Site-3 Toxins and Cardiac Sodium Channels

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Summary

Site-3 toxins are small polypeptide venoms from scorpions, sea anemones, and spiders that bind with a high specificity to the extracellular surface of voltage-gated Na channels. After binding to a site near the S4 segment in domain IV the toxin causes disruption of the normal fast inactivation transition resulting in a marked prolongation of the action potentials of excitable tissues including those of cardiac and skeletal muscle and nerve. In this review we discuss the specific binding interactions between residues of the toxin and those of the Na channel, and the specific modification of Na channel kinetic behavior leading to a change in fast inactivation focusing on interactions deduced primarily from the study of sea anemone toxins and the cardiac Na channel ($Na_V1.5$). We also illustrate the usefulness of site-3 toxins in the study of altered Na channel behavior by drug-modification.

Introduction

An extremely interesting set of toxins, named site-3 toxins (Catterall, 1980), bind to the extracellular surface of voltage-gated Na channels and produce profound slowing of current decay. Site-3 toxins have been particularly useful in helping understand the structural basis of Na channel gating, and we will review them with particular attention to their effects on the cardiac isoform ($Na_V1.5$). Members include the 60–70 residue α-scorpion toxins (from *Leiurus quinquestriatus, Centruroides sculpturatus, Tityus serulatus*, and *Androctonus australis* and others for which a database is available (Srinivasan *et al.*, 2002)) as well as several peptide toxins isolated from sea anenome, including Anthopleurin A (Ap-A) and Anthopleurin B (Ap-B), which were isolated from *Anemonia xanthogrammica* (49 aa), and the *Anemonia sulcata* toxins (ATXI [46aa], ATXII [47aa], and ATXIII [27aa]) (Norton, 1991) as well as a several toxins isolated more recently from spiders, including several from funnel spiders, e.g. *Hadronyche versuta* (Little *et al.*, 1998a; Little *et al.*, 1998b) and *Hadronyche versuta*, (Fletcher *et al.*, 1997), as well as from *Plectreurys tristis* (Corzo *et al.*, 2003) and *Phyllodiscus semoni* (Xiao *et al.*, 2005) (for a review see Nicholson & Graudins, 2002). In addition to an excellent summary published in 1991 (Norton, 1991), two recent reviews are also noteworthy (Blumenthal & Seibert, 2003; Honma & Shiomi, 2006). Also, a recent brief review gives additional details of the various identified spider toxins (Nicholson & Graudins, 2002).

Early Investigations

The anemone toxins were first identified in 1976: (1) by noting skeletal muscle cramps and paralysis after injection of venom in crabs (Beress *et al.*, 1976); (2) after superfusion onto

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isolated cardiac muscle strips with resultant increase in inotrophy associated with action potential prolongation (Shibata *et al.*, 1976); (3) by noting extracellular but not intracellular application of toxin caused inhibition of I_{Na} decay in frog node of Ranvier (Bergman *et al.*, 1976b). Initially, the idea that Na channels were represented by multiple isoforms was not appreciated, although it was noted that several of the site-3 toxins exhibited specificity for cardiac preparations. These included ATXII from *A. sulcata* (Beress & Beress, 1975) and Ap-A, isolated from *A. xanthogrammica* (Norton *et al.*, 1976). In addition, several others have more recently been identified, including Jinghaotoxin I (Xiao *et al.*, 2005) while others have been described that specifically target insect/crustacean channels (Salgado & Kem, 1992).

Early data not only reported very different apparent affinities between preparations but even within a single preparation, which depended on the technique used for the assay. For example, ED_{50}'s for ATXII based on binding/flux in rat brain synaptosomes were estimated to be 150–240 nM, (Vincent *et al.*, 1980) while voltage clamp affinities in myelinated nerve were estimated to be 30 to 100 fold lower, i.e. ED_{50}'s at 5 μM(Schmidtmayer *et al.*, 1982) and 20 μM.(Bergman *et al.*, 1976a). It was then recognized that the action of the toxin was specific for Na channels, and that these toxins have a low affinity for the inactivated state of the channel because of voltage-dependent binding (Wang & Strichartz, 1985; Strichartz & Wang, 1986; Warashina *et al.*, 1988). In binding and flux studies site-3 toxins were used in combination with toxins that promoted activation (usually veratridine) where binding and/or flux was augmented (Lawrence & Catterall, 1981; Frelin *et al.*, 1984). Such data were interpreted to indicate a synergistic effect of the two agents (Corbett & Krueger, 1989), or more often a high affinity of the open state (or an open-like state) for toxin, a conclusion also suggested by early patch clamp studies (Schreibmayer *et al.*, 1987b), but questioned by others in which other promoters of the Na channel open state were unable to mimic the effect of scorpion toxin (Rando *et al.*, 1986).

Determinants of the Specific Interactions between Site-3 Toxins and Na Channels

Here we focus on the major toxins from *A. xanthogrammica* and their kinetic actions principally on cardiac Na channels. Extensive structure-function data are available for Ap-B, one toxin from the venom of *A. xanthogrammica,* which when assayed electrophysiologically exhibits a high affinity for multiple Na channel isoforms with a 50 fold preference for rat cardiac channels over neuronal isoforms (0.1 nM vs. 5 nM), although when assayed by flux the affinities were similar and appeared to be high affinity (9 nM vs. 22 nM) (Gallagher & Blumenthal, 1992; Khera *et al.*, 1995). Ap-A, the major toxin from *A. xanthogrammica,* in which only seven of the 49 amino acids are different from Ap-B, was the first noted to have cardiac effects and to exhibit a 50-fold preference for cardiac over neuronal channels (2.5 nM vs. 120 nM). Ap-A toxin also exhibited isoform preference by flux assay of approximately 30-fold (14 nM to 400 nM) (Khera *et al.*, 1995). Other studies also reported data to support the idea that these toxins display a 20 to 40-fold greater affinity for cardiac over neuronal channels (Vincent *et al.*, 1980; Lawrence & Catterall, 1981; Nagy, 1988; Gallagher & Blumenthal, 1994). It should be noted that Ap-B is generally described as a high affinity variant, but it can also discriminate between isoforms. Cationic residues on these anemone toxins have been identified as underlying the affinity differences (Khera *et al.*, 1995), with Arg12 being the most important, although in combination with Lys-49 it accounts for most of the cardiac specificity of Ap-A over Ap-B for cardiac channels. Although these toxins have a quite high affinity, i.e.ED_{50}'s ~1 nM, their on-rates are extremely slow. Ap-B toxin, for example, which has an $ED_{50}$of 0.1 nM estimated by electrophysiology for rat $Na_V1.5$, has an on-rate of only 1.4 x 106 $s^{-1}M^{-1}$, 2–3 orders of magnitude less than predicted for diffusion of small molecules (Khera *et al.*, 1995). Very slow rates of modification and unmodification require that affinities be measured after exposing preparations to toxin for long periods of time or be estimated from

kinetic analyses of washin and washout experiments at toxin concentrations many times the EC_{50} in order to promote channel modification over a time frame achievable in a typical voltage clamp experiment. This method is illustrated in Figure 1.

Even though site-3 toxins from different species are not similar in their sequence, they share, along with other peptide toxins such as charydotoxin and μ-conotoxin, multiple disulfide bonds (either 3 or 4) that form a tight core. Structural information is available for several of the site-3 toxins, including a 1.2 Å crystal structure for the α-scorpion toxin from *Androctonus australis* (Housset *et al.*, 1994). NMR coordinates for Ap-A and Ap-B, although determined and reported earlier, were released in 1996 (Torda & Norton, 1989; Scanlon & Norton, 1994). For more information see Smith and Blumenthal in this issue.

Almost certainly loop flexibility near Arg-12 contributes to the very low on-rate. Substitution of the glycines at either position 10 or 15 in Ap-B markedly decrease affinity (Seibert *et al.*, 2003). Since only a small subset of loop confirmations can achieve high affinity channel interactions, most toxin/channel interactions will be unfruitful. It appears that most of the contacts in the loop with the channel are likely to be hydrophobic since replacement of Asn-16 or Ser-19 with the smaller alanine has little effect on affinity, while introduction of charge at either site dramatically reduces toxin's ability to modify channel activity (Seibert *et al.*, 2004). In addition, consistent with this idea Leu-18 is critical for toxin action, both for its size and shape as well as its hydrophobicity since it cannot even be replaced by a valine, which reduces toxin affinity 100-fold (Dias-Kadambi *et al.*, 1996b).

Early experiments, primarily in the laboratory of Catterall, used antibodies and photo affinity labeling to identify parts of the channel that interacted with site-3 toxins (Tejedor & Catterall, 1988; Thomsen & Catterall, 1989). These studies were primarily carried out with neuronal channels and α-scorpion toxins, and they identified extracellular regions in domain I and domain IV as controlling binding affinity. Later mutagenesis experiments failed to identify any specific residue in the extracellular loops of domain I, although an anionic residue in the S3-S4 loop of domain IV was identified (Rogers *et al.*, 1996) that affected toxin affinity. At about this time other experiments took advantage of the isoform preference of Ap-A for cardiac Na channels over skeletal muscle Na channels, and showed that the isoform preference for this toxin was associated with domain IV (Benzinger *et al.*, 1997). Recently, additional contacts in the domain IV S3–S4 linker have been suggested based on experiments with site-3 toxins isolated from *Anthopleura fuscoviridis* and *Bunodosoma caissarum* tested against neuronal Na channels (Oliveira *et al.*, 2004).

A strong test of whether the residue identified by channel mutagenesis interacted directly with the toxin, in contrast to mutagenesis causing a general conformational change in the channel, is mutant cycle analysis. With this technique one explores the interactions of wild-type toxin with wild-type channels, and combinations of toxin mutated at the putative interaction residue with the channel mutated at the putative interacting residue (Horovitz & Fersht, 1990). If the two residues of interest do not interact then the change in affinity measured when either the toxin or channel is mutated is similar so that the ratio of the K_D's is approximately unity. If, on the other hand, the residues interact, then the combination of mutated toxin and mutated channel is not much worse than either the wild-type and mutant combinations since the binding site is disrupted by either change. In this case the ratio of the K_D's is significantly different from unity. This analysis established a specific interaction between the anionic residue in the domain IV S3-S4 linker (either an aspartate or glutamate depending on channel isoform) and Lys37, a well conserved, positively charged amino acid on the toxin (Benzinger *et al.*, 1998). In addition to this specific electrostatic interaction between toxin and channel, and interaction (s) involving the hydrophobic residues in the flexible loop of the toxin with, as yet, unidentified

channel binding determinants, there is a tryptophan near the important Lys-37 (Trp-33) that also makes a structural contribution to toxin action (Dias-Kadambi *et al.*, 1996a).

Complementing the slow on-rates, off-rates are also exceedingly slow and state dependent, and determine, for the most part, differences in affinity between Na channel isoforms. For Ap-B and cardiac Na channels k_{off}, for example, from the closed state is only 0.0001 s-1, which means even after an hour an appreciable amount of toxin remains bound to the channel (Khera *et al.*, 1995). On the other hand, depolarization greatly augments off-rate and toxin can be washed out in 8–10 minutes (Hanck & Sheets, 1995). Similar disparities in off-rate between negative and positive potentials is shared among all site-3 toxins. Even when overall affinity is in the 100 to 1000 nM range, the action of the toxin is not reversed even after many minutes following removal of toxin from the bath when the cell is held at negative holding potentials (Wang & Strichartz, 1985). In myelinated nerve when the membrane was depolarized the off-rate was so fast that it could be appreciated in the time course of the current (Wang & Strichartz, 1985; Strichartz & Wang, 1986). However, all forms of these toxins share a high affinity for the closed state of the channel.

Together available data indicate that site-3 toxins bind with highest affinity when the channel is closed. It is also clear that they bind to the domain IV S3–S4 linker best when the S4 voltage sensor is deployed towards the inside of the cell (closed state), and bind with the lowest affinity when S4 voltage sensors are deployed towards the outside (inactivated state). This suggests that the S3–S4 linker is located at the extracellular surface independent of the state of the channel. This seems at odds with the suggestion from the crystal structure of bacterial potassium channels in which the S4 is buried in the bilayer when the channel is closed (Lee *et al.*, 2005). The original structure is now recognized to be non-native, but there is considerable evidence that for bacterial channels the voltage sensor is buried in the bilayer when the channel is closed. It might be the case that the toxin partitions into the bilayer to reach its binding site, although this seems unlikely since these toxins are highly hydrophilic, and they do not bind to phospholipids. This is in contrast to site 4 toxins, which are known to bind to a distinct site, and to affect activation (Smith *et al.*, 2005).

Modification of Na Channel Ionic Current by Site-3 Toxins

The mechanism of action of site 3 toxins has been most extensively studied in cardiac Na channels with anemone toxins, the channels with the highest affinities for toxin. As a consequently, low concentrations of the toxins could be used to modify nearly all Na channels, and the channels remained modified for long intervals even after toxin is removed from the bath solution. Consistent with the observation of long lasting sodium currents in whole cell voltage clamp recordings that suggested these toxins inhibited fast inactivation, single channel studies of ATX II toxin-modified rabbit ventricular Na channels found that mean channel open-time durations were monotonically prolonged as test potentials became more positive with toxin-modified open-times prolonged by a factor of 3.5 at −20 mV compared to control and by 4.5 times at −10 mV (Schreibmayer *et al.*, 1987a; El-Sherif *et al.*, 1992).

Fast inactivation is known, however, to occur for Na channels both from the open state and from closed, non-conductive state(s) (i.e. closed-state inactivation) (Bean, 1981; Lawrence *et al.*, 1991). Ap-A does not slow or inhibit closed-state inactivation of cardiac Na channels. In canine cardiac Purkinje cells, closed-state inactivation remained intact after exposure to Ap-A toxin at conditioning potentials from −80 mV to −120 mV, membrane potentials that are negative to Na current threshold (see Figure 2) (Hanck & Sheets, 1995), even though at positive potentials Ap-A modified Na channels behave as though they are driven into an open state from which inactivation becomes much less likely, at least for short durations of time (Hanck & Sheets, 1995). Figure 3 illustrates the difference in the amount of inactivation developed

during conditioning potentials between −20 mV and +60 mV for durations up to 24 ms. In contrast to unmodified Na channels, as the conditioning potential became more positive, the fraction of inactivated Na channel became less in Ap-A toxin. This relationship is best illustrated in Figure 3C which shows an inverse relationship between the fraction of Na channels undergoing inactivation at conditioning potentials from −50 mV to 100 mV in control solution compared to those in Ap-A toxin.

Conversely, recovery from inactivation is augmented by site-3 toxins. This was established in single channel studies comparing the action of site-3 toxins in preparations expressing cardiac and neuronal channels (Benzinger *et al.*, 1999). This action can be appreciated in Figure 4, where the shorter recovery time causes the development of inactivation to reach steady state more quickly at negative potentials. In summary the fast inactivation state is destablized by both slowing the forward rate constant into it and by becoming non-absorbing, i.e. channels can now recover. In contrast to these effects on fast inactivation, activation parameters remain unaffected. Deactivation, i.e. open to the closed, rested state, remained unchanged (Hanck & Sheets, 1995) and channel mean open times at negative potentials were like those of modified channels (El-Sherif *et al.*, 1992). Consequently, site-3 toxins appear to target a single kinetic transition, the open-to-inactivated state transition, without an apparent change in channel activation, deactivation or closed-state inactivation.

Modification of Na Channel Gating Current by Site-3 Toxins

Further information about the action of site-3 toxins has been gained from gating current (I_g) measurements, the small electrical currents arising from the movement of the voltage sensors formed by the S4 segments in each of the four domains of the α-subunit (Bezanilla, 2000). One of the first studies (Neumcke *et al.*, 1985) to directly investigate the molecular action of site-3 toxins on voltage-gated Na channels using this technique studied the effects of ATX-II toxin on gating currents in myelinated nerve. They found that ATX-II reduced the maximum gating charge (Q_{max}) and shortened the time constant of gating current (I_g) decay in response to step depolarizations. Later, a more detailed analysis of the effects of Ap-A toxin on gating currents found that Q_{max} was reduced by about 33% and resulted from the inhibition of the gating charge associated with the open-to-inactivated state transition (Sheets & Hanck, 1995). Because site-3 toxins only inhibit the open-to-inactivated state transition, subtraction of the gating currents of Ap-A modified Na channels from those in control recorded during step depolarizations represented the gating charge resulting from channels undergoing inactivation from the open state, a transition that was found to have a voltage-dependence (electronic charge) of 0.75 e^-. Assuming symmetrical energy barriers the total gating charge associated with both forward and backward open-to-inactivated state transitions was estimated to 1.5 e^-. An unexpected finding from studies with site-3 toxins (Sheets & Hanck, 1995) resulted from the observation that only two-thirds of the gating charge of voltage-gated Na channels moved before the Na channel opened. This is in sharp contrast to voltage-gated K channels, which are formed from four subunits in which all gating charge moves prior to channel opening (Bezanilla, 2000).

Further gating current experiments, in which the three outermost arginine residues in the S4-DIV were neutralized (one by one) to cysteine residues demonstrated that the reduction in gating charge by site-3 toxins resulted specifically from inhibition of the S4 segment in domain IV (Sheets *et al.*, 1999). The substitution experiments were based upon the concept that if the movement of the basic residues in DIV-S4 were to account for most or all of the reduction in gating charge by Ap-A toxin, then site-3 toxin modification of mutant Na channels that had individual basic residues previously neutralized should demonstrate a smaller reduction in Q_{max} than the nearly 33% found for wild-type Na channels. The studies confirmed that expectation, and additionally they found that the three outermost arginine residues did not contribute an equal amount to the gating charge in the S4-DIV but that the outermost arginine

was responsible for nearly two-thirds of all the gating charge contributed by the S4-DIV (Sheets *et al.*, 1999) and predicted total channel charge to be 5–6 e^-.

Although the above studies involved site-3 toxin interactions with the cardiac Na channel, the findings appear to be applicable to the general class of voltage-gated Na channels. Detailed studies of the effects of site-3 toxins on the gating currents of the human skeletal Na channel demonstrated that Ap-B toxin also reduced Q_{max} by about 33% in that isoform with only minor changes in the half-points and slope factors of the Q-V relationship (Sheets & Hanck, 1999). These findings were nearly identical to those for the cardiac channel. This was somewhat surprising because the channel kinetics of skeletal muscle Na channels are more rapid than those of cardiac Na channels (Chahine *et al.*, 1996; Wang *et al.*, 1996), and it had been suggested that the maximal number of electronic charges (e^-) associated with skeletal muscle channel may be greater than that for cardiac channel (Hirschberg *et al.*, 1995). Recently, a report on the effects of the site-3 α-scorpion toxin, Ts3, using site-specific fluorescence labeling of individual S4 segments in skeletal muscle Na channels has confirmed that site-3 toxins inhibit the movement of the S4-DIV by stabilizing the voltage sensor in its deactivated position accompanied by a slight shift of the voltage-dependent movement of S4-DI to slightly more positive potentials (Campos *et al.*, 2006). Thus the action of site-3 toxins indicates their primary action is to inhibit the outward movement of the S4 segment in domain IV. All experiments with site-3 toxins support the idea that the S4 of domain IV has a unique role in the coupling of Na channel activation with inactivation (Chahine *et al.*, 1994; Yang & Horn, 1995; Chen *et al.*, 1996; Yang *et al.*, 1996; Kontis *et al.*, 1997; Kuhn & Greeff, 1999). The issue of the number of charges associated with channel gating, i.e. whether Na channels gate with > 10 e^- as do K channels or whether they are overall less voltage dependent remains to be settled.

Although the movement of the S4 in domain IV is slower than other three S4 segments, after it has translocated Na channels inactivate quite quickly, i.e. within a few hundred μs (Sheets & Hanck, 1995). Attainment of the open state in wild-type Na channels would be expected to occur after movement of the three S4 segments in domains I, II and III but before full movement of the S4-DIV. Such a configuration for the four S4 segments in normal Na channels would be transient, and it would represent the configuration of the open channel that typically lasts no more than the few ms (Aldrich *et al.*, 1983; Kunze *et al.*, 1985; Grant & Starmer, 1987). However, after modification of the Na channel by site-3 toxins the Na channel would be able to occupy the open state for a prolonged time period thereby permitting other kinetic transitions to become apparent. During short, strong depolarizations toxin-modified Na channels remain principally in the open state, or burst, i.e. transitioning between the open and ultimate closed state (Sheets & Hanck, 1995). During long, sustained strong depolarizations toxin-modified Na channels have been shown to inactivate into a non-absorbing inactivated state in contrast to the absorbing inactivated state in control Na channels (Benzinger *et al.*, 1999). As a consequence, the toxin-modified Na channels display persistent I_{Na} resulting from channels cycling between the toxin-induced destabilized inactivated state, the open state, and closed states. Not surprisingly, the magnitude of the persistent I_{Na} varies between different Na channel isoforms (Figure 4) with a smaller magnitude arising from cardiac Na channels while a larger magnitude occurs in neuronal Na channels (Benzinger *et al.*, 1999)

The action of site-3 toxins to inhibit the outward movement of the S4 of domain IV would also be expected to manifest effects in the recovery from inactivation. As discussed above the recovery of ionic current is faster after toxin modification. It has long been appreciated that some of the gating charge that moved outward during depolarization returns slowly after hyperpolarization. The slow time course of return of charge, which reprises recovery of the ionic current, has been termed charge immobilization (Armstrong & Bezanilla, 1977; Bezanilla & Armstrong, 1977), and it has been shown to originate from the slow return of charge of the S4's in domains III and IV but not those from domains I and II (Cha *et al.*, 1999). When fast

inactivation has been removed either by internal perfusion with proteolytic enzymes or by specific mutagenesis of the putative inactivation particle formed by the intracellular linker between domains III and IV (for example, by the substitution of a cysteine for the phenylalanine in the IFM motif with modification by intracellular MTSET) charge immobilization is either completely eliminated (Armstrong & Bezanilla, 1977) or reduced (Sheets *et al.*, 2000). One might expect if site-3 toxins inhibit the outward movement of gating charge arising from domain IV, and that if a fraction of gating charge normally returns slowly reflecting the recovery of inactivated channels, then toxin modified channels should affect charge immobilization. In order to investigate whether charge immobilization could still occur in site-3 toxin modified Na channels where movement of the S4-DIV was prevented, we recorded gating currents from canine cardiac Purkinje cells before and after modification by Ap-A toxin. As discussed above, Na channels modified by Ap-A toxin are still able to undergo closed-state inactivation, therefore the membrane potential was first stepped to a conditioning potential of −70 mV for 500 ms to inactivate toxin-modified Na channels via closed-state inactivation prior to stepping the membrane potential to a second conditioning potential of 40 mV for 20 ms. The membrane potential was then repolarized to −190 mV, a potential where the fast and slow components of the time course of the recovery of gating charge can be readily differentiated in unmodified Na channels. Figure 5 shows the voltage-clamp protocol and the gating current integrals during repolarization for Na channels before and after modification by Ap-A toxin. Also shown are the gating current integrals during a step depolarization to 40 mV directly from a holding potential of −150 mV. As expected, control Na channels that have been inactivated demonstrate both a fast and slow component in the time course of the gating charge during repolarization. Interestingly, the toxin-modified Na channels also demonstrate two components in the gating charge during repolarization consistent with charge immobilization. However, the total magnitude of the gating charge during repolarization of the toxin-modified Na channel was reduced because the toxin inhibited only the movement of the S4-DIV leaving the gating charge from the slow return of charge of S4-DIII to contribute to charge immobilization. These data also demonstrate that the movement of the S4-DIV during a depolarizing step is not required for the putative inactivation particle to bind to its receptor causing channel inactivation and immobilization of the S4-DIII, and they explain the molecular basis of the augmentation of the rate of channel recovery from inactivation of channels bound to site-3 toxins.

Because the domain IV voltage sensor does not need to move in order for the channel to open, it raises an interesting question about the relationship of voltage sensor movement to the pore. In the available crystal structures of voltage-gated K channels the activation gate appears to be an intracellular structure formed at the bundle crossing of the four M2 (or S6) α helices (Jiang *et al.*, 2002; for review see MacKinnon, 2003; Long *et al.*, 2005). Upon depolarization the movement of all four S4 segments leads to channel opening by causing the intracellular ends of the S6 segments to bend away from the pore at hinge points formed by Glycine residues within the α helices of the S6 segments (MacKinnon, 2003). In contrast, the voltage-gated Na channel opens after movement of the first three S4 segments and before little, if any, movement of the S4-DIV. Although the three dimensional structure of the Na channel remains unknown, the S6 in domain IV differs from the other three S6 segments because it contains a serine at what would be by homology modeling its putative hinge point (Lipkind & Fozzard, 2005) while the other S6 segments have typical glycine residues (Goldin, 1995). In the voltage-gated K channel, the movement of the S4 segment tugs on the S4–S5 linker allowing its S6 segment to bend at the glycine hinge point thereby causing the channel to open (Long *et al.*, 2005). It is tempting to suggest that the unique role of the S4-DIV in coupling activation to fast inactivation may depend upon the fact that the S6 in domain IV has a serine at in its putative hinge. The domain IV S6 may be already in an "open permissive" configuration such that movement of the domain IV voltage sensor produces a movement that promotes rapid binding of the putative inactivation particle (formed by the linker between domains III and IV) to its receptor rather than promoting channel opening.

Site-3 Toxins as Tools for Investigation into Drug-Modified Na Channels

Lastly, not only have site-3 toxins revealed important information about how voltage gated Na channels gate, but they have been used to help understand the action of other compounds, such as local anesthetic drugs, on the Na channel. It has long been proposed that local anesthetic drugs such as lidocaine block Na channel current by stabilization of the drug-bound channel in an inactivated state (Hille, 1977; Hondeghem & Katzung, 1977). If this were the case then it would be reasonable to conclude that the S4-DIV with its role in coupling channel activation to inactivation would be principally involved in the action of lidocaine block. Also supporting such an idea is the fact that the most important amino acid determining local anesthetic block is a Phe in domain IV, S6 (Ragsdale *et al.*, 1994). With respect to gating charge lidocaine (Hanck *et al.*, 2000) and other local anesthetic drugs (for example see Cahalan & Almers, 1979) cause a reduction in maximal gating charge (Q_{max}) by nearly 40%. To determine whether the reduction in Q_{max} by lidocaine was caused by stabilization of the S4-DIV, wild-type cardiac (Nav1.5) Na channels were expressed in HEK293 cells, and gating charge was measured in control, after exposure to lidocaine, and then after exposure to both lidocaine and Ap-A toxin. Consistent with previous studies lidocaine, by itself, decreased Q_{max} by approximately 37%, however in the presence of both lidocaine and Ap-A the decrease in Q_{max} was additive and was nearly 60% (Hanck *et al.*, 2000) (Figure 6). These findings suggested that local anesthetic drugs and site-3 toxins stabilize different voltage sensors. Subsequently, it was shown that local anesthetic drugs appear to completely stabilize the S4 in domain III (Sheets & Hanck, 2003) with only a partial stabilization of the S4-DIV accompanied by a leftward shift in its gating.

Conclusion

Site-3 toxins, studied using whole cell voltage clamp, single channel recordings, and gating currents, have revealed a specific interaction with the voltage sensor of domain IV, and they have revealed the special role this voltage sensor plays in inactivation from the open state for voltage dependent Na channels. Unlike K channels, in which all four voltage sensors contribute to activation, for Na channels voltage sensors have specialized movements. Not only has investigation of toxin action revealed fundamental insights into molecular movements associated with kinetic states, but they have allowed the dissection of the mechanism of action of local anesthetics, which form an important class of therepeutic agents for treating pain and reducing arrhythmias. They provide, therefore, an important class of natural products that can be used as structure function probes.

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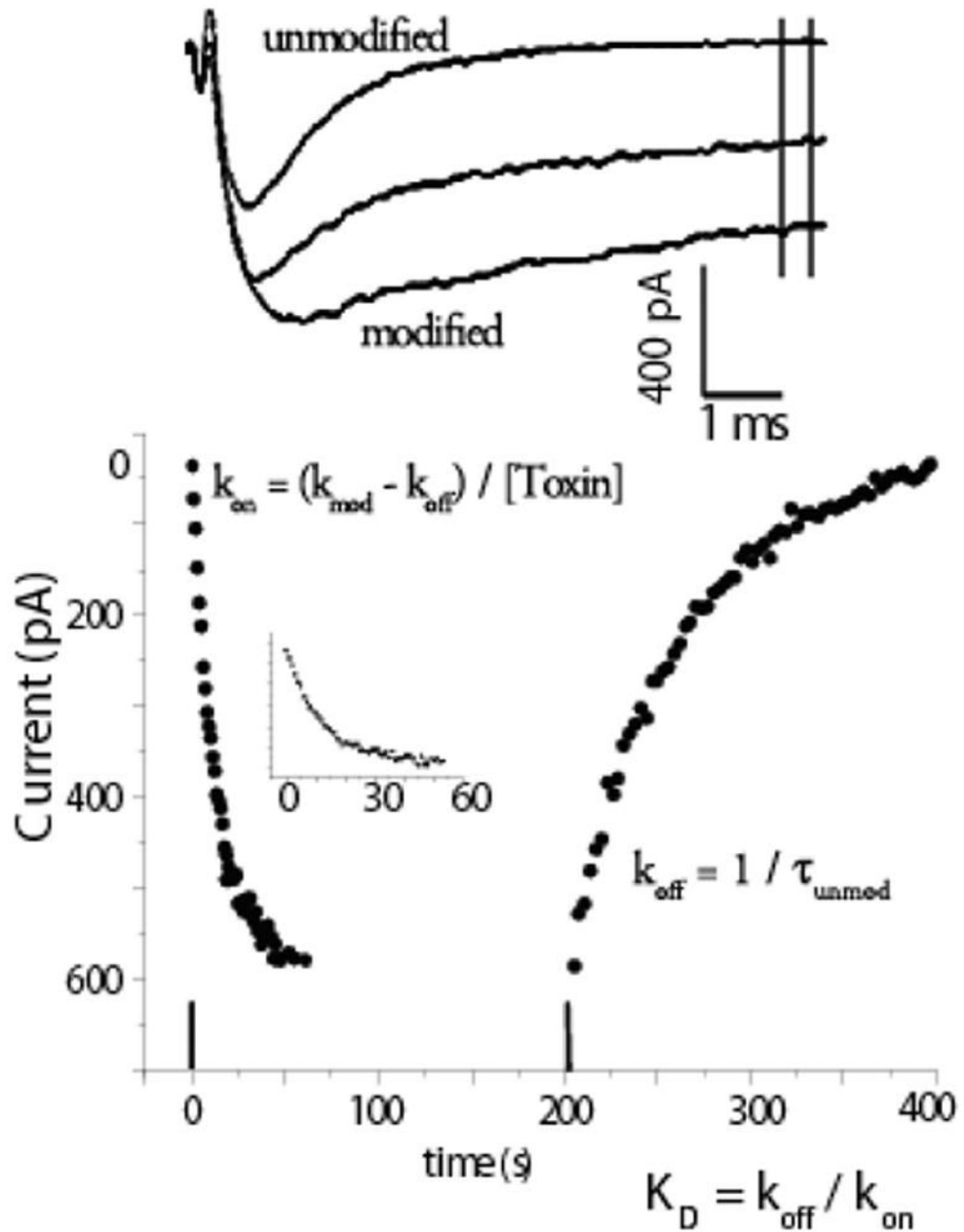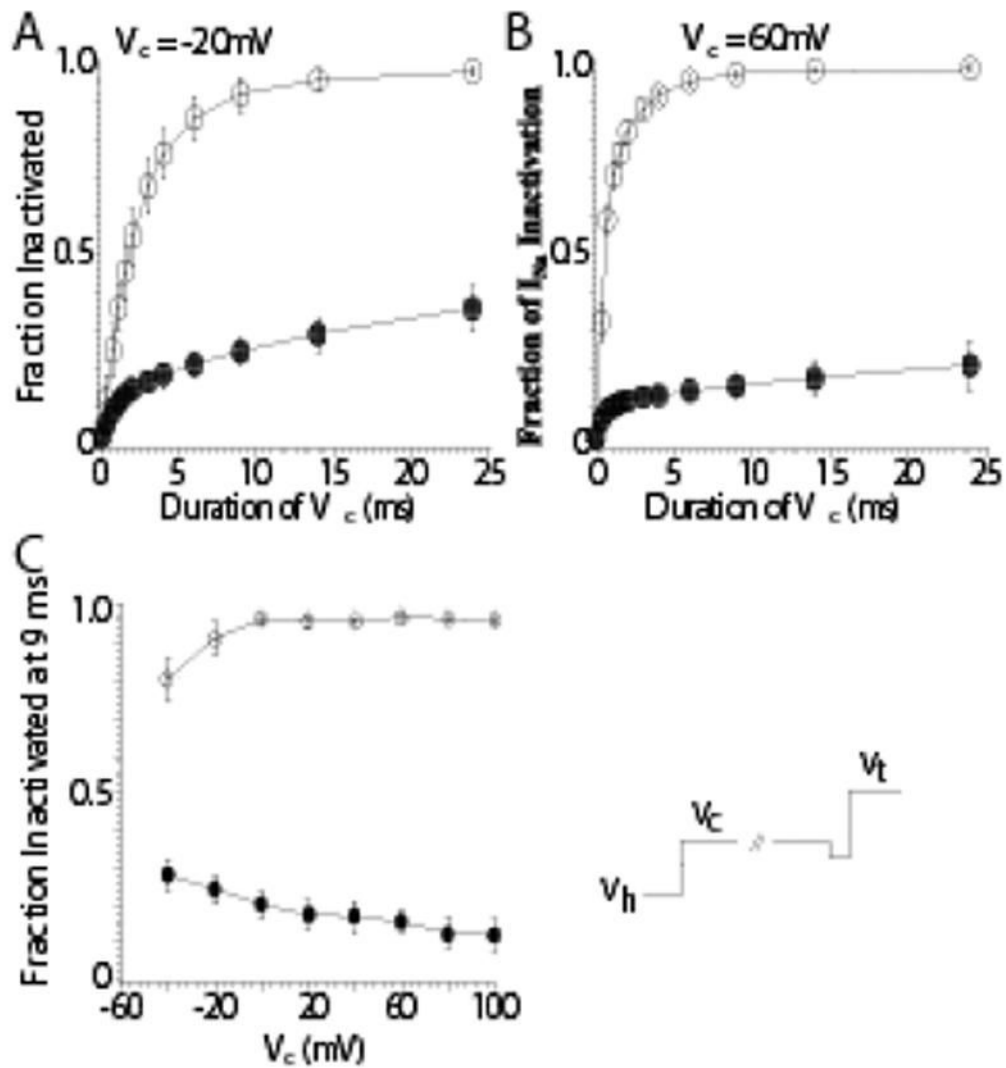
Figure 1.
Calculation of affinity from kinetics. The time course of modification and unmodification of I_{Na} by site 3 toxins occurs so slowly that rate constants can be calculated from currents measured from multiple depolarizations. The upper panel shows superimposed example time course of I_{Na} recorded in a RT4B cell, which expresses cardiac I_{Na} (Zeng *et al.*, 1996) at 0, 7.5, and 25 minutes after 40 nM of an Ap-B mutant toxin (R12S-K49Q) was removed from the bath. Currents were in response to trains of step depolarizations to −10 mV. The mean of the currents between the vertical lines was calculated and these currents are plotted in the panel below. Inset shows washin data with x-axis expanded. Data were fit with single exponentials, $Ae^{-t/\tau} + b$ or $A(1 - e^{-t/\tau}) + b$ as appropriate, and rate constants were calculated as indicated in figure. Modified from Khera *et al.*, 1995 with permission.
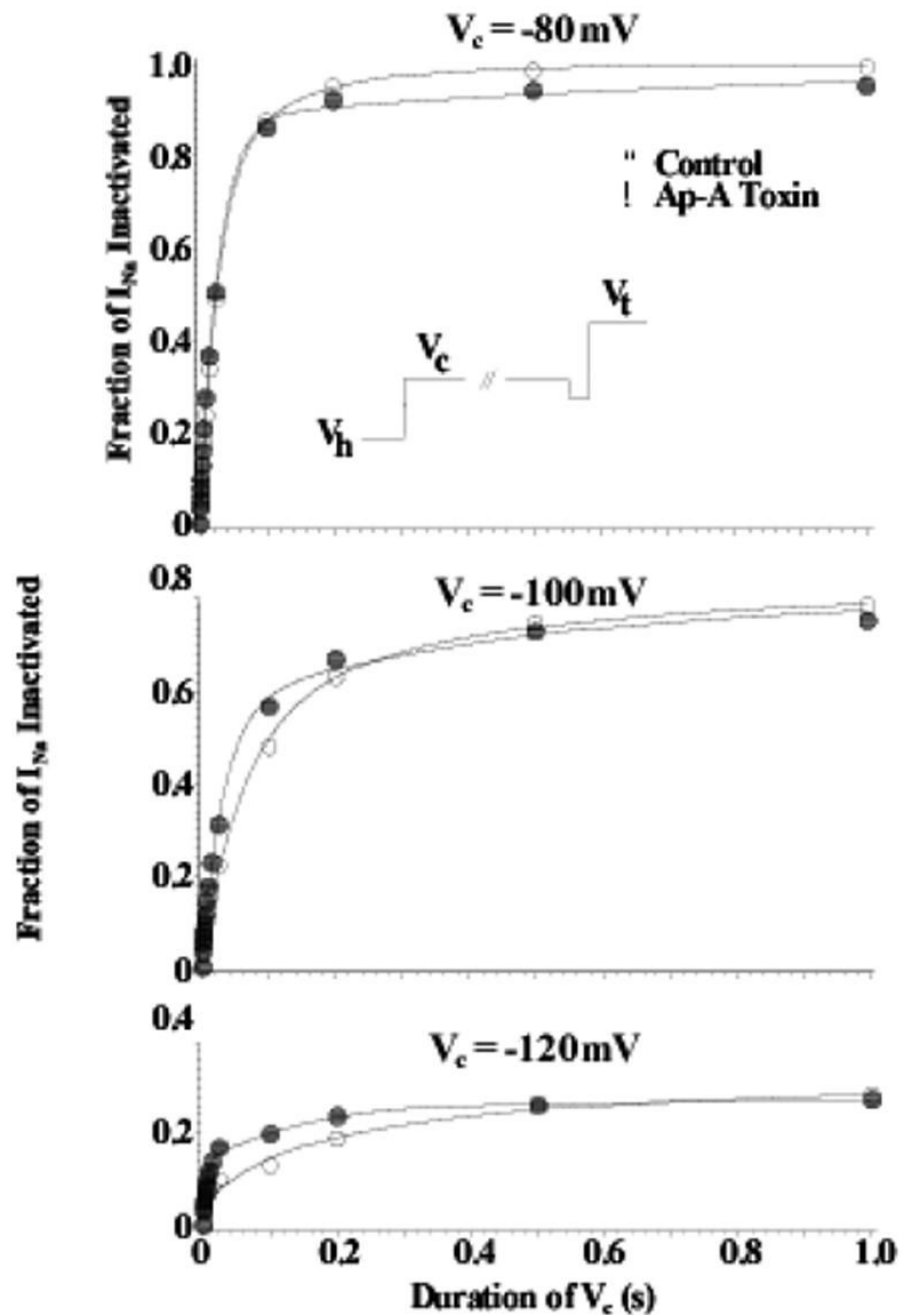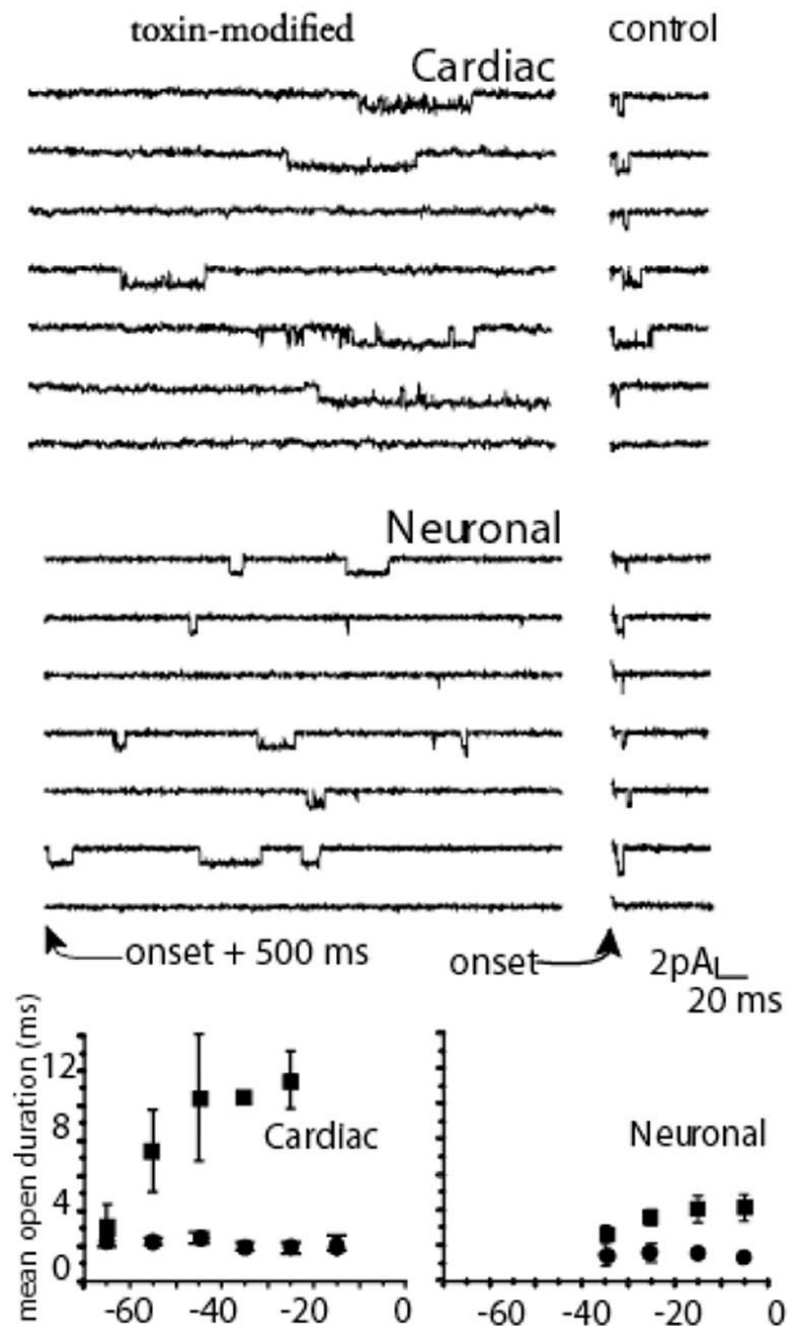
Figure 2.
Closed-State Inactivation Remains Intact after Ap-A Toxin Modification of Na Channels.
Shown are two-pulse development of inactivation relationships for Na channels in canine
cardiac Purkinje cells at conditioning potentials (V_c) of −120 mV (bottom), −100 mV (middle),
and −80 mV (top) in control (○) and after modification by Ap-A toxin (●). The voltage protocol
is shown in the inset where the membrane potential was clamped-back to −110 or −120 mV
for 2 ms before stepping to the test potential, V_t. Note that inactivation after Ap-A toxin occurs
at least as fast as that in control at conditioning potentials negative to channel threshold. From
Hanck and Sheets, 1995 with permission.

Figure 3.
Inhibition of Inactivation from the Open-State after Anthopleurin-A Modification. Shown are two-pulse development of inactivation protocols at more positive conditioning potentials in control (○) and after modification by Ap-A toxin (●;). The voltage protocol (bottom right) is similar to that shown in the previous figure. Prior to stepping to the conditioning potential there was a brief voltage step to −40 mV for 0.2 ms. Peak I_{Na} measurements were normalized to the peak I_{Na} measured in the absence of a conditioning step. Panel A: At a conditioning potential of −20 mV I_{Na} had inactivated by 92% within 9 ms in control solution, but only by 24% after Ap-A toxin. Panel B: At a conditioning potential of 60 mV, 95% of the peak I_{Na} had inactivated at 9 ms in control while only 16% had inactivated after Ap-A toxin. Panel C: The mean fraction of I_{Na} inactivated by 9 ms as a function of potential in control (○) and after Ap-A toxin (●). Note that inactivation became more complete at positive potentials in control solution, while after Ap-A toxin there was less inactivation at positive potentials compared to negative conditioning potentials. From Hanck and Sheets, 1995 with permission.
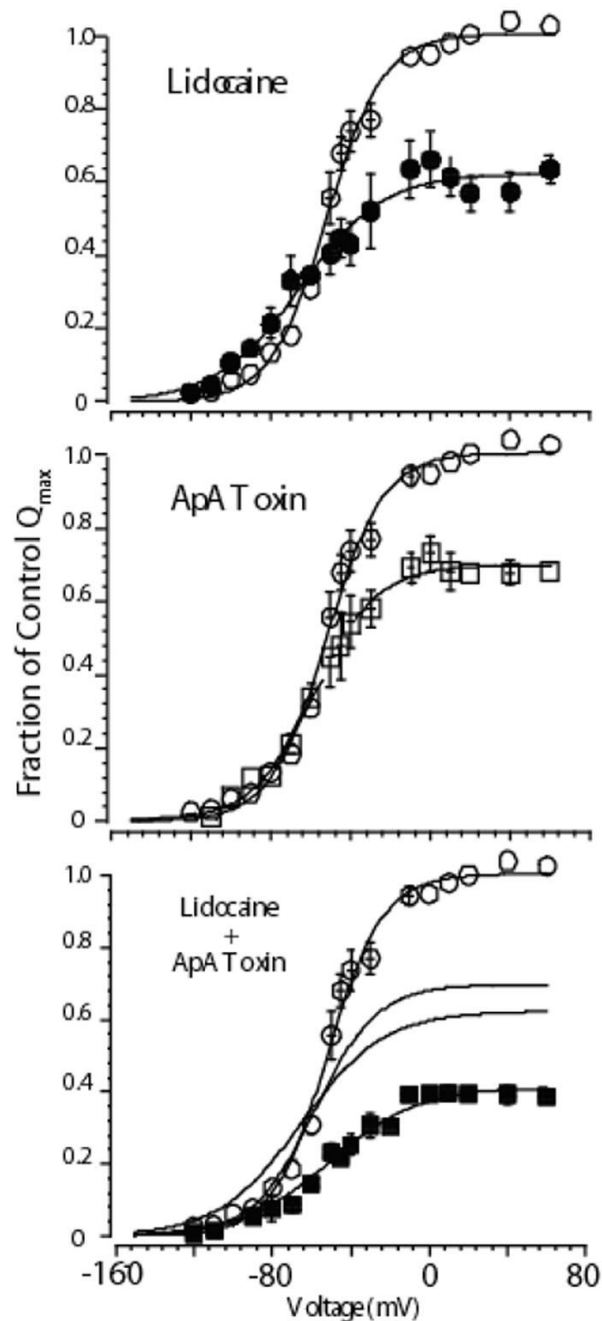
Figure 4.
Mean Channel Lifetime. Upper panels show cell attached single channel recordings with 280 mM Na at 6 C. Right panels shows control (measured immediately after the onset of a step depolarization to −10 mV) in cardiac and neuronal channels. Left panels show records with 1 μM Ap-B in the pipette in which recordings are shown beginning 500 ms after the depolarization. Bottom panels show mean open durations measured from amplitude histograms for multiple patches measured at the potentials indicated. Data analysis techniques are completely described in Benzinger *et al.,* 1999. Mean open time was prolonged by toxin in both isoforms, although more dramatically for cardiac than for neuronal channels.
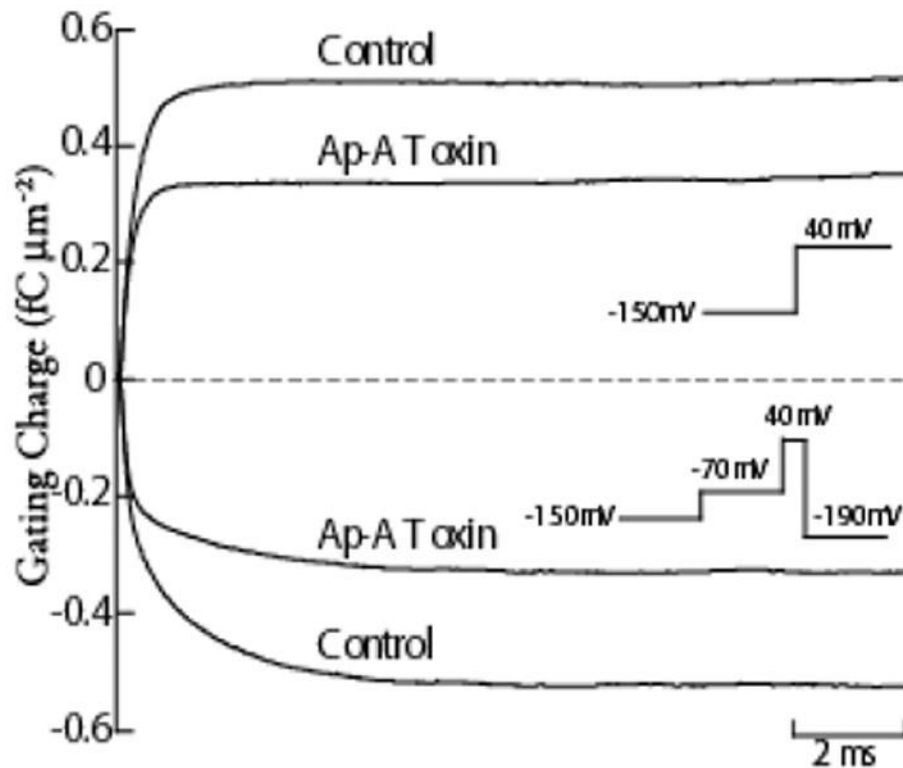
Figure 5.
Charge Immobilization in Ap-A Modified Na Channels. Gating current integrals in response
to a step depolarization to 40 mV from a holding potential of −150 mV are shown for a canine
cardiac Purkinje cell in control and after exposure to Ap-A toxin (top two traces), and gating
current integrals obtained during repolarization to -190 mV in the same cell (bottom two traces).
The voltage protocols are shown as insets, and are adjacent to their corresponding gating charge
traces. To inactivate both control and Ap-A toxin modified Na channels, the membrane
potential was first stepped to −70 mV for 500 ms to allow for closed-state inactivation to occur
before stepping to 40 mV for 20 ms. Note that the time course of the gating charge integral
during repolarization has two readily apparent time constants for both control Na channels and
for Ap-A toxin modified Na channels. In control, the magnitudes of the gating charge in both
the fast and slow time constants are nearly equal while the magnitude of gating charge contained
in the slow component of toxin-modified Na channels is only about one-half that in the fast
component. The total magnitude of the gating charge during repolarization to −190 mV in
control was 0.51 fC μm^{-2}, and it equaled the maximum gating charge obtained during the step
depolarization to 40 mV. After Na channel modification by 340 nM Ap-A toxin (a
concentration that modifies at least 90% of cardiac Na channels) the magnitude of the gating
charge during repolarization was reduced by nearly 35% to 0.33 fC μm^{-2}, and it also equaled
the gating charge at 40 mV.

Figure 6.
Mean charge voltage relationships for cells expressing cardiac Na channels exposed to high concentrations of either lidocaine alone (upper panel), Ap-A alone (middle panel), or both agents together (bottom panel). In each panel open circles show control data. Lidocaine modified data are shown as filled circles, Ap-A toxin modified data as open squares, and when both agents were present as filled squares. Solid lines are Boltzmann fits to the data. When both agents were present the effect on Q_{max} was additive, which can be appreciated from inspection of the fitted lines from each agent alone, which are also graphed in the lower panel. Interestingly, when both agents were present the Q-V relationship did not shift leftward as it did with lidocaine alone (see Hanck *et al.*, 2000). Subsequent experiments determined that the

gating charge responsible for the leftward shift in lidocaine is located in domain IV, the same gating charge that does not move when site-3 toxin is bound (Sheets & Hanck, 2003). Modified from Hanck *et al.*, 2000.