A Structural Rearrangement in the Sodium Channel Pore Linked to Slow Inactivation and Use Dependence

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Abstract Voltage-gated sodium (Na+) channels are a fundamental target for modulating excitability in neuronal and muscle cells. When depolarized, Na+ channels may gradually enter long-lived, slow-inactivated conformational states, causing a cumulative loss of function. Although the structural motifs that underlie transient, depolarization-induced Na+ channel conformational states are increasingly recognized, the conformational changes responsible for more sustained forms of inactivation are unresolved. Recent studies have shown that slow inactivation components exhibit a range of kinetic behavior (from tens of milliseconds to seconds) are modified by mutations in the outer pore P-segments. We examined the state-dependent accessibility of an engineered cysteine in the domain III, P-segment (F1236C; rat skeletal muscle) to methanethiosulfonate-ethylammonium (MTSEA) using whole-cell current recordings in HEK 293 cells. F1236C was reactive with MTSEA applied from outside, but not inside the cell, and modification was markedly increased by depolarization. Depolarized F1236C channels exhibited both intermediate (I1; τ ~ 30 ms) and slower (I2; τ ~ 2 s) kinetic components of slow inactivation. Trains of brief, 5-ms depolarizations, which did not induce slow inactivation, produced more rapid modification than did longer (100 ms or 6 s) pulse widths, suggesting both the I1 and I2 kinetic components inhibit depolarization-induced MTSEA accessibility of the cysteine side chain. Lidocaine inhibited the depolarization-dependent sulfhydryl modification induced by sustained (100 ms) depolarizations, but not by brief (5 ms) depolarizations. We conclude that competing forces influence the depolarization-dependent modification of the cysteine side chain: conformational changes associated with brief periods of depolarization enhance accessibility, whereas slow inactivation tends to inhibit the side chain accessibility. The findings suggest that slow Na+ channel inactivation and use-dependent lidocaine access is linked to a structural rearrangement in the outer pore.

Key words: local anesthetic • gating • cysteine mutagenesis • lidocaine • electrophysiology

Introduction

Local anesthetic compounds, such as lidocaine, suppress the ionic current through Na+ channels. By attenuating central and peripheral neuronal excitability, these compounds enjoy widespread use in the treatment of epilepsy and the relief of pain. In addition, local anesthetic compounds block Na+ channels in skeletal and cardiac muscle, and are used to treat neuromuscular diseases and cardiac arrhythmias. An essential characteristic of the local anesthetic action is use dependence, which is the sustained loss of excitability lasting many hundreds of milliseconds that is induced only when the drug-exposed channel is depolarized (Courtney, 1975). Although studies have identified amino acid residues in the cytoplasmic end of the pore that, when mutagenized, attenuate the action of local anesthetic compounds (Ragsdale et al., 1994; Nau et al., 1999), the structural basis of the sustained, depolarization-dependent loss of excitability associated with use dependence is not understood.

When briefly depolarized, Na+ channels inactivate rapidly within a few milliseconds (fast inactivation), a process mediated by residues situated near the cytoplasmic face of the channel (West et al., 1992; Smith and Goldin, 1997; Filatov et al., 1998). With prolonged depolarization, Na+ channels progressively occupy more stable slow-inactivated states (Rudy, 1978). These nonconductive states have diverse lifetimes ranging from tens of milliseconds (Kambouris et al., 1998; Bentiah et al., 1999) to many seconds (Cummins and Sigworth, 1996; Featherstone et al., 1996; Hayward et al., 1997), and are distinct from fast inactivation that typically recovers within 10 ms between stimuli. Importantly, slow inactivation influences cellular excitability, particularly in the pathophysiological conditions associated with prolonged membrane depolarization such as epilepsy (Feidervish et al., 1996), neuronal or cardiac ischemia (Shander et al., 1995), arrhythmias (Veldkamp et al., 2000), and neuromuscular diseases (Cannon, 1996). Amino acid residues from the P-segments (S5-S6 linkers), donated from each of the four homologous domains, line the extracellular aspect of the Na+ channel pore (see Fig. 1). Although the structural basis of slow inactivation gating is not defined, site-directed
slow inactivation, Na\textsuperscript{+} channels, and lidocaine

mutations and chimeric studies suggest a role for the P-segments (Balser et al., 1996a; Benitah et al., 1999; Todt et al., 1999; Vilin et al., 1999). In addition, P-segment mutations and extracellular cation substitutions that reduce slow inactivation (Townsend and Horn, 1997) seem to inhibit use-dependent lidocaine action (Zilberter et al., 1991; Kambouris et al., 1998; Chen et al., 2000), suggesting a potential mechanistic linkage between these phenomena.

Investigating the structural basis of slow inactivation is hampered by its electrical silence, and requires a means to detect relatively slow changes in the conformational architecture of a nonconducting channel. Cysteine substitution of a P-segment residue in domain III (see Fig. 1, F1236C) yields a Na\textsuperscript{+} channel reactive with the positively charged methanethiosulfonate (MTS)\textsuperscript{1} reagent MTS-ethylammonium (MTSEA) applied from outside the cell, yet the residue is entirely resistant to MTSEA applied from inside the cell (Yamagishi et al., 1997). Adjacent to the domain III lysine (K1237) that contributes to the Na\textsuperscript{+} channel selectivity filter (Heinemann et al., 1992), this cysteine cannot be modified by larger (MTS-ethyltrimethylammonium [MTSET]) or negatively charged (MTS-ethylsulfonate, MTSES) reagents (Yamagishi et al., 1997). We postulated that sulfhydryl modification of a residue harboring such restrictive access might be sensitive to the voltage-gated conformational state of the ion channel. The results show that modification of F1236C is greatly enhanced by brief depolarizations, but is inhibited by more sustained depolarizations that induce slow inactivation. Moreover, lidocaine inhibited depolarization-induced MTSEA modification during sustained, but not brief, depolarizations. We propose that Na\textsuperscript{+} channel slow inactivation and use-dependent lidocaine action are linked to a common structural rearrangement involving the outer pore.

MATERIALS AND METHODS

Molecular Biology and Heterologous Expression

For heterologous expression, the wild-type rat skeletal muscle Na\textsuperscript{+} channel (\(\mu_1\)) \(\alpha\) subunit was subcloned into the HindIII-XbaI site of the vector GFP-IRS for bicistronic expression of the channel protein and GFP reporter as previously described (Johns et al., 1997). The rat skeletal muscle mutant Na\textsuperscript{+} channel F1236C was prepared (Yamagishi et al., 1997) using a PCR-based method (QuickChange site-directed mutagenesis kit; Stratagene). In brief, sense and antisense oligonucleotides (30–34 mer) containing the desired mutation were used as primers for amplification with Pfu DNA polymerase. The PCR product was treated with DpnI to select for the mutant plasmid and transformed into Escherichia coli for subsequent isolation and purification. Wild-type and mutant Na\textsuperscript{+} channels \(\alpha\) subunits were transiently transfected into HEK 293 (human embryonic kidney cell line) cells using lipofectamine (GIBCO BRL), and were cultured in MEM medium supplemented with 10% fetal bovine serum and 1% pen-strep in a 5% CO\textsubscript{2} incubator at 37°C for 1–3 d. In all cases, the HEK cells were cotransfected with the Na\textsuperscript{+} channel \(\beta\) subunit (provided by Dr. Alfred George, Vanderbilt University). Cells exhibiting green fluorescence were chosen for electrophysiological analysis.

**Abbreviations used in this paper:** DTT, dithiothreitol; HEK, human embryonic kidney; I\textsubscript{Na}, Na\textsuperscript{+} channel; MTS, methanethiosulfonate; MTSEA, MTS-ethylammonium; MTSET, MTS-ethyltrimethylammonium.

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**Figure 1.** The Na\textsuperscript{+} channel P-segments influence slow inactivation. The predicted transmembrane topology of the Na\textsuperscript{+} channel \(\alpha\) subunit is shown, with a bold line in each domain indicating the P-segment region that lines the outer pore. The open circles at the base of each P-segment identify the DEKA ring residues that form a putative selectivity filter (Heinemann et al., 1992). Residues are numbered according to the rat skeletal muscle Na\textsuperscript{+} channel sequence (Trimmer et al., 1989). The domain III lysine (K1237) is known to influence slow inactivation gating (Benitah et al., 1999; Todt et al., 1999), and is adjacent to the F1236C residue (filled circle). In addition, substitution of the W402 tryptophan (filled square) in domain I alters slow inactivation (Balser et al., 1996a).
Whole-cell Na\(^+\) currents (I\(_{\text{Na}}\)) were recorded (Axopatch 200B; Axon Instruments) using electrodes with resistances of 1–3 M\(\Omega\) when filled with a pipet solution containing (in mM): 140 NaF, 10 NaCl, 5 EGTA, 10 HEPES, pH 7.40. Replacing the intracellular K\(^+\) with Na\(^+\) eliminated the time-dependent K\(^+\) currents in our HEK cell recordings. Experiments were conducted at room temperature. Current magnitudes were 1–2 nA, and 85% of the series resistance was compensated, yielding a maximum voltage error of \(\sim 1\) mV. The bath solution contained (in mM): 150 NaCl, 4.5 KCl, 1.5 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES (titrated to pH 7.4 with NaOH). MTSEA, MTSES, and MTSET (Toronto Research Chemicals) were kept at 4\(\degree\)C as high concentration stock solutions and were diluted to 25–100 \(\mu\)M in the appropriate bath solution immediately before use. The disulfide reducing agents dithiothreitol (DTT) and glutathione were dissolved directly in the extracellular solution at a concentration of 5 mM (titrated to pH 7.4 with NaOH). Lidocaine HCl (Sigma-Aldrich) or QX-314 (Almone Labs) were diluted from stock solutions to the bath concentrations indicated in the text.

Cells were dialyzed for a 15-min equilibration period before recording data. To avoid junction potentials with solution changes, a 3-M KCl agar bridge was used. Inactivation gating kinetics and use-dependent block were assessed using the voltage-clamp protocols described in the text and figure legends. Whole-cell currents were sampled at 20 kHz (DigiData 1200 A/D converter; Axon Instruments) and low pass–filtered at 5 kHz. The data were acquired and analyzed using pClamp8.0 software (Axon Instruments). The results are expressed as mean \(\pm\) SEM, and statistical comparisons were made using One-Way ANOVA (Microcal Origin) with \(P < 0.05\) indicating significance. Multiexponential functions were fitted to the data using nonlinear least-squares methods (Origin).

**RESULTS**

We first examined the accessibility of the F1236C (Fig. 1) cysteine side chain to sulfhydryl modification using 100 \(\mu\)M MTSEA. A 3-min exposure to MTSEA during hyperpolarization (\(-100\) mV; Fig. 2 A, protocol I) reduced F1236C peak I\(_{\text{Na}}\) by 31 \(\pm\) 3% (after MTSEA washout; summary data Fig. 2 B). This exceeded wild-type modification (13 \(\pm\) 3%; \(P < 0.05\); Fig. 2, A and B), but was less than that previously seen with F1236C using a much higher concentration of MTSEA (49 \(\pm\) 15%, 2.5 mM; Yamagishi et al., 1997). However, modification was substantially increased by clamping to \(-20\) mV during MTSEA exposure for F1236C (73 \(\pm\) 5%, \(P < 0.0001\); Fig. 2 A and B, protocol II) but not wild type (Fig. 2 B), suggesting depolarization increased the accessibility of the cysteine side chain. Identical experiments using the larger, positively charged reagent MTSET, or the negatively charged analogue MTSES, produced reactivity indistinguishable from the wild type, even with sustained (protocol II) depolarization (Fig. 2 B).

![Figure 2. MTSEA modification of F1236C exhibits voltage dependence.](image-url)
The additional covalent modification afforded by depolarization was partially reversed by the hydrophobic reducing agent DTT. Fig. 2 B indicates that after 3 min of depolarization-induced MTSEA modification, the fractional reduction in peak \( I_{\text{Na}} \) (relative to pre-MTSEA) after an additional 20 min of DTT exposure is reduced to 49 ± 5\% (\( P < 0.005 \) versus the 73\% pre-DTT value), indicating the \( I_{\text{Na}} \) reduction associated with MTSEA exposure results from formation of a reducible disulfide bond. The prolonged time (20 min) required for only partial DTT reversal suggests the reducing agent accesses the disulfide bond with some difficulty, perhaps through a hydrophobic pathway. We could not achieve reversal using glutathione, a larger, more hydrophilic, and generally less reactive reducing agent (Fig. 2 B). Considered together, the data (Fig. 2) suggest that although depolarization increases the accessibility of the F1236C side chain (allowing enhanced modification by MTSEA), the residue still lies at a relatively inaccessible position in the outer pore, limiting the effects of the larger sulphydryl modification compounds or reducing agents.

The rate of F1236C \( I_{\text{Na}} \) decay during the depolarizing pulse did not differ from wild type (Fig. 2 A), suggesting the mutant had no marked effect on the fast inactivation gating process. To examine the kinetics of slow inactivation, wild-type and F1236C peak \( I_{\text{Na}} \) were measured after prepulses of incremental duration (Fig. 3, inset, clamp protocol). Each pair of depolarizations was separated by a brief, 20-ms hyperpolarization that allowed channels to recover fully from fast, but not slow, inactivation. Wild-type and F1236C slow inactivation were described by biexponential functions with similar time constants (parameters given in legend), corresponding to intermediate (\( I_{\text{M}} \), \( \tau \sim 30 \) ms) and slower (\( I_{\text{S}} \), \( \tau \sim 2 \) s) kinetic components of slow inactivation described in previous studies of \( \mu \)L expressed in \textit{Xenopus} oocytes (Kambouris et al., 1998; Benitah et al., 1999).

However, the rate of slow inactivation increased in wild-type channels as the membrane potential increased from −20 to +20 mV (Fig. 3 A) because of an increase in the amplitude of the \( I_{\text{M}} \) component (from 0.2 to 8\%) and a shorter \( I_{\text{S}} \) time constant (\( \tau_{\text{S}} \), from 2,519 to 1,255 ms; see Fig. 3 legend). Conversely, the rate of development of slow inactivation in F1236C was similar at −20 mV and +20 mV (Fig. 3 B; amplitude of the \( I_{\text{M}} \) component was ∼10\%). Hence, consistent with previous evidence that P-segment substitutions alter slow inactivation gating processes in Na\(^{+} \) channels (Balser et al., 1996a; Todt et al., 1999), the F1236C mutation did modify (increase) the propensity of the Na\(^{+} \) channels to slow inactivate.

A meaningful analysis of the depolarization-dependent rate of MTSEA modification of the F1236C side chain must recognize the distinctive kinetic features of slow inactivation, including the biexponential characteristics noted in Fig. 3. Therefore, we used parameters derived from the kinetic analysis of F1236C slow inactivation (−20 mV; Fig. 3 legend) to select depolarization pulse widths that would maximize the intermediate and slow kinetic components, \( I_{\text{M}} \) (100 ms; ∼3 × \( \tau_{\text{M}} \)) and \( I_{\text{S}} \) (6 s; ∼3 × \( \tau_{\text{S}} \)). In addition, a brief, 5-ms pulse width was used to examine depolarization-induced MTSEA modification in the complete absence of slow inactiva-
Notably, while the pulse widths are selected to bias the channel toward distinct kinetic components of slow inactivation, caution is needed when relating these components to occupancy of individual gated states. The inactivated state dwell-times depend on many factors, including the detailed connectivity of the kinetic states involved.

Fig. 4 (A and B) shows the time-dependent reduction of \( I_{\text{Na}} \) due to MTSEA modification during trains of either 5- or 100-ms pulses (-20 mV). In all experiments, MTSEA was allowed to equilibrate in the bath for at least 1 min before application of the first depolarization pulse. For these studies, the MTSEA was lowered to 25 \( \mu \text{M} \) (vs. 100 \( \mu \text{M} \) in Fig. 2) to minimize tonic, depolarization-independent modification; as such, the reduction in \( I_{\text{Na}} \) during the first pulse after MTSEA addition was consistently \( \leq 5\% \) (Fig. 4, A and B), compared with 31\% at the higher concentration (Fig. 2 B). In Fig. 4, currents are plotted from every hundredth pulse (A) or every fifth pulse (B), and the currents shown in the two panels are aligned to reflect matching cumulative depolarization time.

A comparison of A and B in Fig. 4 reveals that extending the depolarization duration from 5 to 100 ms markedly reduced the rate of modification. Fig. 4 C plots the time-dependent reduction in \( I_{\text{Na}} \), as a function of cumulative depolarization time using pulse widths (at -20 mV) of 5 ms (n = 7), 100 ms (n = 6), and 6 s (n = 4). All current amplitudes are normalized to that of the first depolarization recorded in MTSEA, and are plotted as the fractional reduction in peak \( I_{\text{Na}} \) (1 - \( I/I_{\text{max}} \)). The solid lines are the best fit of a single exponential function (\( \text{Ae}^{{-\text{rt}}}/\text{t} \)) to the data (every tenth data point plotted for 100-ms pulses, every two hundredth data point plotted for 5-ms pulses). (D) The rate of MTSEA modification, calculated at -20 mV and +20 mV, assuming an irreversible bimolecular reaction: \( k_{\text{on}} = 1/\{\tau \times [\text{MTSEA}]\} \), where \( \tau \) is the exponential fitted as shown in C. At -20 mV (\( \times 10^3 \text{M}^{-1}\text{s}^{-1} \)) for 5 ms, \( k_{\text{on}} = 9.6 \pm 1.4 \); for 100 ms, \( k_{\text{on}} = 3.1 \pm 1.0 \) (\( P = 0.004 \) vs. 5 ms); for 6 s, \( k_{\text{on}} = 0.5 \pm 0.18 \) (\( P = 0.001 \) vs. 5 ms, but not significantly different from 100 ms). At +20 mV: for 5 ms (n = 10), \( k_{\text{on}} = 10.7 \pm 1.7 \); and for 100 ms (n = 5), \( k_{\text{on}} = 3.5 \pm 1.8 \) (\( P < 0.005 \) vs. 5 ms).
spend a much higher percentage of their total depolarized time in the I\textsubscript{k} kinetic component. Nonetheless, the rate of depolarization-dependent MTSEA modification is still reduced compared with the brief, 5-s depolarizations (Fig. 4 C), indicating that pulses recruiting I\textsubscript{k} (like I\textsubscript{Na}) reduce MTSEA accessibility of the cysteine side chain. The data in Fig. 4 C were fitted to an exponential function (solid line) to determine reaction rates (k\textsubscript{on}; Fig. 4 D, see legend) for each depolarization pulse width; increasing the pulse width to either 100 ms or 6 s significantly reduced k\textsubscript{on}. Fig. 4 D also indicates that the effects of pulse width (5 and 100 ms) were insensitive to changing the depolarization voltage from −20 to +20 mV, which is consistent with the similar rate of slow inactivation for F1236C (in contrast to wild type) at these two membrane potentials (Fig. 3 B).

The rate of MTSEA modification changes with the depolarization pulse width; the extent of modification also appears to change. Incomplete elimination of the current could partly result from residual current flow through MTSEA-modified channels. Even under conditions where slow inactivation is prevented (i.e., brief 5-ms pulses), the rate of depolarization-dependent modification is still 100-fold slower than in previous studies examining MTs modification of freely accessible cysteinyl groups (i.e., III–IV linker 1304C; Kellenberger et al., 1996; Vedantham and Cannon, 1998), which is consistent with restricted access of MTSEA to the 1236C intrapore cysteinyl. Given the even slower rate of MTSEA modification at the longer pulse widths, we may speculate that a slow, competing side reaction may gradually oxidize the 1236 cysteine (while the channel remains Na\textsuperscript{+} permeable), as proposed in previous studies of intrapore cysteine disulfide formation (Benitah et al., 1997). Moreover, the modification rate, with long pulses at a low MTSEA concentration (25 \textmu M, Fig. 4 C), is quite slow (relative to the practical limitations on experimental time), making it difficult to determine precisely when modification saturates. Hence, the modification rates for 100-ms and 6 s pulses may be even slower than estimated from the fitting procedure.

We next considered whether accessibility of F1236C to sulfhydryl modification was modified by use-dependent lidocaine block. Since the F1236C mutation alone modifies slow inactivation under control conditions (Fig. 3), we first established whether lidocaine would induce significant depolarization-dependent I\textsubscript{Na} suppression in this mutant. Bath exposure to 100 \mu M lidocaine (Fig. 5 A) reduced I\textsubscript{Na} availability mainly by increasing the amplitude of an intermediate (τ = 68 ms) kinetic component (see Fig. 5 legend, lidocaine increased A\textsubscript{I} from 0.08 to 0.65). Hence, the mutant channel retains use-dependent lidocaine sensitivity typical for skeletal muscle Na\textsuperscript{+} channels expressed in HEK cells (Wang et al., 1996).
To determine whether the effects of lidocaine on use-dependent loss of \( I_{Na} \) availability could be linked to a structural change in the outer pore, we examined voltage-dependent MTSEA modification during lidocaine exposure. Fig. 5 B shows the rate of MTSEA modification induced by 100-ms depolarizing pulses to \(-20 \text{ mV}\) during MTSEA exposure, alone and with lidocaine superfusion. For these experiments, we raised the MTSEA concentration from 25 to 50 \( \mu \text{M} \) to allow significant modification of the cysteinyl side chain using a 100-ms pulse width in lidocaine-free conditions (note, for comparison, only minimal modification using a 100-ms pulse width in Fig. 4 C). Notably, in lidocaine-free solutions, the rate of MTSEA modification \( (k_{on}, 10^3 \text{ M}^{-1}\text{s}^{-1}) \) using 100-ms pulses (Fig. 5 B, 4.4 ± 0.6) was slower than with 5-ms pulses (Fig. 5 C, 9.6 ± 1.4, \( P = 0.004 \) vs. 100-ms pulses) even though the MTSEA concentration was raised in Fig. 5 B. This finding supports the results of Fig. 4, indicating a slower rate of depolarization-dependent modification when the pulse duration is lengthened. The data also reveal a marked reduction in MTSEA modification with lidocaine exposure; in Fig. 5 B (100-ms pulse width), \( k_{on} \) in lidocaine was as decreased to 0.7 ± 0.3 (\( P = 0.0002 \)). In contrast, lidocaine exposure had no effect on the rate of MTSEA modification when a brief pulse width was used (Fig. 5 C, 5 ms). Hence, the protection from sulfhydryl modification afforded by lidocaine is use-dependent and requires a sustained depolarization. This pulse width sensitivity suggests the observed lidocaine effect on MTSEA modification was not a nonspecific, gating-unrelated antagonism between the local anesthetic the MTS reagent.

Based on evidence that enzymes (Cahalan, 1978; Yeh, 1978) and mutations (Bennett et al., 1995; Balser et al., 1996b) that remove fast inactivation also attenuate use-dependent suppression of \( I_{Na} \) by lidocaine and other \( Na^+ \) channel blockers, a high affinity interaction between lidocaine and the fast-inactivated state has been a candidate structural motif for the sustained inactivation induced by these compounds. However, a recent study suggests that amino acid residues critically involved in fast inactivation (West et al., 1992) are not trapped in the fast-inactivated conformational state by lidocaine (Vedantham and Cannon, 1999), raising the possibility that this conformational state may not form a high affinity drug receptor as previously proposed. P-segment mutations that reduce an intermediate kinetic component of slow inactivation (termed \( I_M \)) also attenuate use-dependent lidocaine block (Kambouris et al., 1998), as do cation substitutions that inhibit this component of slow inactivation (Zilberter et al., 1991; Chen et al., 2000). Although these and earlier studies (Khodorov et al., 1976) postulated a linkage between slow inactivation gating and a use-dependent local anesthetic block, unambiguous interpretation of these data has not been possible because the interventions themselves (mutations and cation substitutions) could alter drug binding through gating-independent mechanisms (Hille, 1992). Here, we show that slow inactivation alters the accessibility of an outer pore cysteine to sulfhydryl modification (Figs. 3 and 4). Moreover, lidocaine attenuates MTSEA modification of the same cysteine when exposed to sustained (100 ms) depolarizations (Fig. 5 B), but not brief, 5-ms depolarizations (Fig. 5 C), suggesting the

**DISCUSSION**

Based on evidence that enzymes (Cahalan, 1978; Yeh, 1978) and mutations (Bennett et al., 1995; Balser et al., 1996b) that remove fast inactivation also attenuate use-dependent suppression of \( I_{Na} \) by lidocaine and other \( Na^+ \) channel blockers, a high affinity interaction between lidocaine and the fast-inactivated state has been a candidate structural motif for the sustained inactivation induced by these compounds. However, a recent study suggests that amino acid residues critically involved in fast inactivation (West et al., 1992) are not trapped in the fast-inactivated conformational state by lidocaine (Vedantham and Cannon, 1999), raising the possibility that this conformational state may not form a high affinity drug receptor as previously proposed. P-segment mutations that reduce an intermediate kinetic component of slow inactivation (termed \( I_M \)) also attenuate use-dependent lidocaine block (Kambouris et al., 1998), as do cation substitutions that inhibit this component of slow inactivation (Zilberter et al., 1991; Chen et al., 2000). Although these and earlier studies (Khodorov et al., 1976) postulated a linkage between slow inactivation gating and a use-dependent local anesthetic block, unambiguous interpretation of these data has not been possible because the interventions themselves (mutations and cation substitutions) could alter drug binding through gating-independent mechanisms (Hille, 1992). Here, we show that slow inactivation alters the accessibility of an outer pore cysteine to sulfhydryl modification (Figs. 3 and 4). Moreover, lidocaine attenuates MTSEA modification of the same cysteine when exposed to sustained (100 ms) depolarizations (Fig. 5 B), but not brief, 5-ms depolarizations (Fig. 5 C), suggesting the

![Figure 6. Hypothesis for P-segment rearrangements during slow inactivation and lidocaine block](image-url)

the rate of sulfhydryl modification is also reduced (\( k_b < k_a \)). Lidocaine is bound to site(s) in the aqueous pore on the cytoplasmic side of the selectivity filter (Ragsdale et al., 1994, 1996), but in very close proximity to the selectivity filter P-segment residues (Sunami et al., 1997). The model suggests that the P-segment structural rearrangement associated with slow inactivation may move the deepest P-segment domains into positions that stabilize lidocaine binding (right).
lidocaine effect to suppress MTSEA modification is specifically linked to gated state(s) associated with sustained depolarization. The findings suggest that the use-dependent lidocaine block and slow inactivation share a common, outer pore structural rearrangement that reduces MTS accessibility.

These results do not resolve whether the impeded sulfhydryl modification rate during slow inactivation results from movement of the domain III P-segment to a less accessible position, or rather from movement of other pore structures into positions that somehow protect the F1236C side chain from MTSEA. Nonetheless, a conceptual model based upon recent studies of the Na\(^+\) channel pore demonstrating exceptional mobility of the P-segments (Benitah et al., 1997, 1999; Tsushima et al., 1997) is illustrated schematically in Fig. 6. Whereas the 1236 cysteine side chain is largely inaccessible in the hyperpolarized channel (Fig. 6, left), upon depolarization, we postulate that the P-segments assume positions that increase MTSEA accessibility of the cysteine residue. This would increase the rate of covalent modification (Fig. 6, middle, \(k_0\)) as we observed (Fig. 2). As the depolarization is prolonged, the accessibility of the 1236C residue is once again compromised (Fig. 6, right, \(k_0 < k_s\)), which is consistent with our results (Fig. 4). Hence, Na\(^+\) channel gating during sustained depolarization (i.e., slow inactivation) might involve a constriction of the outer pore, analogous to that envisioned for C-type inactivation of potassium channels (Baukrowitz and Yellen, 1996; Liu et al., 1996). Our results show that pulse widths sufficient to recruit either the intermediate (I\(_{iq}\)) or slower (I\(_s\)) inactivated state kinetic components (Fig. 3) are both effective in reducing depolarization-dependent modification (Fig. 4, C and D), which is consistent with studies linking the P-segments to slow-inactivated states spanning a wide kinetic range (Kambouris et al., 1997; Todt et al., 1999; Vilin et al., 1999). Nonetheless, the marked reduction in MTSEA modification at the 100-ms pulse width is substantial, given the relatively small amplitude of inactivation occurring with 100-ms pulses (Fig. 3 B). We speculate that a gating motion involving the pore may develop with intermediate kinetics closely related to I\(_s\) (recognizing 5-ms pulses do not inhibit modification, whereas 100-ms pulses do), which reduces 1236C accessibility but does not suppress Na\(^+\) permeation (or else, recovers more rapidly than 20 ms upon hyperpolarization and, therefore, is not visible in Fig. 3 B).

The 1236 cysteine side chain is accessible only to modification from outside the cell (Yamagishi et al., 1997), while lidocaine binds to a site in the aqueous pore on the cytoplasmic side (Ragsdale et al., 1994, 1996). Residues forming the P-segment selectivity filter (Fig. 1) of the skeletal muscle Na\(^+\) channel act as electrostatic barriers, preventing extracellular access and escape of the local anesthetic (Sunami et al., 1997). Although mutations in the Na\(^+\) channel domain IV-S6 segment may create an extracellular access pathway for charged lidocaine derivatives (Ragsdale et al., 1994; Qu et al., 1995), we find that the P-segment F1236C mutation does not make the skeletal muscle Na\(^+\) channel sensitive to extracellular QX-314 (1 mM, \(n = 4\), data not shown). Hence, it is unlikely that lidocaine directly inhibits extracellular MTSEA modification of the cysteine residue. At the same time, electrostatic interactions between residue K1237 in domain III (Fig. 1) and lidocaine suggest that drug receptor in domain IV, S6 lies near the P-segment selectivity filter residues (Sunami et al., 1997). Hence, (Fig. 6, right) we postulate that the structural rearrangements associated with slow inactivation may move the Na\(^+\) channel P-segments into positions that stabilize the interaction between lidocaine and the pore.

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