell to slow or delete inactivation. Comparable information was obtained with specific antibodies against porcell to slow or delete inactivation. Comparable information was obtained with specific antibodies against portions of the channel (see sect. ivB). Decisive progress, however, was achieved by molecular genetics enabling site-directed mutagenesis. Mutants are expressed in Xenopus oocytes but most frequently in mammalian cells such as HEK293 (human embryonic kidney cells) and their derivative tsA201, also in Chinese hamster ovary cells (CHO) or in the Escherichia coli strain BL21. Very successful was replacing single amino acid residues by cysteine to which access could then be tested with sulfhydryl reagents (“substituted-cysteine-accessibility method”, SCAM; Ref. 213), whereby access could change with the functional state. Experimental point mutations were employed in most of the papers cited in section iv, B–D, whereas natural mutants found in hereditary diseases (“channelopathies”) are described in section vi. In Figure 1, the localization of the most important mutations is marked by black numbers on white, those of channelopathies as white numbers on black background.

B. Localization of the Gate Mediating Fast Inactivation

The intracellular linker between domains D3 and D4 plays an important role as derived from studies with antibodies directed against the linker which completely blocks fast inactivation (39, 461, 462); also, inactivation is lost or slowed if this linker is cut (432) or bears deletions or mutations (217, 344). In the following description, mutants of Na1.2 are given if not otherwise mentioned. In these Na1.2 channels, the critical motif is the hydrophobic triad I1488, F1489, M1490 (IFM; “h” in Fig. 1). Inactivation is completely blocked in I1488Q-F1489Q-M1490Q (IFM/QQQ, corresponds to I1303Q-F1304Q-M1305Q in Na1.4), but mutant F1489Q alone considerably slows inactivation (502) as does the equivalent F1304Q in muscle channels (Na1.4) for which kinetic modeling led to the assumption of three inactivated states (328). Inactivation is restored by adding short peptides containing IFM (KIFMK-amide) to the intracellular side (117, 118) or in channels in which inactivation is slowed by external application of α-toxin of the scorpion Leiturus quinquestriatus (116). SCAM experiments with the mutant F1489C show that F becomes inaccessible to MTSET on inactivation, suggesting that IFM serves as a hydrophobic latch for a hinged lid formed by the D3-D4 linker (216). The NMR solution structure of the isolated inactivation gate (the linker peptide) has been identified as a stably folded core consisting of an α-helix capped by an NH₂-terminal turn (373). It is supposed that on gate closing the core (the latch) pivots on a more flexible hinge region. Molecular dynamic simulation predicts an additional helical segment in the D3-D4 linker, on the other side of IFM, which possibly helps to guide the inactivation particle towards its receptor (416). Another solution structure study has been done in a medium of low dielectric constant (293).

More recent experiments on mutant muscle channels, Na1.4, revealed that the charge of residues beyond the IFM motif also plays an important role: reduction of positive charge (K1317N,K1318N) accelerates inactivation kinetics in the absence of the β-subunit (280), whereas charge reversal in the presence of the β-subunit (E1314R,E1315R) slows open-state inactivation but accelerates closed-state inactivation (165). It is concluded that clusters of negatively and positively charged residues in the D3-D4 linker differentially regulate the kinetics of fast inactivation.

The D3-D4 linker of Na1.2 (brain) channels also contains a serine, S1506, distal of IFM, whose phosphorylation by protein kinase C (PKC) slows inactivation without an effect on the steady-state curve $h(V)$ (500, 501). Phosphorylation of other sites in the D1-D2 linker reduces $I_{Na}$, an effect that is also observed on phosphorylation by protein kinase A (99, 401). In cardiac Na⁺ channels (Na1.5) phosphorylation by PKC at the equivalent site S1505 causes a strong negative shift of $h(V)$ pointing to stabilized inactivation from closed states; the S1505A mutant is almost unaffected by PKC (352). In muscle μ1 channels (Na1.4), the equivalent serine S1321 does not seem to play a key role in shifting $h(V)$ by PKC, since it is also observed in a S1321A mutant (30).

Fast inactivation of Na1.2 is also interrupted in mutants F1764A/V1774A of D4S6 (corresponding to F1579/80/81 in Na1.4, ① in Fig. 1), originally thought to form part of the hydrophobic latch receptor (278). Later experiments with F1764A/V1774A excluded a direct interaction with the IFM motif, since application of KIFMK-
amide nevertheless restored inactivation (279). KIMFK restores fast inactivation of open but not of closed channels in the F1651A/L1660A mutant in the D4S4-S5 intracellular loop (approximately at ⑦ in Fig. 1). This suggests that the IFM motif interacts with the loop during inactivation of closed channels (280). In Na_{1.4} cysteine substitution, F1579C, inhibits both fast and slow inactivation, whereas Y1586C and I1575C enhance them (21). Interestingly, these residues belong to the LA binding site, which hence appears to be involved in both types of inactivation.

The D3-D4 linker, however, is not the only determinant of fast inactivation. Thus if in the more slowly inactivating heart channel (Na_{1.5}) the linker is replaced by that of the faster brain channel (Na_{1.2}), its properties are not transferred (171). On the other hand, replacing the COOH terminals accelerates inactivation but also magnifies the differences in voltage dependence of the steady-state inactivation (270). Similar results have been obtained with Na_{1.4}/Na_{1.5} chimeras (108, 109). In a more detailed study of the heart channel COOH terminal, it was shown that truncation of its distal part only reduces current density. Truncation of the proximal part, consisting of six helices, additionally shifts the inactivation curve and clearly increases the fraction of noninactivating channels (91). Further experiments with mutants, in particular truncation of the highly charged sixth helix which increases the persistent \( J_N \), suggest that the COOH terminal stabilizes inactivation and minimizes channel reopening (301). The COOH terminal contains a binding site of calmodulin, which is important for the functional expression of channels. In Na_{1.6} calmodulin also slows inactivation in a calcium-dependent manner (176).

Important insights have also been gained from experimental changes of the voltage sensor segment, S4. Point mutations of charged amino acids in S4 to cysteine changed the kinetics of deactivation from open and inactivated states in a domain-specific fashion (164). In heart channels (Na_{1.5}), charge-neutralizing or -reversing substitutions shifted the activation curve \( m_\alpha(V) \) and decreased its slope as had already been demonstrated for D1S4 in the pioneering work of Stühmer et al. (432). Inactivation time constants were markedly decreased only in D4S4 mutations (Ref. 82; see also sect. nB). In rat brain channels (rNa_{1.2}), addition of a positive charge just external of the outermost positively charged residue of D4S4 (F1625R or F1625K) led to a split of \( h_\alpha(V) \) into two components and a large shift towards hyperpolarization but only to a shift in neutral mutants. These and other findings add to the idea that the D4S4 movement controls the inactivation gate, whereby the countercharges may play an important role in the D4S4 position (512).

The still poorly understood connection between sensor movement and gating has recently been subject of several “perspective” papers (44, 134, 189, 243). It should be mentioned that the “paddle” hypothesis leaves the problem of the sensor-gate coupling unresolved.

C. Localization of Site(s) Responsible for Slow Inactivation

The mechanism of slow inactivation is more complicated, located in structures distinct from those of fast inactivation, and it is less well understood (472). Since it bears similarities to the C-type inactivation of potassium channels, which is connected to the external pore lining, corresponding residues in sodium channels have been the target of site-directed mutagenesis. Thus, in the absence of \( \beta_1 \), mutant W402C (P-region of D1, ③; Ref. 22) eliminates slow inactivation, whereas mutation of the adjacent residue, E403C or E403R, seems to favor entry into the slow inactivation state (520) as does W434A (492). E403 together with E758, D1214, and D1532 at comparable positions in domains 2, 3, and 4, respectively, form an outer ring of charges whose structural rearrangement was found to be associated with slow inactivation (507). Also, slow inactivation alters the accessibility by the positively charged MTSEA (2-aminoethylmethanethiosulfonate) to the outer pore cysteine of mutant F1236C (P-region of D3, ④), pointing to a structural rearrangement which, incidentally, may be linked to use-dependent LA action (334). A mutant of the adjacent K1237S or E leads to a very slow (“ultra-slow”) type of inactivation (447). Such very slow inactivation is also enhanced by mutant A1529D in the P-loop of D4, part of the putative selectivity filter (177).

As already mentioned, slow inactivation of human cardiac sodium channels (hNa_{1.5}) develops more slowly and is only 40% complete versus 80% in skeletal muscle channels (Na_{1.4}) (367). Chimeras of domains of these two channels show that slow inactivation can be modulated by all four domains, with D1 and D2 being more prominent (335). Interestingly, a single residue in D2S5-S6 (P-region), V754 of Na_{1.4} and the corresponding I891 of Na_{1.5}, confers their parental properties to the chimera; however, considering the other experimental evidence, it seems unlikely that the P-region is directly involved in slow inactivation (470). Also, mutant V787K (D2S6; ③) markedly enhanced slow inactivation, whereas with V787C it was further slowed, incomplete, and less voltage-dependent than in wild type (336). Mutants Y401C and V787K (D2S6; ③) markedly enhanced slow inactivation, whereas with V787C it was further slowed, incomplete, and less voltage-dependent than in wild type (336). Mutants Y401C and G1530C in the P-region of D1 and D4, respectively, were modified by MTSET at the same rate during slow inactivation as in the noninactivated state, which suggests that the outer mouth of the pore remains open in either state (430).

In mutant channels devoid of fast inactivation, slow inactivation remains intact (127, 463). In such mutants, substitution of positive charges in S4 of D1 or D2 shifts the slow inactivation to more positive potentials, whereas
D. Importance of the β-Subunits

In *Xenopus* oocytes, coexpression of the α-subunit of Na₈,1,2 with β₁-subunit of neuronal and skeletal muscle channels increases the current density, accelerates inactivation, and shifts the steady-state inactivation curve in the hyperpolarizing direction (202, 343) as illustrated by Figure 2. Comparable results have been obtained with Na₈,1,8 and, to a lesser extent, with Na₈,1,7, channels found in dorsal root ganglia (466). Coexpression also accelerates recovery from inactivation (32, 62). It seems that the α-subunit has a slow and a fast gating mode leading to diphasic inactivation. The latter mode is favored on binding of β₁ whereby the inactivation kinetics of either component does not change (296). An opposite effect, slowing of inactivation and inducing a persistent IₚNa, is observed by coexpression of G protein γβ-subunits with Na₈,1,2 (264). α-Subunits expressed alone in mammalian cells inactivate almost as fast as the native preparation (203), which has been attributed to an endogenous splice variant, β₁₂, present in these cells (299). This hypothesis was subsequently rejected on the grounds of experiments with antisense oligonucleotides (297). An additional subunit, β₃, predominant during development (406), has been found that accelerates inactivation but less than β₁ with which it is closely related (300). The distribution of β₃ in human tissues and comparative amino acid sequences of β₁, β₂, and β₃ is described in Stevens et al. (426). Most recently, a novel disulfide-linked subunit, β₄, has been identified which shows similarities with β₂; on coexpression it shifts the activation curve in the hyperpolarizing direction without affecting inactivation (516).

The β₂-subunit does not modulate hH1 (=Na₈,1,5) or IIA (=Na₈,1,2) and β₁/β₂ chimeras, expressed in *Xenopus* oocytes, have served to determine the regions of β₁ necessary for modulation: hH1 channels via the transmembrane portion (“membrane anchor”) plus additional regions, IIA channels via the extracellular region only (276, 277, 522) as has been suggested before (79).

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**FIG. 2.** Effect of coexpression in *Xenopus* oocytes of β₁-subunit with α-subunit of Na₈,1,2 channel. A: normalized whole cell Na⁺ current (IₚNa) on step depolarization from −100 to −10 mV with β₁ + α leading to faster inactivation. B: steady-state inactivation curve is shifted by −19 mV on coexpressing β₁ (squares). C: IₚNa-voltage (V) curves showing a large increase in peak current on coexpression. Note that inactivation is fast when α is expressed in mammalian cells and not further accelerated by β₁. [From Isom et al. (202), copyright 1992 American Association for the Advancement of Science.]
These effects are not yet fully understood, but the functional domains for the interaction seem to be highly conserved (62, 343). Further experiments revealed that only the extracellular domain of β1 is essential for the interaction (79, 277), whereby segment D4S2-S6 of the α-subunit plays an important role (see broken-line connection in Fig. 1; Ref. 351). Earlier studies pointed to this region: inactivation of human heart channels (hH1 = Na1.5), in contrast to skeletal muscle channels (hSkM1 = Na1.4), is not much accelerated on coexpression (in Xenopus oocytes, Ref. 329) with β1, but chimeras containing only D4S5-S6 of hSkM1 show the typical acceleration (268). As for the increased channel density, it is interesting to note that in HEK293 cells the hH1-β1 complex forms already in the endoplasmic reticulum which may facilitate trafficking to the plasma membrane (523).

V. CHEMICAL MODULATION

A. Modulation by Toxins and Local Anesthetics

Various toxins and other chemical agents slow or even abolish inactivation. Several groups of toxins have been characterized by binding studies that originally led to the definition of five binding sites (47, 67, 495) that were eventually extended to nine sites (524). Experiments on mutant channels (see sect. IV) reveal that toxins within one binding group often do not occupy identical but rather overlapping sites. Also, there exists considerable allosteric interaction between the sites. In the following section, toxin binding to sites 2, 3, 5, and 6 will be discussed as well as other chemicals that affect Na+ channel inactivation.

1. Site 2 toxins: veratridine, batrachotoxin, grayanotoxin, and aconitine

The toxins of this group are lipid soluble, which enables them to access binding sites embedded in the membrane. The most important of these compounds are veratridine (VT), an alkaloid from liliaceous plants, BTX, which is secreted by the skin of Colombian arrow-poison frogs, aconitine (AC) from plants of the buttercup family, and grayanotoxin (GTX) from plants of the heather family. Although these toxins differ widely in their structure, their common effect is to keep sodium channels open, hence they are termed “agonists.” The underlying mechanism is a large shift of activation in the hyperpolarizing direction and a slowed or even abolished inactivation. Also, the toxins clearly bind to open channels. Details are found in reviews (64, 106, 224, 294, 455) and in the book of Hille (181).

A typical VT (60 μM) effect is illustrated by Figure 3, which was obtained with long depolarizing impulses on a frog node of Ranvier. It not only shows the slowly developing Na+ inward current followed by a large slowly decaying current tail (cut off) at the end of the pulse but also the slow current reduction on adding benzocaine during the impulse. In contrast, if benzocaine was applied to Na+ channels of a Ranvier node kept open by CT (see sect. v.A5), block was very fast (half-time ~60 ms). From this and other results it is hypothesized that channels kept open by VT cannot be directly blocked, and the current reduction shown in trace 2 in Figure 3 is determined by the rate with which VT-modified channels close during the pulse and become susceptible to the local anesthetic (457).

Site 2 toxins show “use dependence” in that their effects increase on repeated membrane depolarization; measures that open sodium channels (site 3 toxins, mutations) facilitate their action. In contrast to toxins of other groups, they also affect ion permeation, impressively demonstrated with VT by a sudden reduction of single-channel conductance on binding (27, 413, 477) which explains the much reduced macroscopic \( I_{Na} \) in the presence of VT (453). GTX-modified channels also have a reduced conductance (508). Likewise sodium channels from eel electroplax inserted in planar bilayers have reduced conductances in the sequence unmodified > BTX > GTX > VT (115, 364).

Obviously these toxins considerably distort the channel since also selectivity for Na+ is reduced. Thus permeability of ammonium ions increases considerably in the presence of VT (251) and in the presence of AC ammonium ions may even become more permeable than Na+ and the relative permeabilities of Cs+, Rb+, and K+ increase (161, 304). Comparable results have been reported under the influence of BTX and VT (135) and AC (362). On
the other hand, Li\(^+\) that passes native sodium channels readily becomes clearly less permeable under VT treatment (135). Hence, in Li\(^+\) Ringer solution, the typical VT-induced afterpotentials are no longer observed (453). Details of altered selectivity caused by VT including ion flux measurements are found in a review (455), that of other site 2 toxins also elsewhere (181).

2. Site 3 toxins: scorpion, sea anemone, and spider toxins

Another binding site has been characterized and termed site 3 to which \(\alpha\)-scorpion toxins and sea anemone toxins bind (for a review, see Ref. 67). Site 3 toxins have been the subject of many studies so that the reader is referred to a selection of reviews treating toxin structure (156, 218, 326, 347, 370, 498) and/or electrophysiological effects (245, 246, 283, 414, 454). These toxins consist of a polypeptide chain held together by several disulfide bonds; they act from outside the membrane and inhibit inactivation and render it incomplete, but their binding sites seem to be overlapping rather than identical (372). An example of the effect of ATX II, a toxin of the sea anemone *Anemonia sulcata*, is given in Figure 4, which was obtained in a voltage-clamped frog node of Ranvier at a saturating (with respect to the late current) concentration of 5 \(\mu\)M.

There exists a large sequence homology among the many \(\alpha\)-scorpion toxins (347) and sea anemone toxins (326) but little between the two. Not only do the toxins of various scorpion species differ with respect to their effects, but also to the target channel isoforms as in amphibian nodes of Ranvier (285, 305, 485), rat skeletal muscle (80) versus heart muscle (81). TTX-resistant sodium channels of dorsal root ganglion cells are also resistant to sea anemone toxins (and other similarly acting toxins) in contrast to TTX-sensitive channels of this preparation (Ref. 389; see below and sect. v, A3, A5, and B).

As for the toxin molecule, subtle changes may yield largely different effects. Thus in neuroblastoma cells, sea anemone toxins APE 1–1 and 1–2 of *Anthopleura elegans-tissima*, differing by four amino acids, cause nonactivating currents of 20 and 40\%, respectively (54). Likewise, two toxins from another sea anemone (*Anthopleura xantogrammatica*), ApA and ApB, show great differences in affinity for mammalian (tsA201 cells) channels despite a strong sequence homology, arising solely from different rates, \(k_{\text{off}}\) of toxin-channel dissociation (41). In frog nerve ATX I, which differs from ATX II by several amino acids, is ineffective and does not antagonize ATX II (395). Affinity may also be affected: a 20-fold change in \(E_{\text{50}}\) of slowing inactivation of cloned hH1 (=Na1.5) by *Bunodosoma granulifera* toxins II and III which differ by only one amino acid (157). The exclusive targets of some \(\alpha\)-scorpion toxins are insect sodium channels, which makes them potential selective insecticides (155, 524).

Another type of scorpion toxins, termed \(\alpha\)-like, is toxic to both mammals and insects. Typical representatives are LqhIII of *Leiurus quinquestriatus hebraeus* and BomIII and BomIV of *Buthus occitanus mardochei* (72, 80, 137, 138); LqhIII also affects frog axons where, however, it acts like the classical \(\alpha\)-toxin (37). The \(\alpha\)-like toxins seem to bind to a site that is differentially related to site 3 (72). The venom of the scorpion *Tityus serrulatus* contains an interesting toxin, TTX\(\gamma\), which binds to muscle surface channels with a very high affinity (26). In neuroblastoma cells, TTX\(\gamma\) reduces peak \(I_{\text{Na}}\) causes an inward current to flow near the resting potential, but also slows inactivation (468). The toxin thus shows effects of both \(\alpha\)- and \(\beta\)-toxins. Similar results have been observed in *Xenopus* nodes of Ranvier (208).

Binding of the scorpion \(\alpha\)-toxins is weaker on depolarization (36, 71, 285, 403, 429, 496), but the authors do not completely agree as to whether the open and/or inactivated state confers the reduced affinity. Toxin II of the sea anemone *Anemonia sulcata* (ATX II) binding is not reduced on depolarization (285, 473), whereas actions of ATX III and IV on crayfish axons were clearly reduced (497). More recently, voltage dependence of scorpion toxin binding was found in a voltage range where activation and inactivation saturates so that it may originate from other sources (80, 81). Moreover, in steady-state binding experiments to rat brain synaptosomes, depolarization, achieved by high K\(^+\) concentration, yielded different kinetic results than short applications.

The toxins not only slow inactivation but render it incomplete (reviewed in Refs. 106, 454), inducing a per-

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**FIG. 4.** ATX II (5 \(\mu\)M) reduces peak \(I_{\text{Na}}\) slows inactivation, and renders it incomplete. Voltage-clamp record of frog node of Ranvier in the presence of 10 nM TEA to suppress K\(^+\) current, 17\(^\circ\)C, 15-ms impulse of resting potential \((V_{\text{R}}) = +60\) mV. Formal description of diphasic inactivation:\(h(t) = A + B \exp(-Ct_d) + C \exp(-Dt_i)\), with \(A = 0.15, B = 0.83, C = 0.52, t_d = 0.67\) ms, \(t_i = 4.30\) ms compared with \(A = 0, B = 0.65, C = 0.35, t_d = 0.48\) ms, \(t_i = 1.79\) ms in toxin-free solution. [Modified from Ulbricht and Schmidtmayer (456).]

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sistent $I_{\text{Na}}$ component (36) which is more prominent in neuronal than in cardiac Na\(^+\) channels. Also, the toxins enhance the rate of recovery from inactivation through closed states (42).

Slowed and incomplete inactivation is also induced by funnel-web spider toxins that have been shown to bind to site 3 and compete with scorpion α-toxins (258, 259), some of them also act on insect Na\(^+\) channels (163). These toxins are without effect on the inactivation of TTX-resistant channels of rat dorsal root ganglia (321, 322, 434). The spider toxins δ-αtracotoxins form a new family of polypeptides with no similarity to scorpion α-toxins (128). δ-ACTX-Hv1a (formerly versutoxin) is contained in the venom of the funnel-web spider Hadronyche versuta, which also produces the less effective δ-αtracotoxin-Hv1b of no insecticidal effect (434). δ-ACTX-Ar1 (formerly robustoxin) from Atrax robustus is dangerous to humans; it exhibits a 83% amino acid sequence homology with δ-ACTX-Hv1a (321). Another spider, Paracoe- lotes luctuosus, produces the insecticidal δ-palatoxins with effects similar to those of α-scorpion toxins (95). Reviews of spider toxins have been published (94, 162).

3. Site 5 toxins and persistent sodium current

In many preparations a small persistent $I_{\text{Na}}$ is observed already in normal saline; although small, it may be important in regulating excitability (97, 436, 443). Different channel isoforms with comparable inactivation kinetics nevertheless have persistent components of distinctly different size (83, 104, 267, 525). Some such results have been interpreted as “window” currents flowing in the potential range where steady-state activation and inactivation curves overlap (19, 317, 384), but this interpretation does not fit results in ventricular myocytes (388) and especially in mammalian neurons (13, 97, 214, 340).

In addition to site 3 toxins, another group of toxins, binding to site 5, causes persistent currents including ciguatoxin, which is responsible for the ciguatera fish poisoning. Site 5 toxins are lipid-soluble polypeptides produced by dinoflagellates (20, 136). At least in frog nodes of Ranvier, ciguatoxin merely induces late (persistent) currents without affecting inactivation kinetics. Activation of this component is shifted by $-30$ mV towards hyperpolarization. The size of this component depends on the holding potential ($V_{\text{H}}$) being three times larger at $V_{\text{H}} = -70$ than $-120$ mV (38). In TTX-sensitive channels of rat dorsal root ganglia neurons, pacific ciguatoxin-1 shifts the activation curve and the steady-state inactivation curve in the hyperpolarizing direction. In TTX-resistant channels, toxin mainly increases the rate of recovery from inactivation (428).

Another group of dinoflagellate toxins that bind to site 5 are the brevetoxins (also polypeptide molecules), the cause of paralytic or neurotoxic shellfish poisoning. Brevetoxin-3 (PbTx-3), for example, produces a hyperpolarizing shift of the activation curve in TTX-sensitive channels of rat sensory neurons accompanied by an inhibition of inactivation (204). In TTX-sensitive Na\(^+\) channels of rat brain, brevetoxin PbTx-3 causes a similar shift of the activation curve which, together with a slowing of inactivation, leads to hyperexcitability (349). Actions of site 5 toxins on ion channels have been reviewed (20, 102, 274, 505). Studies of brevetoxin derivatives to elucidate active centers of the toxin have appeared (136, 204); some of these derivatives act as antagonists (350).

4. Site 6 toxins: δ-conotoxins

Marine snails of the genus Conus produce a great variety of toxins that act on different ion channels including sodium channels (reviewed in Refs. 125, 445). Many block these channels, but a group of polypeptides, consisting of 26–32 amino acids with 3 disulfide bridges, termed δ-conotoxins, slows inactivation like α-toxins of scorpions but does not bind to site 3. Hence, a new site 6, located at the extracellular side of the membrane, was defined (122; summarized in Ref. 524). The main target of the toxins δTxVIA (C. textile), NgVIA (C. nigropunctatus), and GmVIA (C. gloriamaris) is the sodium channel of mollusk neurons (122, 123, 172, 173, 411). Mammalian cells, as contained in rat brain synaptosomes, generally do not respond (but see below), but \(^{22}\)Na influx stimulated by veratridine is further increased (123). Also, δTxVIA, lacking electrophysiologically detectable effects on insect axons or frog muscle, nevertheless bind to “silent” receptors on these preparations (410).

Toxins of the fish-hunting Conus striatus retard inactivation in mouse neuroblastoma cells (154), in frog sympathetic neurons (55), and in frog nodes of Ranvier (as does δ-EVIA) and shift activation in the hyperpolarizing direction (170). Binding seems to be potential dependent with an optimum between $-100$ and $-60$ mV (154).

Recently, inhibition of inactivation was also reported for mammalian channels; Am 2766, a toxin from C. amadis, was found to affect rat brain channels (433) and δ-EVIA, a toxin from C. ermineus, mammalian neuronal and amphibian Na\(^+\) channels but not muscle or cardiac channels (24). Table 1 recapitulates the neurotoxins mentioned, their electrophysiological effects, and the putative location of their binding sites.

5. Other toxins and agents affecting inactivation

A) OTHER POLYPEPTIDE TOXINS. There are several other toxins slowing inactivation that do not seem to bind to site 3 and are chemically quite different such as Goniopora coral toxin (9.7 kDa; Refs. 152, 307) and pompilidotoxin from solitary wasps (1.5 kDa; Refs. 226, 387). Interestingly, rat heart cells are much less affected by pompilidotoxin (226), and TTX-resistant channels of rat trigeminal

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A review of spider and wasp toxins has recently appeared (273). Binding of these toxins is reduced by depolarization. A recently studied toxin, PnTx-2–6, isolated from the spider Phoneutria nigriventer venom, slows inactivation kinetics in frog muscle channels and does not seem to bind to site 3 (273). A review of spider and wasp toxins has recently appeared (107).

**Table 1. Neurotoxins that modify inactivation**

<table>
<thead>
<tr>
<th>Receptor Site</th>
<th>Toxin</th>
<th>Physiological Effects</th>
<th>Assumed Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Batrachotoxin, veratridine, aconitine, grayanotoxin</td>
<td>Persistent activation, depolarize resting membrane, repetitive firing</td>
<td>S6 in D1–D4</td>
</tr>
<tr>
<td>3</td>
<td>α-Scorpion toxins, sea anemone toxins, δ-atoxatoxins</td>
<td>Keep channel open</td>
<td>Loop D4S3–S4, S5–S6 loops in D1 and D4</td>
</tr>
<tr>
<td>5</td>
<td>Brevetoxins, ciguatoxins</td>
<td>Shift activation</td>
<td>D1S6 D4S5</td>
</tr>
<tr>
<td>6</td>
<td>δ-Conotoxins</td>
<td>Prolong channel opening</td>
<td>?</td>
</tr>
</tbody>
</table>

ganglion cells are not affected at all (387). Binding of these toxins is reduced by depolarization. A recently studied toxin, PnTx2–6, isolated from the spider Phoneutria nigriventer venom, slows inactivation kinetics in frog muscle channels and does not seem to bind to site 3 (273). A review of spider and wasp toxins has recently appeared (107).

**B) Insecticides.** Insect sodium channels are the target of many insecticides, especially dichlorodiphenyltrichloroethane (DDT) analogs and pyrethroids, synthetic analogs of the natural pyrethrin of Chrysanthemum flowers (311). These agents inhibit inactivation (346, 467, 524), but they also affect squid axons (262), lobster axons (312), as well as amphibian (14, 112, 178, 469) and mammalian channels (141, 142), however at higher concentrations and to a varying degree (155, 310, 418). Pyrethroids bind to a separate site allosterically coupled to sites 2 (345) and 3 (451). TTX-resistant channels of rat dorsal root ganglia are more sensitive to pyrethroids than TTX-sensitive channels of the same preparation (141, 435, 441). The differential sensitivity of sodium channel isoforms to pyrethroids has recently been reviewed by Soderlund et al. (418).

Pyrethroids shift the relation between activation and membrane potential in the direction of hyperpolarization and cause a similar shift of inactivation; in their presence, large and prolonged tail currents are observed after repolarization. These tails are drastically further prolonged on lowering the temperature so that the depolarizing afterpotentials increase and with it the deadly repetitive discharges (141, 302, 419). Open channels of squid axons have a higher affinity for pyrethroids (263), and in frog muscle fibers, opening is a prerequisite to the action of the pyrethroid deltamethrin (252). Although many features remind of those caused by veratridine, pyrethroids do not reduce single-channel conductance nor do they change selectivity (509).

**C) Chloramine-T.** Chloramine-T (N-chloro-p-toluensulfonamide; CT) is a mild oxidant (mol wt 228) that irreversibly slows inactivation and renders it incomplete without affecting activation so that, on repolarization, it leaves the tail currents almost unchanged (169, 454, 479). In cloned muscle channels, elimination of fast inactivation by CT even accelerated development of slow inactivation without changing recovery or steady-state slow inactivation (491). CT acts on either side of the membrane in crayfish axons (198) but on rat brain channels, inserted in planar lipid bilayers, only if applied to the inside (98), possibly since the agent cannot penetrate the bilayer in contrast to natural membranes (327). CT is commonly thought to act by modifying methionine groups (479), but this explanation has been questioned (356). Besides, the action of CT cannot be highly specific as it also delays the inactivation of some types of potassium channels (377, 424). CT is preferably applied for only a few minutes, since longer treatments destroy the preparations with the possible exception of squid axons (480).

If shortly applied (to frog nerve fibers) while the membrane is depolarized, CT only reduces peak $I_{Na}$ whereas application during a slight hyperpolarization affects only inactivation, leaving the peak current unchanged as shown in Figure 5, A and B (393). It appears that CT immobilizes the inactivation gate in a portion of channels: in the closed state in Figure 5A, in the open state in Figure 5B. A very similar result has been obtained with an IFM/CFM mutant labeled with a photoactivable cross-linking group with slowed inactivation; on ultraviolet irradiation of the hyperpolarized membrane, a large persistent $I_{Na}$ (see Fig. 5D) was observed, whereas irradiation during a lasting depolarization only reduced the peak current (see Fig. 5C), comparable to Figure 5A (192).

A state-independent effect on inactivation, however, has been observed in crayfish axons in which the blocking action turned out to be reversible on washing (198). CT pretreatment considerably enhances the action of agents that preferably bind to open channels such as batrachotoxin (440), veratridine (454), grayanotoxin (518), or pyrethroids (419). In frog nodes of Ranvier, cooling further slows the modified inactivation and increases the persistent $I_{Na}$ fraction accordingly; this argues against a separate channel population to be responsible for this current component (394).

**D) N-Bromoacetamide.** N-Bromoacetamide (NBA; mol wt 138) is another agent that inhibits inactivation if applied to the inside of squid axons (337, 339) or crayfish axons (390), GH3 cells (194), or cardiac channels (231, 292). External application succeeded in other preparations such as neuroblastoma cells (153), amphibian muscle...
was also found to slow (reversibly) inactivation of certain partially protected in its closed configuration (390). NBA state of these channels leading to an increased Na of cardiac sodium channels and thus prolonging the open exerts a positive inotropic effect by inhibiting inactivation /H11002 100 mV than at penetrated. Removal of inactivation by NBA is faster at the membranes of these preparations can be more easily (325), or myelinated nerve fibers (478), possibly because of S-DPI have been studied in single-channel experiments (233, 234, 324) that have also revealed conductance substates (341, 403). Many other results with this cardiotonic effect are found in a review (106).

BDF 9148 increases action potential duration by delaying inactivation of cardiac sodium channels of guinea pigs (363) and humans (186, 306). Another congener, BDF 9198, delays the slow component of inactivation in guinea pig heart and induces a persistent $I_{\text{Na}}$ component (517). A similar effect has been reported for still another congener, the S-enantiomer of RWJ 24517: casatrin (452). In cultured ventricular myocytes, BDF 9145 proved to interact synergistically with site 2 toxin VT (477).

**H) PROTEOLYTIC ENZYMES.** In preparation for internal perfusion of squid axons, pronase, a mixture of proteolytic enzymes, was employed to facilitate extrusion of the axoplasm. Prolonged enzymatic treatment caused inactivation, but no activation, to be destroyed, which suggested that the inactivation gate is accessible from the axoplasmic side (18, 374). This evidence helped to formulate the ball-and-chain model of fast inactivation (17). Later the active pronase component alkaline proteinase b was identified (375). Removal of squid axon inactivation was employed in studying activation and deactivation (337), steady-state properties of activation (427), and use-dependent block by many local anesthetics (reviewed in Ref. 310). In crayfish axons, pronase treatment revealed that the blocking agent methylene blue, internally applied, binds to open channels (422) and that the rate of removal of inactivation is voltage dependent (390).

The proteolytic enzyme trypsin, which cleaves peptides at lysine and arginine residues, removes inactivation if applied to the interior of excitable cells. This has been observed in GH3 cells (460), N18 neuroblastoma cells (153),
or cardiac myocytes in which, however, α-chymotrypsin is more effective (87). These and other experiments employing agents to remove inactivation were used to study the relation of activation and inactivation. Also, means to prolong the open state improve measurement of currents through single channels inserted in lipid bilayers (3).

1) FREE FATTY ACIDS AND PHOSPHOLIPIDS. Dietary polyunsaturated fatty acids (PUFA), in particular of the n-3 class as contained in fish oil, are antiarrhythmic agents. They exert their effect on cardiac (Na,1.5) Na\(^+\) channels by reducing \(I_{\text{Na}}\) and shifting the steady-state inactivation curve in the hyperpolarizing direction. Since other, structurally unrelated, heart channels are also inhibited, an indirect effect via membrane phospholipids was discussed. However, channels with a mutation in D1S6 (N406K) are significantly less sensitive to PUFAs, and this inhibitory effect is increased on coexpression of \(\beta_1\) (506). This points to a direct effect on the \(\alpha\)-subunit rather than to an effect on the membrane phospholipids such as their packing (348) or fluidity (253).

6. Local anesthetics preferentially bind to inactivated channels

Block by local anesthetics (LA) varies with structure and physical properties of drug (56, 181) and of the type of channel (106, 400) and depends on potential history of the membrane: relatively weakly if resting, but strongly if depolarized. Especially effective are frequent pulses that may lead to increasing block until a new steady level is reached. This phasic block has been termed “use dependent” (96) and has been the subject of numerous studies and several interpretations of which the “modulated receptor hypothesis” has gained most interest. The hypothesis assumes that affinity for LA depends on the conformational state of the channel with the inactivated state showing the highest affinity (179, 188). This energetically favored conformation, then, is stabilized in the presence of LA, resulting in a shift of the steady-state inactivation curve to more negative potentials. A different interpretation (“guarded receptor hypothesis”) assumes that drug binds to a constant-affinity receptor whose access is regulated by channel gates leading to apparently variable affinities (423). Either hypothesis has been used to describe LA results, mostly obtained with antiarrhythmics on cardiac myocytes, however with no clear decision. This has been acknowledged in a critical assessment by one of the proponents of the guarded receptor hypothesis (158) who in a later study showed its limitations, at least in preparations with chemically slowed gating (160). On the other hand, a recent study of mutated cardiac channels offers arguments in favor of this hypothesis (361). It should be mentioned that a more peripheral role of the inactivation gate in LA action has been suggested (Ref. 464; see sect. VB3).

Because block of inactivated channels apparently plays a role in the understanding of LA action, experiments on channels with impaired inactivation have repeatedly been done, however with varying results. Thus in squid axons treated with CT, use dependence persisted, but after pronase treatment it was almost absent (57, 481). CT-treated frog nerve also showed use dependence (519). In this preparation, channels kept open by CT (during 14-s depolarizing pulses) were blocked on sudden application of benzocaine within a fraction of a second. This shows that the LA receptor remains readily accessible (457). If tested with 15-ms impulses after equilibration in benzocaine, the late \(I_{\text{Na}}\) was much more reduced than the peak current. This reflects a weaker resting block, and affinity for resting channels was estimated to be 17 times lower than for inactivated channels (281). In this preparation benzocaine uniformly reduced \(I_{\text{Na}}\) during a test pulse if inactivation was completely abolished by batrachotoxin (398). Benzocaine enhanced the residual steady-state inactivation of batrachotoxin-modified brain channels, rNa,1.2 (486).

Many LA are racemates whose constituents often differ pharmacologically, in particular in binding to the receptor (182, 515) but also in unbinding leading to differences in use dependence (86, 105, 459). No stereoselective action on TTX-resistant channels was observed (51). LA stereoisomers have also been tested on mutant sodium channels to elucidate the structure of the receptor as shown in section VB3.

It may be mentioned here that partial block of TTX-resistant channels of dorsal root ganglia by La\(^{3+}\) leads to a slowed inactivation of the remaining inward \(I_{\text{Na}}\), whereas block and slowing are much less if \(I_{\text{Na}}\) flows outward. The kinetic effect appears to reflect that blocked channels cannot inactivate, which becomes visible as the blocking-unblocking reaction at a site near the selectivity filter is significantly faster than the inactivation rate (242).

B. Toxins and Receptor Sites

1. Molecular determinants of toxin binding site 2

Site 2, to which the lipid-soluble toxins VT, BTX, AC, and GTX bind (64, 65; for a review, see Ref. 495), turned out to very likely consist of overlapping receptors. Attempts to localize the binding site(s) employed point mutations. Thus it was found that if residues in the middle of S6 of several domains were changed to the positively charged lysine, the channel, expressed in a mammalian cell, became resistant to BTX: N433K, N434K and N437K in D1 (493), N784K and L788K in D2 (489), S1276K and L1280K in D3 (490), and F1579K and N1584K in D4 (494); other mutants in D4, F1764A and I1760A, caused affinity to be reduced 60- and 4-fold, respectively (257). These mutants showed little change in gating. In mutant N434K

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on the other hand, VT binding was not abolished but seemed to reduce channel conductance (as in WT, Ref. 252) and affect gating (493). Similar observations were also made with F1579K and N1584K, which led to the hypothesis of VT action that in a first step peak $I_{Na}$ was reduced and then VT trapped within the D1S6 and D4S6 domain interface (482). More recently, the assumed close alignment of the VT receptor and the LA receptor (see also sect. vB3), already suggested earlier (see Fig. 3; Ref. 457), was studied by cysteine substitutions of three residues constituting the LA receptor: N434C/L1280C/F1579C (S6 of D1, D3, and D4; rNaᵥ1.4). This mutant remained susceptible to LA block, whereas VT not only failed to keep the channel open but progressively blocked it on repetitive pulsing. This block was prevented by simultaneous application of LA (488). The authors suggest that the VT and LA receptors overlap extensively with that of VT being situated in the inner channel vestibule. VT blocks also wild-type channels, although imperfectly, which may lead to the drastically reduced unitary conductance, in contrast to the interpretation of Barnes and Hille (27).

The moderately BTX-resistant mutant F1710C in D4S6 of Naᵥ1.3 channels (expressed in Xenopus oocytes) allows rapid dissociation of toxin (which in wild type binds almost irreversibly) from its receptor, but only in open channels. This led to the conclusion that BTX reaches its receptor from the cytoplasmic side only when the activation gate is open (256), but access and dissociation occur probably only during the initial steps of depolarization (103).

The BTX-resistant mutants I433K, N434K, and L437K of D1S4 and their matches in rat heart muscle, V406K and L410K, turned out to be resistant to GTX as well (201). Other completely GTX-resistant mutants were found in D4S6: N1575A and Y1586K, whereas Y1586A and F1579K were partially resistant. It is suggested that GTX and BTX have overlapping but not identical binding sites. The mutants were partially resistant. It is suggested that GTX and BTX have overlapping but not identical binding sites. In mutant F1579A, on the other hand, GTX was more effective than in wild type (225). Recent binding studies on mutant Naᵥ1.4 channels revealed two more GTX binding sites on D2S6 (N784) and D3S6 (S1276). Also, systematic substitutions of F1579 (D4S6) showed that the smaller the substituent residue, the more both rates $k_{on}$ and $k_{off}$ were increased, leading to almost unchanged values of $K_p$, whereas substitution of Y1586 (D4S6) selectively increased $k_{off}$ thus increasing $K_p$ (266). The authors conclude that all four S6 segments contribute to the binding site but F1579 appears to control access and Y1586 binding. Less is known about AC action on mutant channels. In human heart muscle, the related alkaloid lappaconitine which, in contrast to the agonist AC, blocks (irreversibly) sodium channels, fails to bind to mutants F1760K or N1765K of D4S6, corresponding to the BTX-resistant mutants F1579K and N1584K in skeletal muscle (503).

2. Molecular determinants of toxin binding site 3

Site 3 toxins act only if applied to the external side of the membrane, and earlier experiments employing photoaffinity labeling placed the receptor site on the extracellular loop S5-S6 of D1 (444). This was confirmed by antibody mapping that revealed another spot on the equivalent loop of D4 (446). Experiments with modified toxins (see summary in Ref. 372) suggested acidic residues to be required for toxin binding. In experiments on mutant α-subunits (Naᵥ1.2 expressed in mammalian cells) several residues in D4S3-S4 could be identified, with E1613R and E1613H showing considerably reduced binding of scorpion (Leiurus quinquestriatus) toxin and sea anemone (Anemonia sulcata) toxin ATX II, however to a somewhat different extent for the two groups of toxins. Extensive studies have been done testing three types of Leiurus quinquestriatus hebraeus toxins on rNaᵥ1.4 channels into which D4S3-S4 linkers of various isoforms had been incorporated. These studies show that recombining a few amino acid residues in site 3 leads to phenotypes of great toxicological variety (254). Wild-type cardiac channels have a much reduced affinity for scorpion toxin, and substitution of cardiac D4S3 into skeletal muscle α-subunits rendered them rather insensitive to toxin (372). TTX-resistant Naᵥ1.8 is resistant to Leiurus quinquestriatus hebraeus toxin as well and is so a chimera of the sensitive muscle channel Naᵥ1.4 containing, in the D4S3-S4 linker, four additional amino acids (SLEN) existing in Naᵥ1.8 (385).

Although binding affinities of wild-type and mutant (E1613R) α-subunits differ largely, the voltage dependence of scorpion toxin binding at equilibrium, $K_p(V)$, is quite similar. It is suggested that the affinity changes are due to a conformational change required for fast inactivation (see sect. vB) that follows the voltage-dependent transitions among closed states (372). It was found that the increase in $K_p$ is predominantly due to an increased dissociation rate ($k_{off}$) of scorpion toxin, whereas association ($k_{on}$) appears to be independent of potential, at least between -120 and -80 mV. Comparable results have been obtained with different scorpion toxins (80). However, the situation is more complicated since in steady-state binding studies on synaptosomes with toxin II of Leiurus quinquestriatus hebraeus (LqhII), $k_{on}$ turned out to be far more affected by depolarization than $k_{off}$. Fast depolarization led to a biphasic unbinding whose first phase revealed a fast $k_{off}$ as observed in electrophysiological experiments on rBII sodium channels (rNaᵥ1.2) expressed in mammalian cells; the second slower phase resembled the almost voltage-independent off-rate observed in the steady-state binding experiments attributed to binding to the slow-inactivated state (139). Strong depolarizing pulses also weaken the effect of Ts3, a toxin.
from the scorpion Tityus serrulatus, assumedly due to dissociation from its binding site (58).

Point mutations in Na,1.2, away from the putative receptor in the central part of D4S4, that slow inactivation by slowing S4 movement, also reduce the effect of ATX II without changing its affinity. This has been interpreted as an electrostatic interaction of the positively charged toxin with the outermost S4 residue (240); it helps to understand how a toxin that binds to the external side eventually influences the inactivation “lid” at the internal side. Inhibition of D4S4 movement by a site 3 toxin has also been observed in Na,1.5 channels (408).

3. **Molecular determinants of the binding sites of local anesthetics**

The importance of inactivation for phasic block by local anesthetics (LA) prompted experiments with inactivation-deficient mutants with varying results depending on the LA. Thus in IFM/QQQ mutants (in D3-D4 linker, “h” in Fig. 1) use dependence of lidocaine block was absent (Na,1.5, ventricular myocytes; Ref. 331) as with disopyramide, whereas it was retained with the lidocaine derivative RAD-243 or flecainide (159). In F/Q mutants of rat muscle channels, rNa,1.4, expressed in Xenopus oocytes, use-dependent action of lidocaine was observed, at least at higher concentrations, and lidocaine blocked the plateau $I_{Na}$ much more efficiently than the peak current; this was not observed in IFM/QQQ mutants (23). In their attempt to describe these effects by a kinetic model, the authors concluded that lidocaine acts as an allosteric effector to enhance inactivation. A mutant of this F, F1304C, reacts with MTSET applied to the inside in noninactivated (hyperpolarized) channels thereby blocking inactivation. However, if the channels are depolarized, the cysteine becomes unreactive and may thus indicate the gate position also during depolarization-dependent block by lidocaine. After a depolarizing pulse, the gate reopens with the same kinetics independent of whether or not lidocaine is present, but in the latter case, $I_{Na}$ recovers very slowly as the anesthetic slowly unbinds (464). This unexpected result suggests that the inactivation gate may play a more marginal role in local anesthesia.

Other experiments to determine the LA binding site were done by site-directed mutations in the middle of segment S6 of domains D1 to D4. Mutations F1764A and Y1771A in D4S6 of rat brain IIA channels, rNa,1.2, expressed in Xenopus oocytes, facing the pore (® in Fig. 1), drastically decreased the affinity of open and inactivated channels for etidocaine, leading to a nearly complete loss of use and voltage dependence (357). The authors concluded that the hydrophobic residues F1764 and Y1771 are determinants of the LA binding site, and substitution with alanine destabilizes drug binding. Mutation N1769A, oriented away from the pore, considerably increased the affinity only of the resting channel, possibly by an indirect effect. Mutant I1760A, closer to the extracellular side of D4S6, enabled the quaternary LA QX314 to block on external application, which would be ineffective in the wild type. In subsequent experiments, these authors found comparable results with lidocaine, the anticonvulsant phenytoin and, to a lesser extent, with quinidine and flecainide, suggesting an overlapping common receptor site (358). In rat muscle channels, rNa,1.4, expressed in HEK293 cells, etidocaine, and the neutral LA benzocaine were tested on mutants N1584A and F1579A (equivalent to N1769A and F1764A of Na,1.2), revealing an increased block of the former and a decreased block of the latter by both LA (483). This suggests a common receptor for the two types of LA, already deduced from kinetic experiments on wild-type channels (397). Also, mutant I1575A (equivalent to I1760A in Na,1.2) allowed the channel to be blocked by externally applied QX314. Substitution by hydrophilic lysine did not change resting block by benzocaine at F1579K but decreased it at N1584K and increased it at Y1586K (504).

BTX-resistant mutants N434K and L437K (D1S6, near ® in Fig. 1) bind LA significantly weaker than wild type, whereas N434D restored or even enhanced binding (484). Cysteine substitutions N434C enhanced inactivation (shift of $V_h$ to more negative potentials) and increased sensitivity to lidocaine, whereas I436C shifted $V_h$ in the opposite direction (235). The authors also observed a reduction of use-dependent block in D2S6 mutants like I782C and V786C and concluded that determinants of LA binding in D1S6 and D2S6 are subsidiary to those in D4S6. This confirms the extensive study on alanine-substituted residues in D1S6 and D2S6 (514) in Na,1.2 channels and those in D3S6 (513). The results of these two papers, together with earlier findings, suggest that the LA receptor site is formed by residues in D3S6 and D4S6 with the contribution of a single amino acid in D1S6.

The BTX-resistant mutants S1276K and L1280K (D3S6) showed much reduced bupivacaine binding to the inactivated state, but clearly less to the resting state. It appears that LAs interact with these S6 segments when the channel is in its inactivated state (490). BTX-resistant mutants N784K and L788K (in D2S6) remain sensitive to bupivacaine (489). Incidentally, the BTX-resistant N434R shows a clear stereoselectivity, decreasing S(−)-bupivacaine potency definitely more than of its enantiomer; similar results were obtained with cocaine but not with RAC 109 (314). Stereoselective bupivacaine block of inactivated Na,1.4 channels was also found with mutant L1280 (D3S6), leading to the assumption that this residue and N434 interact directly with LA, facing each other in the pore (316). Weak stereoselectivity for bupivacaine was also observed in heart channel (Na,1.5) mutants...

Other experiments on mutants of heart sodium channels, Na$_v$1.5, such as F1760K (expressed in HEK293 cells) revealed that block of resting and inactivated states by the antidepressant amitriptyline (a use-dependent blocker of sodium channels) is greatly reduced and use dependence eliminated (313).

Finally, experiments have been done on mutants causing hereditary muscle diseases (see sect. viB) to test whether LA could restore normal inactivation behavior. This was not completely achieved in equine hyperkalemic periodic paralysis (386) or paramyotonia congenita (124, 438). Incidentally, two agents of possible clinical significance, the preservative 4-chloro-m-cresol and the bacteriostatic diluent benzylalcohol, have been tested on these mutants, showing typical LA behavior (167, 168).

4. Binding sites for agents of low molecular weight

A) CT. Little is known of the precise location of the CT target, generally assumed to be a methionine group (see sect. viA5). The M of the IFM triad (in the D3-D4 linker) forming the inactivation lid could be a likely candidate as it stabilizes the inactivation state via hydrophobic interactions (373); oxidation of M would disrupt these interactions (195). However, this interpretation may be questionable, since an IFI mutant remains sensitive to CT (487). Quiñonez et al. (355), working on muscle fibers, postulate at least two critical methionine groups.

B) NBA. The incomplete inactivation of cardiac Na$^+$ channels after treatment with NBA and iodate was indistinguishable from that on applying an antibody targeting a portion of the D3-D4 linker, which provided a substrate for NBA but not for the action of iodate (39). In myelinated frog nerve fibers, application of near-saturating concentrations of ATX II after iodate pretreatment yielded additive effects, suggesting different mechanisms by which these two agents act (396).

VI. GENETIC MODULATION: CHANNELOPATHIES

Mutations of single amino acid residues in the channel molecule are not only produced in the laboratory but are recognized as the cause of many hereditary diseases called “channelopathies.” The first channelopathies were identified in skeletal muscle by electrophysiological experiments that later led to genetic and functional expression studies. Defects of sodium channel inactivation, mostly leading to hyperexcitability, are also found in heart muscle and in the central nervous system leading to a great variety of clinical symptoms. An extensive review of genetics, chemistry, and symptoms is available (248), also in a shorter version (249).

A. Heart Muscle

The most serious disorder in heart muscle caused by ion channel defects is the long Q-T syndrome (LQT) due to disturbed myocardial repolarization that may lead to ventricular arrhythmias or even sudden death (215, 227, 271). However, only one (rare) type, LQT3, is linked to a sodium channel gene, SCN5A, encoding the α-subunit. One defect in hH1 (hNa$_v$1.5) channels is the deletion ΔKPQ of three consecutive amino acids (K1505, P1506, Q1507) in the D3-D4 linker (not far from the inactivation “lid”) accompanied by a persistent current (34, 77, 476). A persistent I$_{Na}$ component was also observed in the point mutations N1325S (in D3S4-S5) and R1644H (in D4S4; Ref. 476); slowed inactivation was seen in R1623Q (in D4S4, Ref. 212). N1325 and R1644 are located near the docking sites of the inactivation gate, whereas R1623 seems to be involved in activation-inactivation coupling.

In section ivB, it was mentioned that the COOH terminal stabilizes inactivation. It is therefore to be expected that inherited mutations in the COOH terminal of cardiac sodium channels may cause arrhythmias. For example, E1784K in the acidic domain within the early portion of the COOH terminal is accompanied by a small persistent I$_{Na}$ (499). Y1795C (B in Fig. 1) produces LQT3 with the onset of inactivation slowed, whereas Y1795H speeds inactivation leading to the Brugada syndrome, an ST elevation in ECG leads V1 through V3 (369). It is suggested that in Y1795C a disulfide bond is formed with a partner cysteine in the channel (442). Another mutant, 1795insD, in which an aspartate is inserted, may lead to both LQT3 and Brugada syndrome (46), possibly because fast inactivation is disrupted, but slow inactivation is augmented which could reduce I$_{Na}$ at rapid heart rates (465).

B. Skeletal Muscle

A great number of hereditary skeletal muscle diseases are due to mutations in the sodium channel α-subunit (gene SCN4A) that lead to slowed inactivation. Many reviews have appeared (25, 59, 146, 187, 199, 209, 229, 248, 250). The inactivation defects induce trains of action potentials or even sustained depolarization of the sarcolemma, with the former causing myotonic symptoms and the latter paralyses due to persistent inactivation. The most common diseases are paramyotonia congenita (PMC), hyperkalemic periodic paralysis (HyperPP), and potassium-aggravated myotonia (PAM). PMC is a paradoxical myotonia with cold-induced muscle stiffening during exercise followed by weakness or paralysis. HyperPP shows episodic attacks of generalized weakness triggered by rest after body exertion or K$^+$ intake, accompanied by hyperkalemia. Hypokalemic periodic paralysis (HypoPP-2) is a rare disorder characterized by intermit-
tent attacks of weakness accompanied by a decrease in serum K⁺ concentration. PAM is a generalized myotonia aggravated by K⁺ intake, showing no weakness.

The underlying mutations are found in expected locations such as the D3-D4 linker containing the “lid” (PMC, PAM) or D4S6 (HyperPP, PAM) but also in other locations as S4-S5 linker of D2 (1 in Fig. 1; PMC) or in D4S3 and D4S4 for PMC (2) and the intracellular linker S4-S5 in D3 (3; HyperPP, PAM); the mechanisms are not always understood. Lists of mutations are found in several reviews (60, 61, 229). Often the mutations were studied in heterologously expressed α-subunits (hNav1.4) rather than in native muscle fibers or even on rat channels (rNa₁.4; Ref. 298). More recent papers and some containing particularly interesting mutations are mentioned below.

Thus cold aggravation of PMC has recently been studied again; that caused by mutation R1448H (in D4S4) showed a clearly increased “window” current on cooling (295), R1448C a disproportionally slowed deactivation on repolarization (110). Mutation R1441P in rat (corresponding to R1448 in humans) also slowed deactivation, which opposes action potential repolarization and thus exacerbates PMC; this double cause could contribute to cold aggravation as was shown by computer simulation (126). Mutation T1313A/M (4 in D3-D4 linker, distal to “h” in Fig. 1), another cause of PMC, does not change temperature sensitivity per se but further slowing of inactivation on cooling may push it over the threshold of PMC (50).

The most common HyperPP mutations are T704M in D2S5 and M1592V in D4S6; they cause a small persistent cooling may push it over the threshold of PMC (50).

Potassium-aggravated myotonia (PAM) has been observed in mutations with impaired fast inactivation such as S804F (in D2S6), I1160V (in D3S4-S5), G1306A/E/V (5 in D3-D4 linker), and V1589M (in D4S6), extensively described in the review of Kleopa and Barchi (229). Possibly K⁺ aggravates indirectly by depolarizing the membrane (187, 368).

C. Central Nervous System

Mutations in the α-subunit of brain sodium channels (Na₁.1 and others) may cause some forms of childhood-onset epilepsy: generalized epilepsy with febrile seizures plus (GEFS⁺2) and severe myoclonic epilepsy of infancy (SMEI) with GEFS⁺ being considered “benign” but SMEI “intractable.” Mutations in the β₁-subunit have been identified as cause of GEFS⁺1, rendering this subunit ineffective in accelerating inactivation (see sect. IV), which may thus lead to hyperexcitability (474). Such “gain-of-function” changes, for instance due to persistent Iₙ, may be associated with either GEFS⁺ or SMEI as no simple correlation exists between clinical symptoms and electrophysiological behavior (366). Even “loss-of-function” mutations, associated with reduced current densities, may cause GEFS⁺2 or SMEI (260), an unexpected effect that could be indirect by eliminating inhibition.

An increasing number of mutations causing GEFS⁺2 have been detected (146), and some have been heterologously expressed in human cells to study their electrophysiology (9, 255, 260, 261). For instance, R1648H (in D4S4) induces a sizable, T875M (in D2S4) and W1204R (cytoplasmic D2-D3 linker, close to D3S1) a small persistent current (261). Also, T875M enhances slow inactivation, another example of reduced excitability resulting in seizures (10, 420).

VII. CONCLUSION

Fast inactivation is a highly important feature of sodium channel kinetics as it helps to repolarize the excitable membrane during an action potential, in some preparations such as mammalian nodes of Ranvier being the only repolarizing force. Inactivation modulates the affinity of the channels for various chemical agents, in particular for local anesthetics and similarly acting drugs, leading to increasing block during trains of action potentials that may lead to a beneficial antiarrhythmic effect. The molecular mechanism of fast inactivation as an intrinsic block of the intra-axonal pore entrance is fairly well understood but, less well, why some subtle changes away from this location have drastic effects on inactivation. Although the secondary structure of the channel...
molecule is known, much is still to be learned about the tertiary structure and higher orders, in particular the exact conformational changes underlying gating. Such information is now increasingly becoming available through X-ray and NMR studies that eventually will also clarify the more complicated mechanism of slow inactivation.

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