Molecular Action of Lidocaine on the Voltage Sensors of Sodium Channels

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ABSTRACT Block of sodium ionic current by lidocaine is associated with alteration of the gating charge-voltage (Q-V) relationship characterized by a 38% reduction in maximal gating charge (Q_{max}) and by the appearance of additional gating charge at negative test potentials. We investigated the molecular basis of the lidocaine-induced reduction in cardiac Na channel–gating charge by sequentially neutralizing basic residues in each of the voltage sensors (S4 segments) in the four domains of the human heart Na channel (hH1a). By determining the relative reduction in the Q_{max} of each mutant channel modified by lidocaine we identified those S4 segments that contributed to a reduction in gating charge. No interaction of lidocaine was found with the voltage sensors in domains I or II. The largest inhibition of charge movement was found for the S4 of domain III consistent with lidocaine completely inhibiting its movement. Protection experiments with intracellular MTSET (a charged sulfhydryl reagent) in a Na channel with the fourth outermost arginine in the S4 of domain III mutated to a cysteine demonstrated that lidocaine stabilized the S4 in domain III in a depolarized configuration. Lidocaine also partially inhibited movement of the S4 in domain IV, but lidocaine's most dramatic effect was to alter the voltage-dependent charge movement of the S4 in domain IV such that it accounted for the appearance of additional gating charge at potentials near −100 mV. These findings suggest that lidocaine's actions on Na channel gating charge result from allosteric coupling of the binding site(s) of lidocaine to the voltage sensors formed by the S4 segments in domains III and IV.

KEY WORDS: gating current • $Na_V1.5$ • heart • mutant • local anesthetic drug

INTRODUCTION

Voltage-gated sodium (Na) channels are largely responsible for excitability in cardiac muscle as well as in other tissues, making them common targets of antiarrhythmic, antimyotonic, anticonvulsant, and local anesthetic drugs (Catterall, 2002). Consequently, they have been frequently the focus of studies on drug action, and one drug, lidocaine, a tertiary amine, is arguably the canonical example of a local anesthetic drug. Drugs in this class achieve their therapeutic benefit by virtue of use dependence, a feature that is generally believed to result from the variable affinity for the drug based on different kinetic states or conformations of the channel (i.e., open, closed, or inactivated). Because Na channel states are largely determined by their voltage sensors, the affinity of local anesthetic drugs should be influenced by their positions, as well as by the chemical properties of the individual local anesthetic drugs. Together, the channel and drug determine the overall equilibrium constant and, therefore, the use dependent profile of any given agent. This idea, termed the modulated receptor hypothesis, was proposed in the late 1970s (Hille, 1977; Hondeghem and Katzung, 1977), although other explanations for use dependence have also been proposed, including multiple binding sites (e.g., for review see Lee-Son et al., 1992) and a single binding site with a fixed affinity but variable accessibility (i.e., the guarded receptor hypothesis) (Starmer et al., 1984).

It is likely that lidocaine and lidocaine-like drugs bind in the pore of the channel, and mutagenesis experiments have probed for determinants of binding in regions thought to line the pore on the intracellular side of the selectivity filter. Although the three dimensional structures of Na channel pores remain unknown, the structure of bacterial KcsA K channel (Doyle et al., 1998) and the MthK channel (Jiang et al., 2002) suggest that the S6 segments form helices that line the inner vestibule of the pore. The first of the mutagenesis studies looking for the local anesthetic drug-binding site implicated the residues in the S6 segment of domain IV, particularly Phe-1764 and Tyr-1771 located near the middle of the S6 segment of the rat brain II Na channel (Ragsdale et al., 1994), where affinity changes between one and two orders of magnitude were produced. A subsequent study in the rat brain III Na channel reported variable effects on rested and in-

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activated state block depending on the amino acid substituted at an analogous position of the Phe in the S6 segment of domain IV (Li et al., 1999). Mutations in the S6 of domain I of the rat skeletal muscle Na channel (at Asn-434) produced channels with increased affinity for bupivacaine of up to one order of magnitude (Nau et al., 1999), although mutations in the S6 of domain II did not affect drug block (Wang et al., 2001). Most recently, mutations in the S6 of domain III have been shown to decrease drug affinity by about an order of magnitude in skeletal muscle Na channels (Wang et al., 2000) and brain IIa Na channels (Yarov-Yarovoy et al., 2001).

No clear picture of the local anesthetic drug binding site has yet emerged from the mutagenesis experiments. Many of the mutations produced changes in affinity of less than an order of magnitude, and studies used different drugs and channel isoforms. Interpretation of the results is further complicated because the mutations themselves produced alterations in channel kinetics, making it difficult to differentiate direct from indirect effects. We, therefore, chose to approach the problem from a different perspective.

Alterations in gating of Na channels bound to local anesthetics can be directly assayed by measuring gating currents (Ig), small electrical signals resulting from movement of the channel's voltage sensors formed by the fourth transmembrane-spanning segments (S4) in each of four domains of the α subunit of the channel (Bezanilla, 2000). Reductions in gating charge by lidocaine have been reported in embryonic chick ventricular myoctes (Josephson and Cui, 1994), canine cardiac Purkinje cells, and human heart Na channels expressed in HEK293 cells (Hanck et al., 2000) and by quaternary lidocaine derivatives in squid giant axon (Cahalan and Almers, 1979; Bekkers et al., 1984; Tanguy and Yeh, 1989), frog node of Ranvier (Guselnikova et al., 1979; Khodorov, 1981), and canine cardiac Purkinje cells (Hanck et al., 1994). Importantly, dose-dependent block of cardiac I_{Na} by local anesthetic drugs is correlated with dose-dependent alteration of gating charge movement (Hanck et al., 1994, 2000), suggesting that they both result from the same drug binding process. Moreover, the concentration at half-maximal effect (EC_{50}) for reduction in gating charge by lidocaine is comparable to therapeutic plasma levels of ~5 μM (Opie, 2001).

We postulated that insight into Na channel–local anesthetic drug interactions would benefit by a better understanding of the allosteric consequences of drug binding to the channel as measured by its effects on movement of the voltage sensors. For example, because lidocaine has an increased affinity for inactivated Na channels (Khodorov et al., 1976; Bean et al., 1983; Grant et al., 1989; Zamponi et al., 1993b), a preferential effect on the S4 segment in domain IV might be expected because this voltage sensor has been shown to participate in the coupling of channel activation to inactivation (Chahine et al., 1994; Hanck and Sheets, 1995; O'Leary et al., 1995; Chanda and Bezanilla, 2002). However, simultaneous modification of human heart Na channels (hH1a)* by both lidocaine and a site-3 toxin, a peptide that inhibits movement of the S4 in domain IV (Sheets and Hanck, 1995; Sheets et al., 1999), produced almost additive reductions in gating charge, suggesting that the S4 in domain IV could make only a small contribution to the reduction in charge produced by local anesthetics.

To investigate the origin of the drug-induced modifications in gating charge, we sequentially tested the contribution of each of the four S4 segments to modification of the Q-V relationships by lidocaine. Na channels with neutralization of basic residues in each of the four S4's segments were expressed in fused tsA201 cells cotransfected with the β1 subunit, and the ability of lidocaine to modify the Q-V relationship was assayed. The results demonstrated that the largest effect of lidocaine was to stabilize the S4 in domain III in a depolarized conformation. No interactions with voltage sensor movement in domains I and II were evident. Drug binding also partially restricted the movement of S4 in domain IV while altering its voltage dependence causing the appearance of gating charge at potentials near −100 mV. These experiments help further understand the state-dependent action of local anesthetic drugs, and they also give us an additional approach to investigate allosteric coupling between ligand binding and conformational changes in a channel protein.

MATERIALS AND METHODS

cDNA Clones

In hH1a Na channels ($Na_V1.5$, provided by H. Hartmann and A. Brown; Hartmann et al., 1994) individual basic amino acid residues in the S4 segments were mutated by 4-primer PCR (Benzinger et al., 1998) to either a cysteine or glutamine at the following positions: (a) 219 in domain I (the outermost arginine mutated to DI-R1C), (b) 808 in domain II (the outermost arginine mutated to DII-R1Q, (c) 1299, 1302, 1305, and 1308 (the outermost basic residue lysine, the second outermost basic residue arginine, the third outermost residue arginine and the fourth outermost residue arginine mutated to DIII-K1C, DIII-R2C or DIII-R2Q, DIII-R3C, DIII-R4C, respectively), and (d) 1622 (the outermost arginine DIV-R1C). The equivalent positions in the hH1 Na channel (Gellens et al., 1992) are the same for the mutations in domains I and II, but are 1300, 1303, 1306, and 1309 for mutations in domain III, and is 1623 in domain IV. All cDNA inserts were confirmed by sequencing. Because our anecdotal experience suggested that block of I_{Na} by tetrodotoxin increased

Abbreviations used in this paper: hH1a, human heart sodium channel $Na_V1.5$; $MTSET_i$, intracellular 2-trimethylammonium ethyl methanothiosulfonate; STX, saxitoxin; TTX, tetrodotoxin.

the survival of cultured cells transiently transfected with Na channel cDNA, the sensitivity of mutant channels and wild-type channel to block by tetrodotoxin (TTX) was increased by mutating the cysteine at position 373 to a tyrosine (C373Y) (Satin et al., 1992). The cDNAs were subcloned directionally into the mammalian expression vector pRcCMV (Invitrogen) as was the cDNA for the rat β1 subunit (Satin et al., 1994). For all studies, both α and β1 subunits were cotransfected with a mole ratio of α to β1 of ~1:2.

Cell Preparation

Multiple tsA201 cells (SV40 transformed HEK293 cells) were fused together using polyethylene glycol as described previously (Sheets et al., 1996). After fusion, the cells were placed in culture for several days to allow for membrane remodeling, and then they were transiently transfected using a calcium phosphate precipitation method (GIBCO BRL). TTX (300 nM) was added to the culture media 1 d after transfection. 3–6 d after transfection fused cells were detached from culture dishes with trypsin-EDTA solution (GIBCO BRL) and studied electrophysiologically.

Recording Technique, Solutions, and Experimental Protocols

Recordings were made using a large bore, double-barreled glass suction pipette for both voltage clamp and internal perfusion as described previously (Sheets et al., 1996). Currents were recorded with a virtual ground amplifier (Burr-Brown OPA-101) using a 2.5 M3 feedback resistor. Voltage protocols were imposed from a 16-bit DA converter (Masscomp 5450; Concurrent Computer) over a 30/1 voltage divider. Data were filtered by the inherent response of the voltage-clamp circuit (corner frequency near 125 kHz) and recorded with a 16-bit AD converter on a Masscomp 5450 at 200 kHz. A fraction of the current was fed back to compensate for series resistance. Temperature was controlled using a Sensortek (Physiotemp Instruments, Inc.) TS-4 thermoelectric stage mounted beneath the bath chambers, which typically allowed temperature to vary less than 0.5°C during an experimental set. Cells were studied at 13°C.

A cell was placed in the aperture of the pipette, and after a high resistance seal formed between the cell and glass pipette, the cell membrane inside the pipette was disrupted with a manipulator-controlled platinum wire. For I_{Na} experiments, voltage control was assessed by evaluating the time course of the capacitive current and by the steepness of the negative slope region of the peak current-voltage relationship (Hanck and Sheets, 1992). To allow for full Na channel availability, the holding membrane potential was set between −150 and −180 mV, and depolarized once per second. I_g protocols contained four repetitions at each test voltage that were 1/4 of a 60 Hz cycle out of phase to improve the signal to noise ratio. Steady-state voltage-dependent Na channel availability was assayed from the magnitude of I_{Na} in a step to 0mV after conditioning for 500 ms at potentials between −170 and −50 mV. Cells were allowed to recover for 1.5 s between conditioning pulses.

The control extracellular solution for I_{Na} measurements contained (in mM) 15 Na^+, 185 TMA^+, 2 Ca^{2+}, 200 MES^- and 10 HEPES (pH 7.2), and the intracellular solution contained 200 TMA^+, 75 F^-, 125 MES^- 10 EGTA, and 10 HEPES (pH 7.2). For one cell expressing DIII-R4C, 200 mM extracellular Na^+ was used to increase the magnitude of I_{Na}. For measurement of I_g under control conditions the extracellular Na^+ was removed and replaced with TMA^+, and 10 μM saxitoxin (STX; Calbiochem Corp.) was added to the extracellular solution. 1 mM 2-trimethylammonium ethyl methanothiosulfonate ($MTSET_i$; Toronto Research Chemicals) was dissolved in the intracellular solution approximately 1 min before the start of internal perfusion. After appropriate control recordings had been obtained, the cell was perfused for 5 min with the intracellular solution containing MTSET before switching back to the control internal solution and waiting an additional seven minutes. For experiments with DIII-R4C no measurements were obtained during these 12 min, and the membrane potential was held constant at either −150 or 0 mV. Lidocaine-HCl (Sigma-Aldrich) was added to extracellular solutions at a high concentration of 10 mM to ensure nearly complete modification of Na channels, a concentration that is three orders of magnitude greater than the EC_{50} for cardiac Na channels under these conditions (Hanck et al., 2000).

Data Analysis

Peak I_{Na} was taken as the mean of four data samples clustered around the maximal value of current that had been digitally filtered at 5 kHz and leak corrected by the amount of the extrapolated time-independent linear leak calculated from the linear conductance measurements obtained between −190 and −110 mV. Data were capacity corrected using 4–16 scaled current responses recorded from voltage steps of 30 or 40 mV negative to the holding potential.

I_gs were leak corrected by the mean of 2–4 ms of data typically beginning at 8 ms after the depolarizing step and then integrated to calculate charge. Q-V relationships were fit with a simple Boltzmann distribution:

$$Q = Q_{max}/(1 + e^{(Vt - V_{1/2})/s}), \quad (1)$$

where Q is the charge during depolarizing step, V_t is the test potential, and the fitted parameters are Q_{max}, the maximum charge, $V_{1/2}$, the half-point of the relationship, and *s*, the slope factor in mVs. This relationship was a convenient descriptor for comparison between cells and conditions; fractional Q was calculated as Q/Q_{max} for each cell in the control solution.

Steady-state voltage-dependent Na channel availability was calculated from fits of peak I_{Na} by a Boltzmann distribution:

$$I_{Na} = I_{max}/(1 + e^{(Vc - V_{1/2})/s}), \quad (2)$$

where I_{Na} was the peak current after a conditioning pulse (V_c), and the fitted parameters were $V_{1/2}$, the half-point of the relationship and *s*, the slope factor in mVs.

Data were analyzed and graphed on a SUN Sparcstation using SAS (Statistical Analysis System). Unless otherwise specified summary statistics are expressed as means ± one SD, and figures show means ± SEM. Experimental parameters for mutant channels were compared using paired *t* tests, and data were considered significantly different at $P < 0.05$.

RESULTS

We have reported previously that lidocaine's alteration of the Q-V relationship in wild-type hH1a (in HEK293 cells without coexpression of β1) was characterized by a reduction in Q_{max} of nearly 38% accompanied by a shallower voltage-dependence (i.e., a reduction in the slope factor of the Boltzmann fit), and a shift of $V_{1/2}$ to more negative potentials (Hanck et al., 2000). We confirmed these changes in the control experimental preparation used in this study, a fused tsA201 cell expressing wild-type hH1a but with a pore mutation, C373Y, and coexpressed with the β1 subunit. Fig. 1 shows the mean

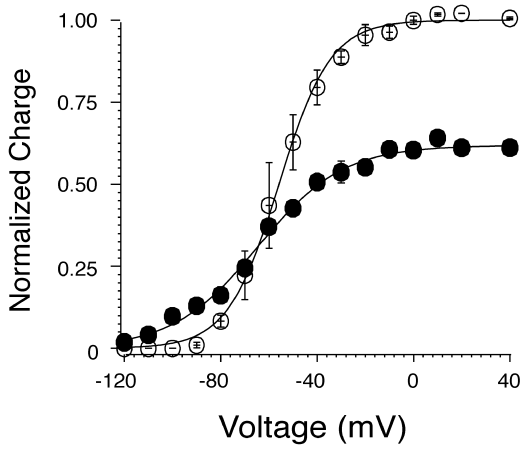

FIGURE 1. Effect of lidocaine on the Q-V relationships of wild-type hH1a (with C373Y) coexpressed with β1. Data plotted are means ± SEM for cells in control (○) and after lidocaine (●). The solid lines represent the mean of the best fits to each cell by a Boltzmann distribution (Eq. 1). Gating charge in lidocaine was normalized to the Q_{max} determined for each cell in control. The parameters from the best fits to the data are given in Table I.

Q-V relationships for two cells cotransfected with wild-type hH1a (with C373Y) and β1. Similar to previous findings, Q_{max} was decreased by 38% and the Q-V relationship exhibited a marked shift in $V_{1/2}$ and reduction in slope factor in the presence of lidocaine (Table I). The change in slope factor and half-point by lidocaine resulted, in part, because of the appearance of additional gating charge at test potentials near −100 mV.

Lidocaine Blocks I_{Na} in Mutant Na Channels

In general, the outer basic residues in S4 segments of voltage-gated channels have been shown to make the greatest contribution to gating charge. For example, the outermost basic residue makes a large contribution to gating in *Shaker* K channels (Aggarwal and MacKinnon, 1996; Seoh et al., 1996) and in the domain IV of hH1a (Sheets et al., 1999), although this is not the case for domain III in hH1a (Sheets and Hanck, 2002). Therefore, we constructed mutant hH1a channels in which the outermost basic residues (or the second outermost arginine in the S4 of domain III) were neutralized to either a cysteine or glutamine. All of the Na channel mutations expressed well in fused tsA201 cells. Examples of families of I_{Na} traces in response to step depolarizations are shown in Fig. 2 for four Na channels, each with a S4 segment mutation in a different domain. For all of these mutant channels onset of I_{Na} was similar, whereas R1C-DIV, as shown previously (Yang and Horn, 1995; Chen et al., 1996), had a slowed I_{Na} decay. The addition of 10 mM lidocaine to the extracellular solution effectively blocked I_{Na} in all Na channels (Fig. 2, right).

The Movement of the S4s in Domains I and II Is Not Inhibited by Lidocaine

To identify which voltage-sensor(s) movement was inhibited by lidocaine, we compared the effect of lidocaine on the Q-V relationship in the control construct (Fig. 1) with those that had one of the basic residues in the S4 of each of the four domains neutralized. If a basic residue in an S4 were to contribute to Q_{max} but its movement were not restricted by lidocaine, then the fractional reduction of Q_{max} caused by lidocaine would be greater than in wild-type, because the magnitude of gating charge from the domain(s) inhibited by lidocaine would remain unchanged while the overall Q_{max} of the mutant channel would have been decreased by the neutralization. Conversely, if a basic residue were to make a contribution to Q_{max} in the wild-type channel and its movement were inhibited by lidocaine, then the fractional reduction in Q_{max} by lidocaine in the neutralized channel would be less than wild-type hH1a because the amount of the remaining gating charge that could be inhibited by lidocaine would be proportionally less than the decrease in the mutant's overall gating charge. Lastly, if a charged residue made little or no contribution to gating charge then the Q_{max} and the amount of gating charge inhibited by lidocaine would be the same for the mutated channel and the wild-type channel.

In domains I and II, DI-R1C and DII-R1Q were studied. Although the magnitude of the contribution of the

TABLE I

Comparison of Boltzmann Parameters (Mean ± SD) from Fits of Q-V Relationships in Control and After Lidocaine

Parameter	D1-R1C	D2-R1Q	D3-K1C	D3-R2C and D3-R2Q	D3-R3C	DIV-R1C	W.T.
	$n = 4$	$n = 5$	$n = 3$	$n = 5$	$n = 3$	$n = 5$	$n = 2$
V1/2 (mV) control	−59 ± 5	−52 ± 7	−56 ± 4	−57 ± 8	−55 ± 4	−55 ± 8	−56 ± 6
s (mV) control	−15 ± 1	−15 ± 1	−16 ± 1	−14 ± 2	−15 ± 2	−15 ± 1	−11 ± 1
V1/2 (mV) lidocaine	−80 ± 5[a]	−70 ± 8[a]	−75 ± 2[a]	−70 ± 9[a]	−71 ± 9[a]	−60 ± 10	−65 ± 1
s (mV) lidocaine	−18 ± 2[a]	−25 ± 7[a]	−24 ± 2[a]	−22 ± 1[a]	−19 ± 1[a]	−18 ± 1[a]	−18 ± 1
Reduction in Q_{max} by lidocaine (%)	47 ± 3[a]	44 ± 4[a]	38 ± 2[a]	23 ± 3[a]	29 ± 3[a]	32 ± 3[a]	38 ± 2
Difference from wild-type (%)	9	6	0	−15	−9	−6	—

[a]Significance $P < 0.05$ for paired *t* test for each channel in control solution compared to same channel in lidocaine.

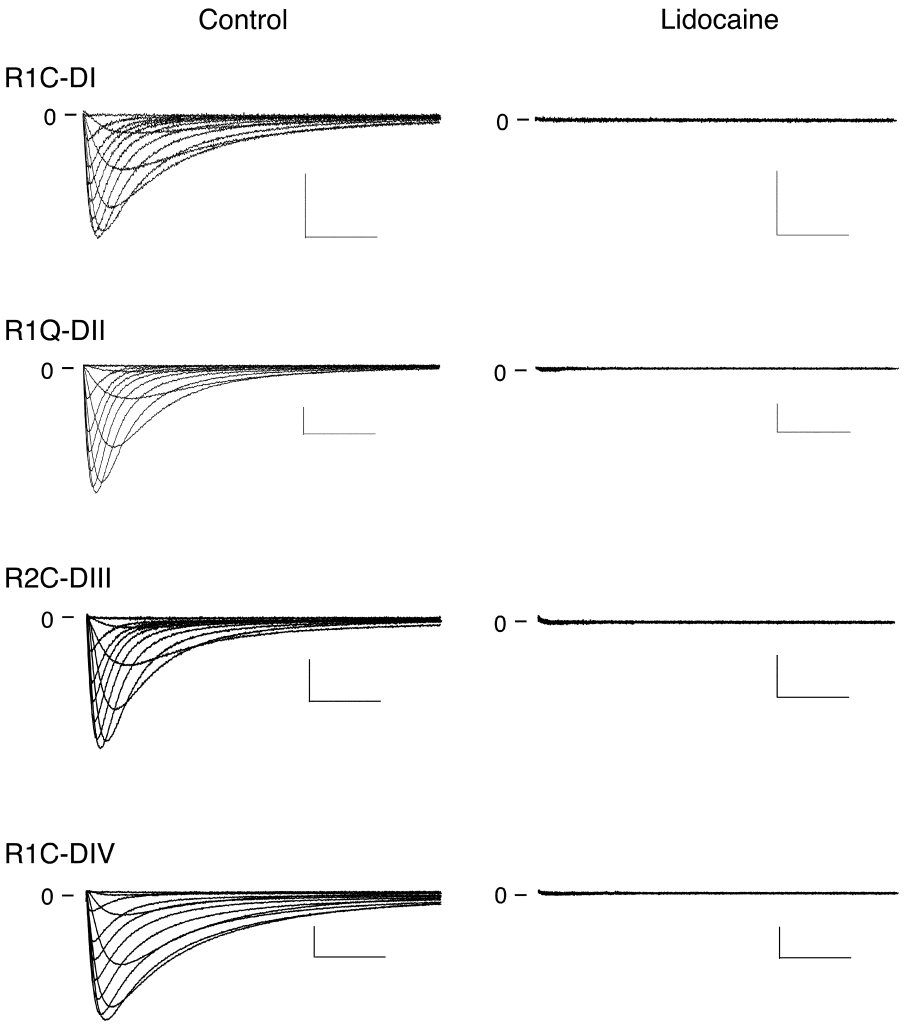

FIGURE 2. Families of leak and capacity-corrected I_{Na} during step depolarizations to potentials between −100 and 20 mV for four mutant channels, D1-R1C, DII-R1Q, DIII-R2C, and DIV-R1C (from top to bottom, respectively), in control solutions (left) and in 10 mM lidocaine (right). The extracellular solution contained 15 mM Na^+ and the intracellular solution contained 200 mM TMA^+ without Na^+. Bars, 10 nA and 10 ms.

outermost basic arginines in domains I and II to Q_{max} in Na channels has not been reported, if the outermost arginines in these domains made a significant contribution to gating charge in Na channels, then the fractional reduction in Q_{max} by lidocaine should differ from that of wild-type hH1a, demonstrating either a larger or smaller reduction. Only if the outermost arginine residues were to make little or no contribution to gating charge would there be no difference in the fractional reduction in Q_{max} by lidocaine. I_g was recorded from Na channels by removing extracellular Na^+ and adding 10 μM STX. Fig. 3 A shows an example of a family of capacity and leak-corrected I_g traces and their corresponding integrals in control solutions (left) and in 10 mM lidocaine (right) for DII-R1Q. Lidocaine produced a greater fractional reduction in Q_{max} in both mutant channels (Fig. 3, B and C), consistent with both of these residues contributing to gating charge, and also suggesting that lidocaine does not inhibit the movement of the S4s in domains I or II. Q_{max} was reduced by 47 ± 3% for D1-R1C and by 44 ± 4% for DII-R1Q, 9% and 6%, respectively, greater than the decrease seen in wild-type (see Table I). The overall characteristics of the Q-V relationships for the neutralized channels modified by lidocaine were also similar to wild-type with the half-points shifted to more negative potentials and gating charge less steeply dependent upon voltage. These findings are consistent with the outermost basic residues in domains I and II making a significant contribution to Q_{max} but not to the modifications of gating charge caused by lidocaine.

The Movement of S4 in Domain III Is Inhibited by Lidocaine

We next investigated the role of the voltage sensor of domain III in the reduction of gating charge by lidocaine. Recently, we demonstrated that the second outermost basic residue, an arginine, in domain III rather than the outermost basic residue, a lysine, makes the greatest contribution to Q_{max} (Sheets and Hanck, 2002). In fact, the outermost basic residue in the S4 of domain III made little or no contribution to overall gating charge (Sheets and Hanck, 2002). Therefore, it was not unexpected that the reduction in gating charge by lidocaine in DIII-K1C was the same (38%) as that for

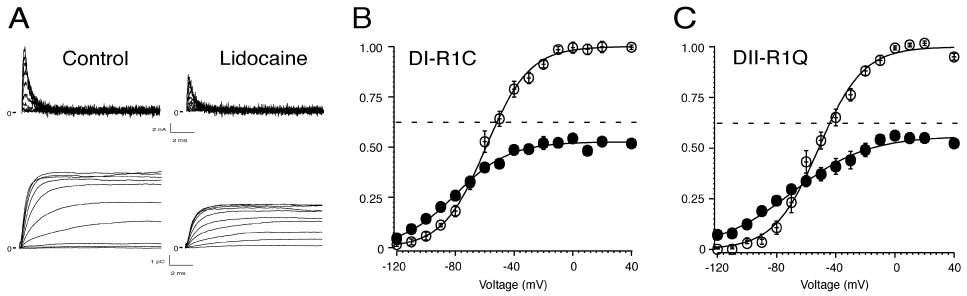

FIGURE 3. Effect of lidocaine on gating currents of DII-R1Q (A) and the Q-V relationships of DI-R1C (B) and DII-R1Q (C). An example of a family of gating currents (A, top) and their integrals (A, bottom) in a fused tsA201 cell expressing DII-R2Q. Recordings were in control solutions (A, left) and in 10 mM lidocaine (A, right), and the traces were in response to step depolarizations from a holding potential of −150 mV to test potentials between −120 and 40 mV. Lidocaine reduced the Q_{max} from 5.5 to 3.2 pC, a reduction of 42%. The data are shown capacity and leak corrected and digitally filtered at 15 kHz with every fourth point plotted. (Cell A7.20) In B and C the data are means ± SEM for cells in control (○) and after lidocaine (●). The solid lines represent the mean of the best fits to each cell by a Boltzmann distribution (Eq. 1) after gating charge in lidocaine was normalized to the Q_{max} determined for each cell in the absence of lidocaine. The dashed lines represent the 38% decrease in Q_{max} seen for wild-type hH1a (see Fig. 1). The number of cells and the parameters from the best fits to the data are given in Table I.

wild-type Na channels (Fig. 4 A and Table I). However, data from mutant channels in which the second outermost basic residue, an arginine, was neutralized indicated that this charge makes the largest contribution to that portion of gating charge inhibited by lidocaine (Fig. 4 B). We mutated R2 to both a cysteine and glutamine because glutamine is similar in size to arginine, and perhaps is less affected by the surrounding pH compared with cysteine. However, the results for the two mutants were indistinguishable. Q_{max} was reduced in the presence of lidocaine by 21 ± 2% for R2C (n = 3 cells) and by 23 ± 3% for R2Q (n = 2 cells) and consequently the data for both mutations were combined.

Additional evidence in support of the inhibition of the movement of the S4 in domain III by lidocaine was provided by the mutant channel, DIII-R3C. Lidocaine produced a smaller decrease in Q_{max} in R3C (29%) compared with the 38% seen for wild-type (Fig. 5 C). Comparison of the differences between the magnitudes of reduction in Q_{max} by lidocaine in the control construct to R2C and R2Q (15%) and to R3C (6%) is consistent with the finding that R2 contributes almost twice as much gating charge to Q_{max} as does R3 (Sheets and Hanck, 2002). In addition, because R2 and R3 in domain III account for most all of the gating charge contributed to Q_{max} by the S4 in domain III (Sheets and Hanck, 2002), and because both residues contribute to the reduction in Q_{max} by lidocaine, it suggests that the S4 in domain III may be completely stabilized by lidocaine. It is interesting to note that the Q-V relationships of the domain III mutant channels continued to demonstrate characteristic changes caused by lidocaine with a negative shift in the half-points, shallow dependence of charge on voltage, and additional gating charge appearing at potentials near −100 mV.

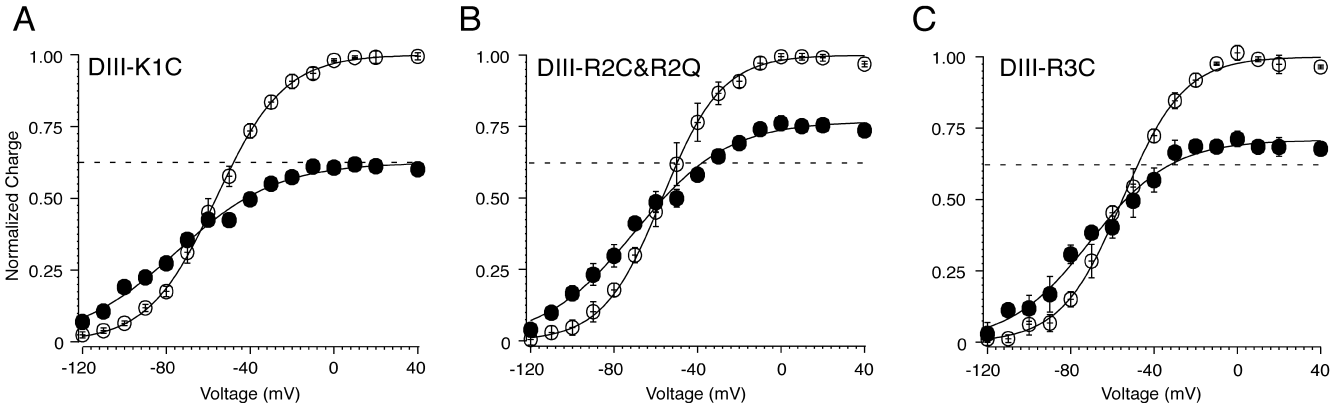

FIGURE 4. Effect of lidocaine on the Q-V relationships of DIII-K1C (A), the combination of DIII-R2C and DIII-R2Q (B, see text), and DIII-R3C (C). Data plotted are means ± SEM for cells in control (○) and after lidocaine (●). The solid lines represent the mean of the best fits to each cell by a Boltzmann distribution (Eq. 1), and gating charge in lidocaine was normalized to the Q_{max} determined for each cell in the absence of lidocaine. The dashed line in each panel represents the 38% decrease in Q_{max} seen for wild-type hH1a (Fig. 1), and the number of cells and the parameters from the best fits to the data are given in Table I.

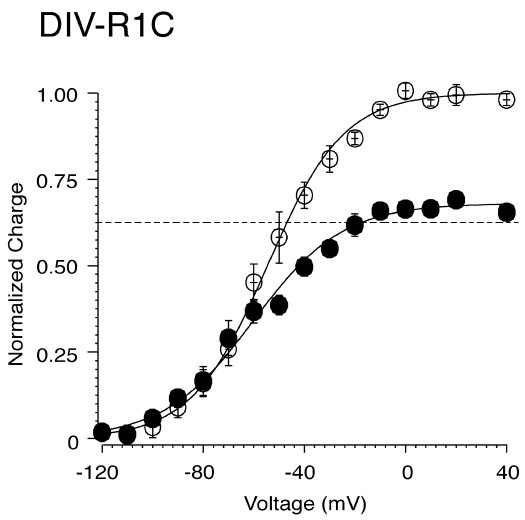

FIGURE 5. Effect of lidocaine on the Q-V relationships of DIV-R1C. Data plotted are means ± SEM for cells in control (○) and after lidocaine (●). The solid lines represent the mean of the best fits to each cell by a Boltzmann distribution (Eq. 1), and gating charge in lidocaine was normalized to the Q_{max} determined for each cell in the absence of lidocaine. The dashed line represents the 38% decrease in Q_{max} seen for wild-type hH1a while the number of cells and the parameters from the best fits to the data are given in Table I.

The S4 in Domain IV Contributes the Additional Gating Charge at −100 mV in Lidocaine

Lastly, the contribution of the domain IV voltage sensor was investigated by neutralizing the outermost arginine in its S4. We chose DIV-R1C because previous studies have shown that this charge makes the largest contribution to gating of DIV-S4 (Sheets et al., 1999). Consistent with lidocaine affecting gating of DIV-S4, it reduced Q_{max} by only 32%, 6% less that the 38% for wild-type hH1a (Fig. 5). However, the reduction was less than either neutralization tested in domain III. This was somewhat surprising since we have shown previously that DIII-R2 and DIV-R1 contribute nearly identical amounts to overall gating charge in wild-type hH1a (Sheets et al., 1999; Sheets and Hanck, 2002). Therefore, the lower fractional reduction in charge by lidocaine in DIV-R1C (32%) versus DIII-R2C (23%) suggests that lidocaine only partially inhibited the movement of the S4 in domain IV. In addition, comparison of the Q-V relationships of DIV-R1C in control and after lidocaine revealed that the DIV neutralized channel exhibited only a small shift (−5 mV) in the half-point of gating. This was only marginally greater than that expected from the small time-dependent background shift of Na channel kinetics (Sheets and Hanck, 1999) which, based on the cells in Fig. 5, would be ~3 mV. Most impressively, additional gating charge did not appear at test potentials near −100 mV. These findings suggest that lidocaine partially inhibited the movement of the S4 in domain IV in wild-type hH1a while simultaneously altering the voltage dependence of the remaining gating charge domain IV that still moves in the presence of lidocaine.

The S4 of Domain III Is Stabilized in a Depolarized Conformation by Lidocaine

The large contribution of both R2 and R3 in the S4 of domain III to the gating charge inhibited by lidocaine raises the question as to whether the S4 in domain III is stabilized by lidocaine in a conformation associated with either depolarization or hyperpolarization. To determine the position of the S4 of domain III in the presence of lidocaine, we designed a protection experiment using a mutant channel (DIII-R4C) and intracellular MTSET. Previous studies have demonstrated that the third outermost arginine, R3, of the S4 in domain IV of the human skeletal muscle Na channel was protected from modification by $MTSET_i$ when the membrane potential depolarized but not when the membrane potential was hyperpolarized (Yang et al., 1996). After this approach we investigated the voltage-dependent modification of DIII-R4C, a residue that has been shown not to appreciably contribute to gating charge (Sheets and Hanck, 2002). We first confirmed that lidocaine at 10 mM effectively blocked I_{Na} in cells expressing this mutant channel (Fig. 6 A). Then in the absence of lidocaine, if the cell were held at −150 mV while 1 mM MTSET was internally perfused (see MATERIALS AND METHODS), Na channel availability was dramatically shifted rightward by nearly 30 mV (Fig. 6 B). In contrast, when the membrane potential was held at 0 mV during exposure to 1 mM $MTSET_i$, channels were protected from modification by MTSET (Fig. 6 C). Subsequent exposure to $MTSET_i$ in these cells, but with the holding membrane at −150 mV, confirmed that MTSET could modify the previously protected channels and would produce a large, rightward shift in Na channel availability (unpublished data).

If lidocaine were to stabilize the S4 of domain III in a hyperpolarized position, then exposure to lidocaine and intracellular MTSET together should result in a large rightward shift of Na channel availability. However, if lidocaine stabilized the S4 in a depolarized position, then simultaneous exposure to both lidocaine and $MTSET_i$ should protect R4C from modification, and therefore, produce no shift in Na channel availability. Fig. 6 D shows the data comparing Na channel availability for DIII-R4C before and after exposure to 10 mM extracellular lidocaine and $MTSET_i$. To maximize the number of lidocaine-bound channels, channels were first exposed to lidocaine for 2 min and then pulsed for 100 cycles to 0 mV for 10 ms from a holding potential of −150 mV (or −170 mV for one cell) at 9.1

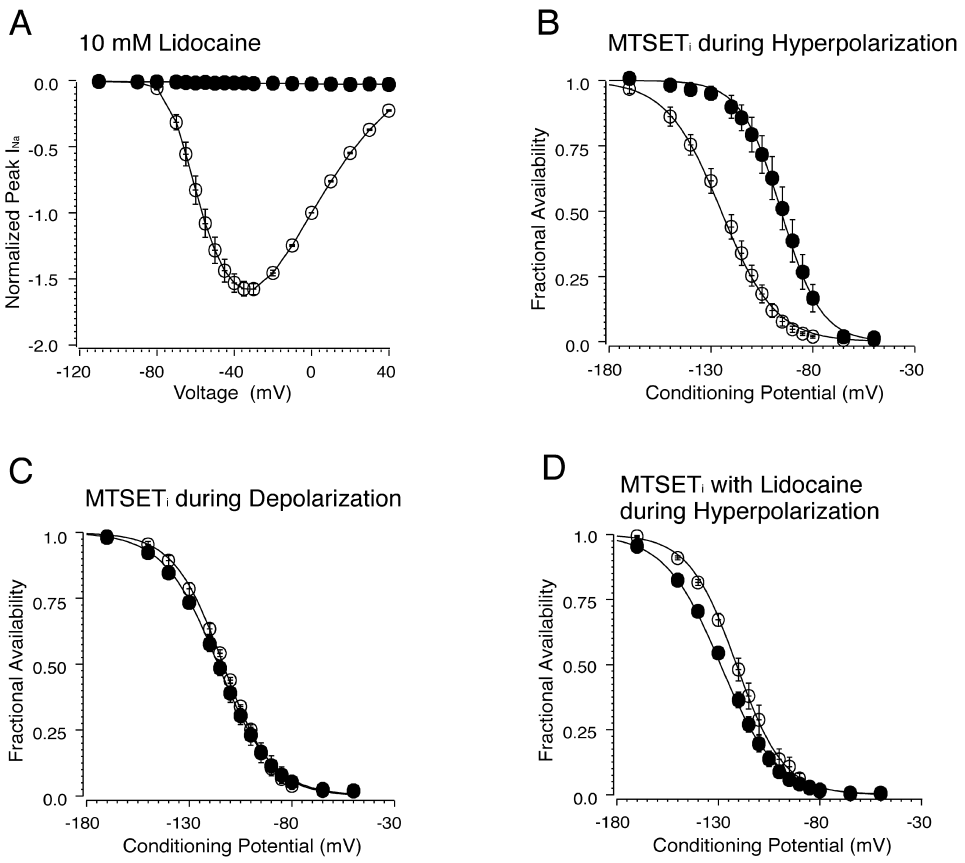

FIGURE 6. Protection of DIII-R4C from modification by intracellular MTSET by lidocaine and membrane depolarization. (A) Peak I_{Na}-voltage relationships for five cells in control solutions (○) and in 10 mM lidocaine (●) indicate that lidocaine blocks I_{Na} in this mutant channel. The lines connect the points. (B) Voltage-dependent Na channel availability for DIII-R4C ($n = 4$ cells) in control (○) and after exposure to 1 mM MTSETi while the cells were held at a hyperpolarized holding potential of −150 mV (or −170 mV in one cell) (●). The $V_{1/2}$ of the availability curve shifted from −125 ± 5 mV to −96 ± 7 mV ($n = 4$ cells). (C) Voltage-dependent Na channel availability for DIII-R4C ($n = 4$ cells) in control (○) and after the cells had been held at a depolarized potential of 0 mV (●) while exposed to 1 mM $MTSET_i$. The half-points were −114 ± 1 mV and −116 ± 2 mV ($n = 4$ cells), respectively. The small leftward shift in half point resulted from the well described background shift in Na channel kinetics (Sheets and Hanck, 1999). (D) Voltage-dependent Na channel availability for DIII-R4C ($n = 4$ cells) in control (○) and after simultaneous exposure to 10 mM lidocaine (bath) and 1 mM $MTSET_i$ while the cells were held at a hyperpolarized potential (●). The half-points were −121 ± 4 mV and −128 ± 3 mV ($n = 4$ cells), respectively, and the leftward shift is consistent with the background shift in channel kinetics. In all protocols Na channel availability was assayed by using 500-ms conditioning steps to the indicated potentials followed by a test depolarization to 0 mV. Data plotted are means ± SEM. The lines in B–D represent the mean of the best fits of the data for each cell by a Boltzmann distribution (Eq. 2).

Hz. Then the cell was then held at a hyperpolarized potential of −150 mV (or −170 mV for one cell) without pulsing in the presence of both 10 mM lidocaine and 1 mM $MTSET_i$. After washout of $MTSET_i$ lidocaine was then washed from the bath, and Na channel availability was again determined in control solutions (Fig. 6 D). The Na channel availability curve remained unchanged, suggesting that $MTSET_i$ could not react with DIII-R4C in the presence of lidocaine. However, a second exposure to $MTSET_i$ after washout of lidocaine and while the membrane potential was hyperpolarized caused a large, rightward shift in Na channel availability demonstrating that R4C could still be modified (unpublished data). Together, these data suggest that lidocaine stabilized the voltage sensor of domain III in an outward, depolarized conformation.

DISCUSSION

We investigated the contribution of each of the four voltage sensors to the modification of gating charge caused by the local anesthetic drug, lidocaine, in the human cardiac Na channel by comparing the action of lidocaine in wild-type channels with that of a series of mutant channels in which basic residues in the S4 segments from each of the four domains were neutralized. The I_{Na} of the mutant channels could be effectively blocked by 10 mM lidocaine, a high concentration of drug chosen to ensure a uniform population drug-bound channels. Initially, we confirmed that lidocaine modification of wild-type Na channel gating charge in this study's experimental preparation (i.e., wild-type hH1a with the C373Y mutation coexpressed with β1 in fused tsA201 cells) was comparable to that previously reported for native cardiac Na channels and wild-type hH1a without coexpression of β1 in fused HEK293 cells (Hanck et al., 2000) and for native cardiac Na channels blocked with either the quaternary ammonium lidocaine derivative or the neutral compound benzocaine (Hanck et al., 1994). The hallmarks of modification of the Q-V relationship by lidocaine include a reduction in overall Q_{max}, a lesser voltage de-

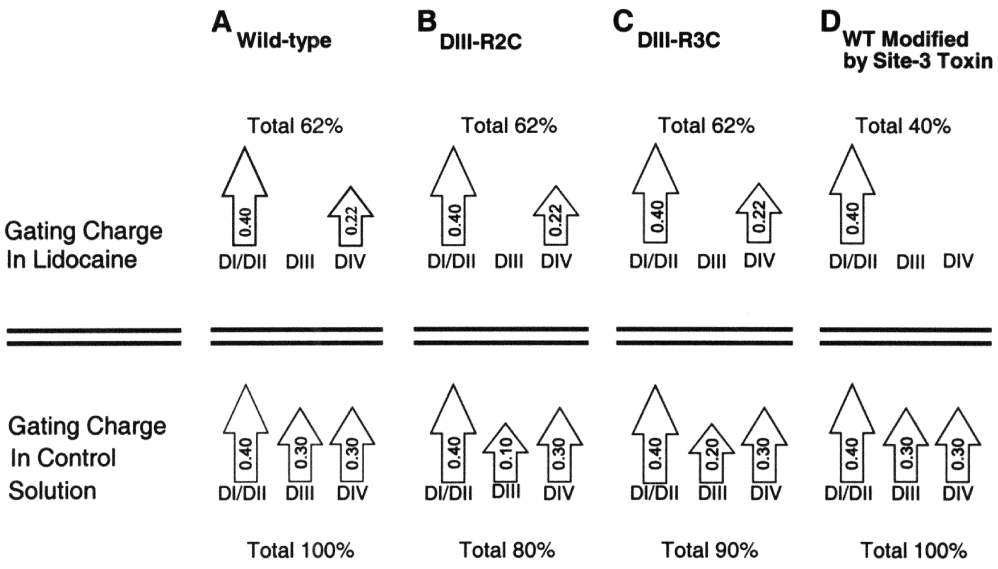

FIGURE 7. Algebraic consideration of the effect of lidocaine on Q_{max} in wild-type hH1a (A), DIII-R2C (B), DIII-R3C (C), and site-3 toxin modified wild-type hH1a (D). Above the double line represents the channel in lidocaine while below the double line the channel in control solution. The values inside each arrow represent the fraction of gating charge from each domain compared with the Q_{max} for wild-type hH1a in control except the charge for both domains I and II was combined into one arrow. The percentages above and below the lines are compared with wild-type Na channels with a value of 100%. For A the percentage reduction in Q_{max} by lidocaine is 38% (100% − 62%/100%). For B the reduction in Q_{max} by lidocaine would be 22% (80% − 62%/80%. For C the reduction by lidocaine would be 31% (90% − 62%/90%). For D the reduction in Q_{max} by both lidocaine and site-3 toxin in wild-type hH1a would be 60%. See text for details.

pendence (a shallower slope factor), and a shift in the half-point to more negative potentials with the appearance of additional gating charge near −100 mV. Similar findings of a reduction in the magnitude of gating charge and its voltage dependence have been described for other Na channel isoforms (Cahalan and Almers, 1979; Guselnikova et al., 1979; Khodorov, 1981; Bekkers et al., 1984; Tanguy and Yeh, 1989); consequently it is likely that the findings reported here are applicable to voltage-dependent Na channels in general.

Effects of Lidocaine on Voltage Sensor Movement Are Domain Specific

To identify those S4s that contribute to the decrease in Q_{max} we measured the ability of lidocaine to modify the Q-V relationships of Na channels that had basic residues neutralized in each of the four S4 segments and compared these data with those from wild-type hH1a. It is not straight forward to establish the absolute reduction in gating charge associated with each neutralized residue because expression levels between cells can vary markedly, and the prediction of the number of channels from ionic current data depends on multiple assumptions, such as single channel conductance and the probability that channels will be open at peak I_{Na}. However, in order to evaluate whether or not gating charge from the individual S4s is affected by lidocaine, it is only necessary to compare the relative reduction in gating charge caused by lidocaine for each mutant channel. Using this strategy we established that lidocaine did not inhibit movement of the voltage sensors in domains I and II, although the outermost arginines in both of these S4s did make a substantial contribution to Q_{max}.

When the outermost charged residue in the S4 of domain III was neutralized (DIII-K1C), lidocaine decreased Q_{max} to the same extent as it did in wild-type Na channels, as would be expected if this charge did not appreciably contribute to the gating charge of the channel. This finding was consistent with a previous study that also suggested that this residue does not appreciably contribute to gating charge in the wild-type Na channel (Sheets and Hanck, 2002). In contrast, when the basic residues at positions R2 and R3 in the S4 of domain III were neutralized, the fractional reduction in gating charge by lidocaine was less than in wild-type channels, indicating that lidocaine restricted movement of this voltage sensor. For channels in which the outermost charged residue, an arginine, in the S4 of domain IV was neutralized, lidocaine also produced a lower fractional reduction in gating charge than for wild-type channels.

Further insight into the action of lidocaine on the voltage sensors can be inferred if the results are combined with data from previous studies with this channel isoform in which each of the S4s in domains III and IV were estimated to contribute ~30% to the overall gating charge of wild-type cardiac Na channels (Sheets et al., 1999, 2000; Sheets and Hanck, 2002). Because domains III and IV contribute ~60% to Q_{max}, then domains I and II must contribute the remaining 40%. If lidocaine were to inhibit completely the movement of gating charge in the S4 of domain III, then the 38% re-

duction in total gating charge observed in wild-type channels would be predicted to have contributions of 30% of Q_{max} from the S4 of domain III and 8% from the S4 of domain IV (Fig. 7 A). Addition of lidocaine to the wild-type channel would leave domains I and II charge intact (40%) and inhibit all of the charge from domain III and almost one-third of gating charge from domain IV (leaving 22%).

The results presented here for charge neutralizations in domain III are quantitatively consistent with these predictions. R2 and R3 in domain III have been previously estimated to account for nearly all of the charge in domain III, and contribute 19% and 10%, respectively, to Q_{max} (Sheets and Hanck, 2002). Consequently, the Q_{max} of DIII-R2C (Fig. 7 B) would be predicted to equal ~80% (40% from domains I and II and 10% from domain III and 30% from domain IV) of that for wild-type hH1a. In the presence of lidocaine the Q_{max} of DIII-R2C would be predicted to be only 62% (40% from domains I and II and 22% from domain IV) of that of wild-type hH1a. As a result, for DIII-R2C, the fraction of Q_{max} in lidocaine compared with Q_{max} in control solutions would be 62%/80% (=0.78), making a fractional reduction of 22%, which approximates the observed 23% reduction. Similarly, DIII-R3C (Fig. 7 C) would be predicted to have 90% of the Q_{max} of wild-type hH1a (40% from domains I and II and 20% from domain III and 30% from domain IV), and in the presence of lidocaine its overall gating charge would be 62% (40% from domains I and II and 22% from domain IV). Thus, the fraction of Q_{max} in lidocaine would be 62%/90% (=0.69), i.e., a fractional reduction of 31%, which is similar to the 29% reduction found for DIII-R3C (Fig. 4 C).

The experimental strategy of neutralization of basic residues within each of the S4s assumed that minimal secondary changes in channel conformation occurred due to redistribution of charge from adjacent basic residues in the same S4 or from the other three S4s. Also the strategy assumed that lidocaine did not cause an increase in the gating charge of either domains I or II while simultaneously causing a decrease in gating charge from domains III and IV. Although both these assumptions are difficult to verify experimentally, previous experiments on wild-type hH1a channels have been consistent with the finding reported here on Na channels with neutralized basic residues. Earlier studies have reported on the simultaneous modification of the Q-V relationship of wild-type hH1a by both lidocaine (Hanck et al., 2000) and Anthopleurin-A (ApA) toxin, a site-3 toxin that selectively inhibits movement of the S4 in domain IV, a voltage sensor that contributes ~30% to Q_{max} (Sheets et al., 1999). Simultaneous modification of wild-type hH1a by both lidocaine and ApA toxin reduced Q_{max} by 59%, a value that would be predicted from the results in this study (Fig. 7 D). The similarity between this prediction and the observed results on wild-type hH1a suggests that large secondary changes in channel conformation did not occur due to the neutralization of basic residues, and that lidocaine did not cause an increase in gating charge in either domains I or II that was offset by an even greater decrease in charge from either domains III or IV.

Domain III-S4 Charge Is Stabilized in a Depolarized Configuration

Our experimental data indicate that the voltage sensor in domain III failed to move in response to a change in membrane potential when lidocaine was bound to the Na channel. This raised the question as to whether the sensor was held in an inward or outward position. To address this question, we studied a mutant channel with the fourth outermost arginine replaced with a cysteine in domain III (DIII-R4C), making it possible to design a protection experiment with intracellular MTSET in order to evaluate the position of the stabilized voltage sensor. Our experiments were consistent with the S4 in domain III being stabilized in an depolarized "outward" conformation by lidocaine. However, the kinetic consequences of this are not immediately obvious. An outward position of the S4 in domain III has been clearly implicated in activation of the channel. However, an outward position of both S4s in domains III and IV has also been associated with the slow time course of recovery of gating charge during repolarization. During repolarization of a fast-inactivated channel the S4s in these two domains return slowly and are responsible for the slow component of gating charge, i.e., the charge that becomes "immobilized" (Bezanilla, 1977; Cha et al., 1999). An outward conformation of the voltage sensor in domain III might favor an inactivated-state conformation of the channel, i.e., with the putative inactivation particle, formed by the intracellular linker between domains III and IV bound to its intracellular receptor (for reviews see Bezanilla, 2000; Catterall, 2000). However, elegant studies using MTSET's ability to modify the unbound inactivation particle (Vedantham and Cannon, 1998, 1999) determined that lidocaine does not appear to stabilize the inactivation particle in a bound position. Furthermore, if lidocaine were to stabilize the channel in an inactivated state then the movement of both S4s in domains III and IV might have been expected to be prohibited. Instead, we found that the S4 in domain IV was only partially inhibited (see below). Thus, it appears that lidocaine's action on gating charge cannot be interpreted as stabilizing the Na channel in a fast-inactivated state. Recent studies have implicated that slow inactivated states may also contribute to lidocaine's action (Chen et al., 2000; Ong et al., 2000), and it is possible

that stabilization of the domain III voltage sensor in an outward position may favor entry into a slow inactivated state.

Lidocaine Alters the Voltage-dependent Movement of the S4 in Domain IV

The action of lidocaine on the gating charge from the S4 in domain IV was complex. Although part of the gating charge contributed by the S4 in domain IV was inhibited by lidocaine, this S4 was also responsible for the appearance of the additional component of gating charge occurring at potentials around −100 mV. We found that lidocaine failed to induce any additional gating charge at negative potentials in DIV-R1C, a mutation where nearly 2/3 of the gating charge in the S4 is neutralized (Sheets et al., 1999). This result explains why modification of wild-type hH1a by both lidocaine and Anthopleurin-A toxin, a toxin that inhibits movement of the S4 in domain IV (Sheets and Hanck, 1995; Sheets et al., 1999) also did not induce the appearance of additional gating charge near −100 mV (Hanck et al., 2000). In contrast to the effects on the S4 in domain III, lidocaine binding did not simply inhibit the movement of the voltage sensor in domain IV, it also altered the voltage-dependence of its remaining gating charge. As a consequence, the straightforward calculations used to predict the reduction of gating charge by lidocaine for amino acid residues neutralized in domain III would not be expected to apply to DIV-R1C, and indeed they do not. Previous studies have predicted that R1 in the S4 of DIV to contribute ~19% to Q_{max} while the entire S4 contributes ~30% (Sheets et al., 1999). The neutralized channel would, therefore, be predicted to be able to contribute up to 11% to Q_{max}. However, lidocaine produced a 32% reduction of Q_{max} in RIV-R1C (see Fig. 5), which requires the S4 in domain IV to contribute nearly 14% to gating charge (68% = [the 40% from domains I and II + the amount from domain IV]/79%). Moreover, the discrepancy cannot be resolved by assuming that less than 100% of the gating charge in domain III is stabilized by lidocaine because that would result in the S4 of domain IV in DIV-R1C having to make an even greater contribution than 14%. Consequently, it is likely that other basic residue(s) in the S4 of domain IV that did not contribute to overall gating charge in wild-type hH1a may contribute to Q_{max} in the presence of lidocaine.

Previously, it has been shown that the S4 in domain IV facilitates coupling of inactivation to channel activation (Chahine et al., 1994; Hanck and Sheets, 1995) by moving slowly, therefore after the translocation of the other S4's and after the channel has opened (Sheets and Hanck, 1995; Cha et al., 1999; Sheets et al., 1999; Chanda and Bezanilla, 2002). One can speculate that the movement of the S4 in domain IV may be controlled by the conformation of the S4 in domain III. When the S4 in domain III is in a "retracted" hyperpolarized position, the movement of the S4 in domain IV may remain inhibited. However, when the S4 in domain III is stabilized in a depolarized conformation by lidocaine, the movement of the S4 in domain IV might be facilitated, as demonstrated, by an increase in gating charge at potentials near −100 mV. Additional experiments may be able to test whether this is indeed the case.

Relationship of Gating Charge Studies to the Putative Binding Site for Local Anesthetic Drugs

The experiments presented here do not speak directly to the location of the binding site for lidocaine and lidocaine-like drugs. As summarized in the INTRODUCTION, mutagenesis experiments have not produced a clear picture of the location of the binding site(s), although it seems likely that lidocaine binds in the pore of Na channels. Our experiments approached the problem from a different perspective, i.e., we sought to develop insight into the interaction of local anesthetic drugs with Na channels by characterizing the allosteric consequences of lidocaine binding in the pore on movement of the voltage sensors. A simple interpretation of our data suggests that the binding site within the S6 segment of domain III may be most important because lidocaine has its greatest effect on the voltage sensor in that domain. Although our data do not rule out lidocaine binding to domain I or II, they do indicate that there does not appear to be coupling between the binding site and the voltage sensors in those domains.

Although our experiments used high concentrations of lidocaine in order to produce a uniform population of drug-bound channels, therapeutic concentrations of lidocaine also result in similar changes to Q-V relationships (Hanck et al., 2000). Local anesthetic drugs have been shown to cause both a "slow" block (or high affinity block) that stabilizes Na channels into a long-lived, nonconducting state(s) (Grant et al., 1989; Benz and Kohlhardt, 1991; Gingrich et al., 1993; Zamponi and French, 1993), as well as a "fast" block (or flicker block of open channels) that becomes apparent when fast inactivation has been removed (Cahalan, 1978; Uehara and Moczydlowski, 1986; Benz and Kohlhardt, 1991; Gingrich et al., 1993; Zamponi et al., 1993a; Balser et al., 1996). Slow block has been shown to have EC_{50}s close to the clinical therapeutic range for lidocaine of 5 μM (Nilius et al., 1987; Grant et al., 1989) and to depend on the aromatic ring of local anesthetic drugs (Zamponi and French, 1993; Haeseler et al., 2002). In contrast, fast block has EC_{50}s 2–3 orders of magnitude greater than that for slow block (Gingrich et al., 1993; Zamponi et al., 1993a; Kimbrough and Gingrich,

2000). Because we have shown previously that the amount of block of wild-type hH1a I_{Na} by local anesthetic drugs directly correlated with alteration of the Q-V relationship with EC_{50}s comparable to that slow block, and that the gating current changes occurred irrespective of whether the drug was positively charged (QX-222), neutral (benzocaine) or both (lidocaine) (Hanck et al., 1994, 2000), it is likely that lidocaine's effects on the S4 segments of domains III and IV result from the high affinity binding characteristics associated with slow block.

The experiments here provide important insight into how lidocaine affects specific structures involved in the Na channel gating apparatus apparently through an allosteric effect between the pore of the channel and its voltage sensors. Evidence for allosteric coupling between pore residues and channel conformation are beginning to emerge. For example, an external basic residue of the S4 in the *Shaker* K channel positions itself closer to the pore during activation (Elinder et al., 2001), and an analysis of pore mutations on conductance in the context of the closed (KcsA) and open (MthK) channel structures suggest energetic coupling between the pore and voltage sensors at distances of up to 15 Å (Yifrach and MacKinnon, 2002). Studies of drug interactions should provide an additional tool for probing how channels link permeation and/or occupancy in the pore with important channel kinetic conformations including the positions of the voltage sensors.

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