The Na Channel Voltage Sensor Associated with Inactivation Is Localized to the External Charged Residues of Domain IV, S4

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ABSTRACT Site-3 toxins have been shown to inhibit a component of gating charge (33% of maximum gating charge, Q_{max}) in native cardiac Na channels that has been identified with the open-to-inactivated state kinetic transition. To investigate the role of the three outermost arginine amino acid residues in segment 4 domain IV (R1, R2, R3) in gating charge inhibited by site-3 toxins, we recorded ionic and gating currents from human heart Na channels with mutations of the outermost arginines (R1C, R1Q, R2C, and R3C) expressed in fused, mammalian tsA201 cells. All four mutations had ionic currents that activated over the same voltage range with slope factors of their peak conductance-voltage (*G-V*) relationships similar to those of wild-type channels, although decay of I_{Na} was slowest for R1C and R1Q mutant channels and fastest for R3C mutant channels. After Na channel modification by Ap-A toxin, decays of I_{Na} were slowed to similar values for all four channel mutants. Toxin modification produced a graded effect on gating charge (*Q*) of mutant channels, reducing Q_{max} by 12% for the R1C and R1Q mutants, by 22% for the R2C mutant, and by 27% for the R3C mutant, only slightly less than the 31% reduction seen for wild-type currents. Consistent with these findings, the relationship of Q_{max} to G_{max} was significantly shallower for R1 mutants than for R2C and R3C mutant Na channels. These data suggest that site-3 toxins primarily inhibit gating charge associated with movement of the S4 in domain IV, and that the outermost arginine contributes the largest amount to channel gating, with other arginines contributing less.

INTRODUCTION

Systematic conformational changes of protein structure in response to changes in the potential field across the cellular membranes are characteristic of voltage-gated ion channels. The ability of channels to respond to changes in membrane potential was proposed to result from movements of specialized charged portions of the channel called *voltage sensors* (Hodgkin and Huxley, 1952) and was first demonstrated in 1973 (Schneider and Chandler, 1973; Armstrong and Bezanilla, 1973). The putative voltage sensors have been shown to reside, in large part, in the fourth transmembrane-spanning segment (S4) of a six-transmembrane motif in voltage-gated channels that include K channels (Aggarwal and MacKinnon, 1996; Seoh et al., 1996; Mannuzzu et al., 1996) and Na channels (Stuhmer et al., 1989; Yang and Horn, 1995; Yang et al., 1996). The voltage-gated K channel is formed from the association of four α-subunits (Mackinnon, 1991; Liman et al., 1992), and for many K channels such as the *Shaker* K channel, the four subunits are identical. In contrast, the Na channel is composed of a single α-subunit that comprises four homologous domains (Noda et al., 1984; Gellens et al., 1992). As a consequence of the different domains in Na channels, it is possible that the individual domains have developed specific roles in channel kinetic transitions.

Previous studies of Na channels have, in fact, suggested that the putative voltage sensor formed by S4 in domain IV (DIV) may have a unique role in channel inactivation (Stuhmer et al., 1989; Krafte et al., 1990; Chahine et al., 1994; Chen et al., 1996; Kontis et al., 1997). Recently, we identified a component of cardiac Na channel gating charge (33% of Q_{max}) that was suppressed when channel inactivation from the open state (O↔I) was inhibited by the use of the site-3 polypeptide toxin anthopleurin-A (Ap-A) (Sheets and Hanck, 1995). Site-3 toxins have been shown to inhibit inactivation from the open state in Na channels with little effect on channel activation or on inactivation from closed states (Kirsch et al., 1989; El-Sherif et al., 1992; Hanck and Sheets, 1995), although site-3 toxins may also affect transitions between inactivation states (Benzinger et al., 1999). In addition, site-3 toxins have been shown to bind extracellularly to regions in domain IV (Thomsen and Catterall, 1989; Benzinger et al., 1997, 1998; Rogers et al., 1996).

We postulated that site-3 toxins exert their effect by inhibiting movement of the S4 of domain IV, and to test this hypothesis we investigated the effects of Ap-A toxin modification on the gating currents (I_g) of cardiac Na channels that had undergone single amino acid mutagenesis of each of the three outermost charged residues (all arginines) in the S4 of domain IV in the human heart Na channel (Fig. 1) that represent amino acids at positions 1623, 1626, and 1629 in hH1 Na channels (Gellens et al., 1992). These studies confirm that site-3 toxins inhibit movement of the S4 of domain IV and demonstrate that the outermost basic residue makes the greatest contribution to the gating charge arising from the voltage sensor formed by the S4 of domain IV. Some of

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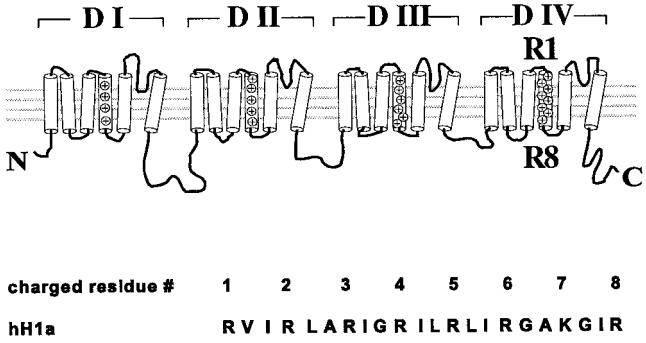

FIGURE 1 Model of the hH1 sodium channel showing the four domains (DI–DIV), each with six transmembrane segments, and the intracellular locations of both the N and C termini (*top*). The outermost arginine in the fourth subunit in domain IV is labeled R1, and the innermost arginine is labeled R8. The amino acid sequence for the S4 of domain IV with numbering of the eight basic residues from the extracellular surface to the intracellular surface is shown (*bottom*). The sequence corresponds to amino acid positions 1623–1644 in the hH1 Na channel (Gellens et al., 1992) or to 1622–1643 in the hH1 Na channel (Hartmann et al., 1994).

these data have been published in abstract form (Sheets et al., 1998).

MATERIALS AND METHODS

cDNA clones

In hH1a (kindly provided by H. Hartmann and A. Brown; see Hartmann et al., 1994) the arginines at positions 1622, 1625, or 1628 (referred to as R1, R2, R3, respectively) were mutated to a cysteine by 4-primer polymerase chain reaction (Benzinger et al., 1998) to R1C, R2C, R3C, respectively, and the insert including the single mutated site was confirmed by sequencing. The equivalent positions in the hH1 Na channel are 1623, 1626, and 1629. At position 1623 in hH1 (Gellens et al., 1992) the arginine was mutated to a glutamine (referred to as R1Q). For expression of the mutations of hH1a, cDNA was subcloned directionally into the mammalian expression vector pRcCMV (Invitrogen), and the mutation of hH1 was subcloned into the expression vector pcDNA (Invitrogen). The wild-type human heart Na channel was hH1a subcloned into pRcCMV. The rat β1-subunit (Satin et al., 1994) was also subcloned directionally into pRcCMV. In all studies, both the α-subunit and β1-subunit were cotransfected, because cotransfection may increase expression levels of the α-subunit. Unless specifically stated, the abbreviation hH1 will refer to either hH1a or hH1.

Cell preparation

Multiple tsA201 cells (SV40-transformed HEK293 cells) were fused together into large mammalian cells, using polyethylene glycol as previously described (Sheets et al., 1996). After fusion, the cells were placed in cell culture for several days to allow for membrane remodeling before they were transiently transfected with calcium phosphate (GIBCO, Grand Island, NY) or lipofectamine (GIBCO). Three to six days after transfection, fused cells were detached from culture dishes with trypsin-EDTA solution (GIBCO) 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 previously described (Sheets et al., 1996). I_{Na} was measured with a virtual ground amplifier (Burr-Brown OPA-101), using a 2.5 MΩ feedback resistor. Voltage protocols were imposed from a 16-bit DA converter (Masscomp 5450; Concurrent Computer, Tinton Falls, NJ) 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 or 300 kHz. A fraction of the current was fed back to compensate for series resistance.

A cell was placed in the aperture of the pipette and transferred to one of four experimental chambers. After a high-resistance seal had formed, the cell membrane inside the pipette was disrupted with a manipulator-controlled platinum wire. Voltage control was assessed by evaluation of the time course of the capacitive current and 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 typically -150 mV, although in some cells holding potentials as negative as -180 mV were used to confirm that full channel availability had been obtained at -150 mV. To maximize the signal-to-noise ratio, I_g protocols contained four repetitions at each test voltage that were one-fourth of a 60-Hz cycle out of phase.

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). Intracellular solution contained 200 TMA^+, 75 F^-, 125 MES^-, 10 EGTA, and 10 HEPES (pH 7.2). Tetramethylammonium (TMA) and 2-(*N*-morpholino)ethanesulfonic acid (MES) were used because they minimized current through either Na channels or any of the low-density background conductances in the tsA201 cells; hypertonicity compensated for the lower conductivity of the solutions containing these substitute ions. For measurements of I_g, Na^+ was replaced with TMA^+, and saxitoxin (STX) (Calbiochem Corp., San Diego, CA) was added to the extracellular solution at a concentration of at least 2.5 μM. The site-3 toxin used to modify hH1 channels was Ap-A toxin (Sigma Chemical Co, St. Louis, MO) at a concentration of 1 μM, which is at least three orders of magnitude greater than the K_D (Hanck and Sheets, 1995; Khera et al., 1995). After control measurements of I_{Na} and I_g were obtained, the cell was transferred to an extracellular solution containing STX and site-3 toxins, and I_g measurements of toxin-modified Na channels were obtained. To conserve Ap-A toxin and because site-3 toxins unbind extremely slowly at normal holding membrane potentials (Hanck and Sheets, 1995; Khera et al., 1995), I_{Na} recordings of toxin-modified Na channels were typically obtained in control solutions containing 15 mM Na_o after wash with STX and before there was appreciable unbinding of toxin. In some cells transfected with R1C, an Ap-A toxin concentration of 10 μM was used to confirm that full channel modification had been achieved with 1 μM.

Changes in bath solution were achieved by placing the pipette with the cell adjacent to the inlet of one of four parallel experimental chambers containing the experimental solution, and cells were exposed to site-3 toxins, maintaining a V_h of -150 mV. To wash site-3 toxins from Na channels, the membrane potential was depolarized to -10 mV for ~8 min in control solution. This procedure, which took advantage of the lower affinity of toxin for inactivated channels, allowed for dissociation of toxin from the channel (Hanck and Sheets, 1995; Khera et al., 1995). The temperature was controlled with a Sensortek (Physiotemp Instruments, Clifton, NJ) TS-4 thermoelectric stage mounted beneath the bath chambers and typically varied by less than 0.5°C during an experimental set. Cells were typically studied between 12°C and 13°C.

Data analysis

Peak I_{Na} was taken as the mean of four data samples clustered around the maximum value of data digitally filtered at 5 kHz and leak corrected by the amount of the calculated time-independent linear leak. Data were capacity corrected using 4–16 scaled current responses between the holding potential and 40 mV negative to it. Leak resistance (RL) was calculated as the reciprocal of the linear conductance between -190 mV and -110 mV, and cell capacitance was measured from the integral of the current responses to voltage steps between -150 mV and -190 mV. To determine time

constants of I_{Na} decay, the current traces were fit by a sum of exponentials with DISCRETE (Provencher, 1976), a program that provides a modified *F*-statistic to evaluate the number of exponential components that best describes the data. For gating charge measurements data were leak-corrected by subtracting the mean of the current typically taken between 8 and 10 ms for test potentials (V_t) < 0 mV, and between 6 and 8 ms for $V_t \geq 0$ mV. Running integrals exhibited a stable plateau except occasionally at the most positive potentials, when a small outward ionic current, which developed after a delay of several milliseconds, was sometimes present.

Data were analyzed and graphed on a SUN Sparcstation, using SAS (Statistical Analysis System, Cary, NC). Unless otherwise specified, all summary statistics are expressed as means ± one standard deviation (SD). Regression parameters are reported as the estimate and the standard error of the estimate (SEE).

RESULTS

Ionic currents

Ionic currents in response to step depolarizations are shown in Fig. 2 for representative cells expressing wild-type and mutant hH1 channels, R1C, R1Q, R2C, and R3C (in DIV-S4). All ionic current recordings were obtained at 12–13°C with 15 mM Na_o and no intracellular Na^+ both before and after modification by 1 μM Ap-A toxin. Each of the mutant channels expressed well, and I_{Na} kinetics were not grossly disrupted, although I_{Na} decay for R1C and R1Q was obviously slowed compared to R2C and R3C mutant channels and to wild-type Na channels. Despite the differences in the time courses of decay of I_{Na} in control solutions, after modification by Ap-A toxin all currents looked similar, with a markedly slowed I_{Na} decay, i.e., channel mutants could no longer be readily identified by the rate of I_{Na} decay.

To better compare the decay rates of the ionic current traces, they were fit by a sum of up to two exponentials. Two time constant fits were accepted when 1) they produced a statistically significant *F*-statistic (Provencher, 1976), 2) the longer time constant was not greater than the duration of the data fitted (~40 ms), and 3) the amplitude of a second time constant contributed to greater than 10% of the overall current amplitude. In control wild-type currents I_{Na} decays were better fit with two time constants 78% of the time. R3 mutant channels were similar, fitting two time constants 70% of the time. In contrast, R2 mutant channels were better fit with two time constants only 50% of the time, and R1 mutant channels only 9% of the time. When detected, the longer time constant was 12 ms in wild-type and 20 ms in R2C and R3C channels, it was not voltage dependent, and it contributed ~25% to the overall amplitude. After modification by Ap-A, all I_{Na} decays were almost always best fit by single time constants, with two time constants fitting best less than 10% of the time (7% for R1, 9% for R2, and 5% for R3). Fig. 3 graphically summarizes these data. The time constants for R1C and R1Q were similar and were the longest (Fig. 3 *A*). Neutralization of other arginines (R2 and R3) produced currents with much shorter time constants (Fig. 3 *B*) closer to those of wild-type currents. However, after modification of I_{Na} by site toxins, the decay time constants were similar for wild-type and all mutant channels (Fig. 3 *B*, *solid symbols*).

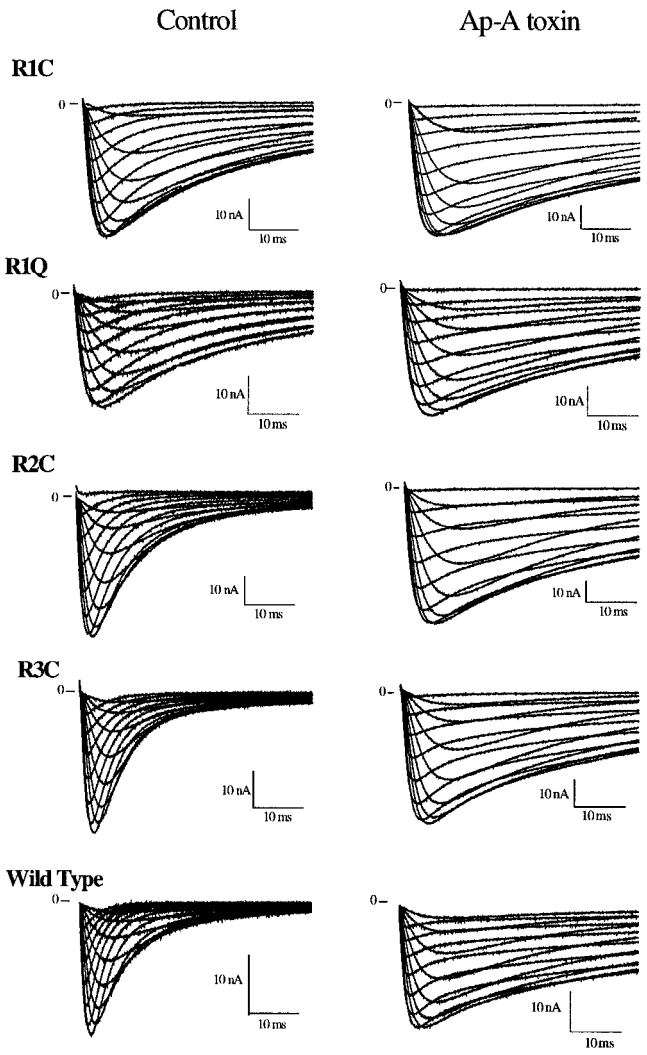

FIGURE 2 Family of I_{Na} responses for hH1 sodium channels mutations of R1C, R1Q, R2C, and R3C, and for wild-type Na channels. The holding potential was −150 mV (or −170 mV) with step depolarizations from −80 mV to 30 mV. The external solution contained 15 mM Na^+, and the internal solution contained TMA^+ without Na^+. The I_{Na} current traces were capacity-corrected but not leak-corrected and were digitally filtered at 5 kHz. On the left are shown I_{Na} in control, and the right panels show I_{Na} after modification by Ap-A toxin.

Conductance-voltage (*G-V*) relationships reflect voltage-dependent changes in peak I_{Na}, which, in turn, depend upon the overlap in the time course between channel activation and inactivation during a step depolarization. As a consequence, the differences in the rates of decay of I_{Na} under control conditions between Na channels mutated at R1, R2, and R3 may be manifested in their *G-V* relationships. Despite their large differences in the I_{Na} decay time course, *G-V* relationships in control solutions for all four mutants and wild-type hH1 were remarkably similar (Fig. 4), with no statistical differences in half-point or slope factor from Boltzmann fits (Table 1). After modification by toxin there was only a small hyperpolarizing shift in half-point and modest steepening of slope factor for each mutant (Fig. 5,

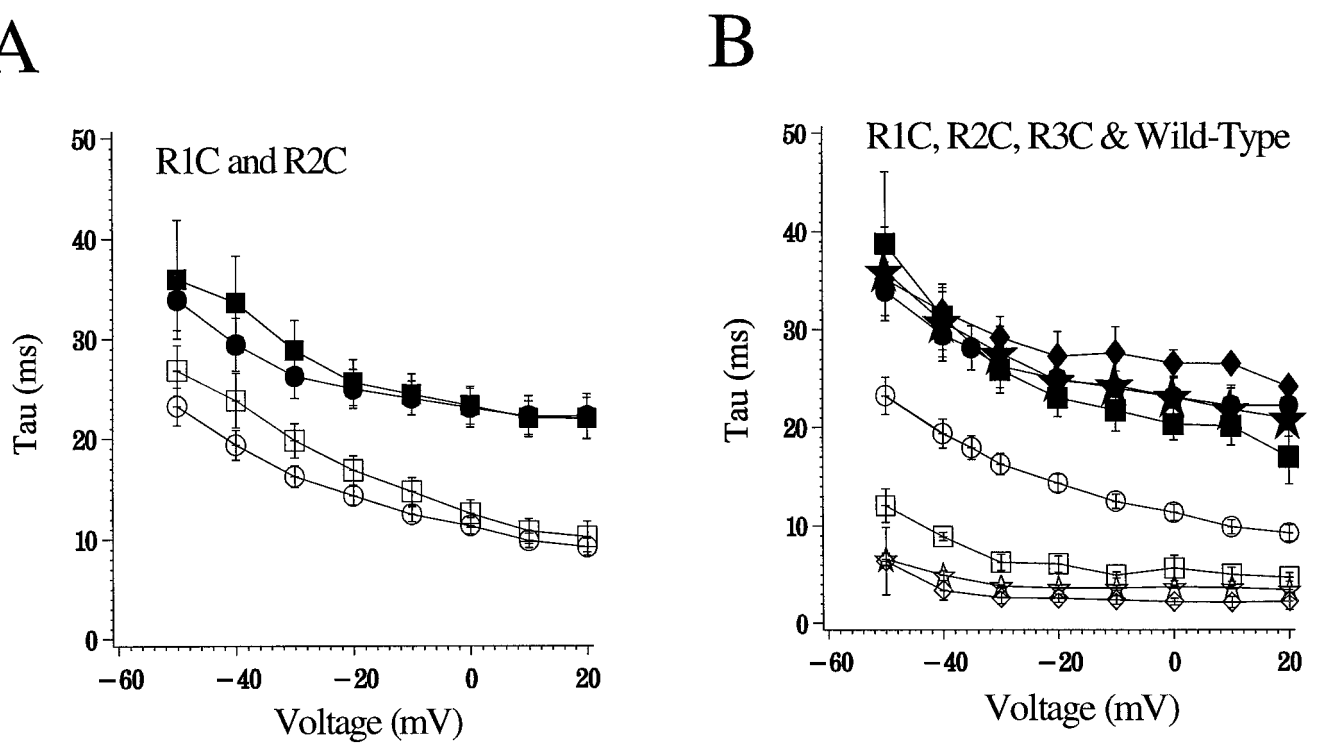

FIGURE 3 Voltage dependence of I_{Na} decay. (*A*) Mean time constants (± SEM) as a function of voltage based on single time constant fits to I_{Na} decay as described in the text for R1C ($n = 7$) and R1Q ($n = 4$). Data are shown for control (○, □) and after modification with Ap-A toxin (●, ■) for R1C (○, ●) and for R1Q (□, ■). (*B*) Mean time constants (± SEM) as a function of voltage for R1C (as in *A*), R2C ($n = 4$), R3C ($n = 6$), and wild type ($n = 2$). For wild-type, R2C, and R3C Na channels, if two time constants described the data, the tau making the largest contribution to the amplitude was graphed. For R1C and for all data obtained in the presence of site-3 toxin, the tau from the single exponential fit was used. Data are shown for control (○, □, ◇, ☆) and in toxin (●, ■, ◆, ★) for wild-type (◇, ◆), R3C (☆, ★), R2C (□, ■), and R1C (○, ●).

Table 1). These data were similar to those we have previously observed for both native cardiac Na channels and wild-type hH1a channels (Hanck and Sheets, 1995; Sheets and Hanck, 1999) and suggest that there is little overlap in the time course between activation and inactivation for both wild-type and mutant channels, and that Ap-A toxin exerts its most prominent effect on channel inactivation.

Gating current studies

We have already shown for native heart Na channels in canine cardiac Purkinje cells (Sheets and Hanck, 1995) and for wild-type hH1a Na channels (Sheets and Hanck, 1999) that site-3 toxins reduce Q_{max} by ~30%. If inhibition of the movement of the basic residues in DIV-S4 were to account for most or all of the reduction in Q_{max} caused by Ap-A toxin, then mutant channels with neutralization of basic residues in DIV-S4 should undergo a reduction of Q_{max} of less than 30% after modification by site-3 toxins. In addition, if each arginine were to contribute a similar amount of charge to the voltage sensor, then we would expect the reduction in Q_{max} by site-3 toxins to be equal for the four mutations. However, if the three outermost arginines in DIV-S4 do not contribute an equal amount to the voltage sensor, then the magnitude of reduction in Q_{max} after modification by site-3 toxins should be should be different for each of the three mutant channels. Consequently, the mutant channel with the neutralization of the arginine that makes the largest contribution to gating charge in wild-type channels should undergo the smallest reduction in Q_{max} after modification by Ap-A toxin.

Fig. 6 shows an example of a family of capacity and leak-corrected I_g traces and the corresponding integrals in control and after modification by Ap-A toxin for a cell expressing R3C mutant. For this cell, toxin modification reduced Q_{max} to 3.5 pC from 4.8 pC, a reduction of 27% that is similar in magnitude to the 31% reduction in Q_{max} found for wild-type hH1a (Sheets and Hanck, 1995).

The mean *Q-V* relationships for R3C and the other three mutant Na channels are shown in Fig. 7, and the values from the fits of a Boltzmann distribution (Eq. 2) to the *Q-V* relationships are summarized in Table 2. Also included in Table 2 are the parameters obtained from the *Q-V* relationships of wild-type hH1a recorded under similar conditions both before and after site-3 toxin modification (Sheets and Hanck, 1999). For R1C Ap-A toxin resulted in a small reduction in Q_{max} of 12% ($n = 7$ cells), whereas the reduction for R3C was 27% ($n = 6$ cells), which was not statistically different from wild type (31%). R2C had a reduction in Q_{max} after modification by Ap-A toxin of 22% ($n = 4$ cells), a value intermediate between those of R1C and R3C. To confirm that the smaller reduction in Q_{max} did

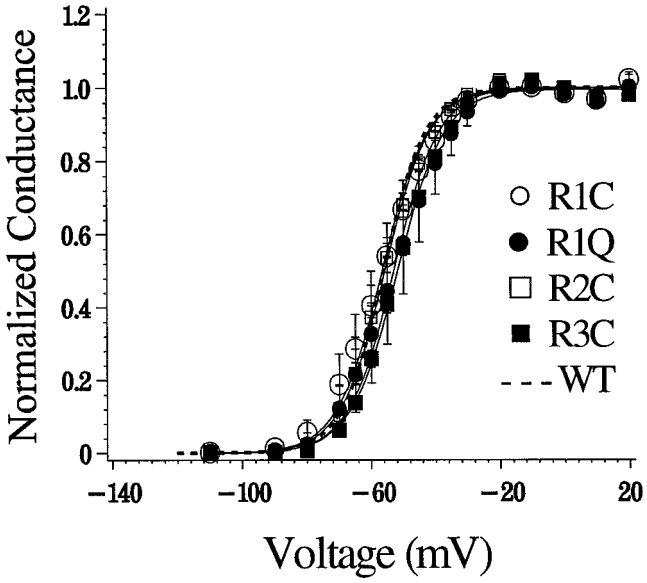

FIGURE 4 Normalized peak *G-V* relationships for R1C, R1Q, R2C, and R3C in control solutions. Also shown is the *G-V* relationship for wild-type hH1 recorded under similar control conditions (from Sheets and Hanck, 1999). The lines represent the mean of the best fits to each mutant channel by a Boltzmann distribution:

$$I_{Na} = \frac{(V_t - V_{rev})G_{max}}{1 + e^{(V_t - V_{1/2})/s}} \quad (1)$$

where I_{Na} is the peak current in response to a step depolarization, and V_t is the test potential. The parameters from the best fits were $V_{1/2}$, the half-point of the relationship; s, the slope factor (in mV); and V_{rev}, the reversal potential. Parameters are given in Table 1. G_{max}, the maximum peak conductance, was normalized to a value of 1 for each cell.

not result from incomplete modification of mutant channels by 1 μM Ap-A, 10 μM Ap-A toxin was applied to a subset of cells transfected with R1C, but the higher toxin concentration did not cause a further reduction in Q_{max} (data not shown). In addition, we studied neutralization of R1 to glutamine, a neutral residue that is similar in size to arginine and, therefore, may be less disruptive of secondary structure. However, the 13% reduction ($n = 4$ cells) in Q_{max} for R1Q was not distinguishable from that for R1C, confirming that the results were not specific to a cysteine substitution.

We have previously observed for wild-type channels that Ap-A toxin selectively reduced charge at positive potentials where channel inactivation is rapid (Sheets and Hanck, 1995), and this action was reprised for each of the four mutant channels (Fig. 7). Similar to the modest shift in the half-point of the *Q-V* relationship for wild-type channels after modification by Ap-A toxin, the *Q-V* relationships for R2C and R3C also demonstrated a small shift of their half-points (Table 2). In the mutants with the smallest effect of toxin on Q_{max} (R1Q, R1C), no shift in half-point was apparent. For both wild-type and mutant Na channels, there was no change in their slope factors of the *Q-V* relationships both before and after modification by Ap-A toxin, although the slope factors for all four mutant channels were less than that for wild-type channels. Possible causes for differing slope factors are raised in the Discussion.

Q_{max} compared to G_{max} for S4, DIV mutant Na channels

In addition to a smaller reduction in the magnitude of Q_{max} by Ap-A toxin, those Na channel mutants for which an arginine made a large contribution to gating charge should have less total gating charge per channel compared to wild-type hH1 and to the other mutant channels. Although a direct measurement of the total electronic charge per Na channel has not been made to date, we can compare Q_{max} to G_{max} for each of the four mutations and to wild-type hH1 recorded under similar conditions (see Sheets and Hanck, 1999). If the total electronic charge were decreased in the mutant channels compared to wild type, then it would be expected that the slope of the Q_{max} versus G_{max} relationship for the mutant channels would be less steep. Although such a comparison has limitations (see the Discussion), the relationships should allow for a qualitative comparison between mutant Na channels and wild-type hH1 Na channels. Fig. 8 shows a comparison of the four mutant and wild-type Na channels. Not surprisingly, the slopes of the relationships are nearly identical for R3C and wild-type Na channels, and they are nearly identical for R1C and R1Q, which have the shallowest slopes. R2C is intermediate between the two groups. These relationships agree with results from the effects of Ap-A toxin on Q_{max} of the four mutant channels and the wild-type Na channel. Also of note, it is readily apparent that in the 18 cells expressing R1C or R1Q, almost none of them had a Q_{max} much greater than 3.5 pC, whereas 50% of cells expressing the R3C mutant had Q_{max} magnitudes greater than this, suggesting that both R1 mutant Na channels have less total charge per channel.

DISCUSSION

Mutations of the three outermost arginine amino acids of DIV-S4 in hH1 Na channels (R1C, R1Q, R2C, and R3C) produced currents that activated over the same voltage range as wild-type channels, i.e., normalized peak *G-V* relationships were similar in slope factor and half-point (see Fig. 4), although the decay of I_{Na} was slowed by varying degrees. R1C and R1Q mutant channels had the slowest decays, whereas R3C mutant channels had decays of I_{Na} similar to that of wild type. The decay of R2C channels was intermediate between those for R3C and R1C channels. The effects of these mutations appeared similar to those observed in mutated human skeletal muscle Na (hSkM1) in which analogous residues were neutralized (Yang and Horn, 1995; Yang et al., 1996) and where slowing of I_{Na} decays was shown to reflect a slowing of inactivation from the open state (Chahine et al., 1994).

Regardless of the different rates of I_{Na} decay under control conditions for the four mutations, after modification by

TABLE 1 Comparison of Boltzmann parameters (mean ± SD) to fits of *G-V* relationships for domain IV mutant Na channels and wild-type hH1 channels in control and after Ap-A toxin

Parameter	R1C ($n = 7$)	R1Q ($n = 4$)	R2C ($n = 4$)	R3C ($n = 6$)	Wild type*
$V_{1/2}$ (mV) control	−57 ± 9	−53 ± 10	−58 ± 2	−52 ± 2	−56
Slope (mV) control	−7.1 ± 0.8	−7.1 ± 0.3	−7.0 ± 0.3	−7.3 ± 0.5	−6.6
$V_{1/2}$ (mV) toxin	−60 ± 9	−55 ± 8	−60 ± 2	−58 ± 2	−61
Slope (mV) toxin	−6.9 ± 0.5#	−6.8 ± 0.5#	−5.9 ± 0.2	−6.7 ± 0.5#	−5.8

*Taken from Sheets and Hanck (1999).
#Difference in toxin values compared to control for each channel is significant at $p < 0.05$.

Ap-A toxin the decay rates for the four mutant channels and wild-type channels were all slowed to a similar amount. There were only small changes in the *G-V* relationship after toxin modification, and these changes were similar in magnitude and direction to those observed for native and wild-type channels. The comparable effects of Ap-A toxin on the mutant Na channels suggest that toxin binding and channel modification were similar to toxin effects on both wild-type Na channels and native cardiac Na channels (Hanck and Sheets, 1995).

Q_{max} versus G_{max} relationships for mutant and wild-type hH1 Na channels

To directly measure the gating charge associated with a single ion channel, the total gating charge and the number of channels must be determined in the same preparation. So far, this has not been accomplished for Na channels. However, a relative comparison of gating charge can be made between mutant Na channels and wild-type hH1 by comparing Q_{max} to G_{max}. When the measurements are performed under similar experimental conditions, differences in G_{max} will be proportional to the number of channels if conductance and the probability of being open at peak I_{Na} are similar. It is unlikely that single-channel conductance of the four mutant channels is altered by mutations of the outermost arginines of DIV-S4 in hH1 Na channels. In hSkM Na channels with mutations of R1 of DIV-S4, single-channel conductance was similar to that of wild-type channels (Chahine et al., 1994), which is not surprising, because the mutations were in a segment of the channel that is not thought to be in the permeation path (Fozzard and Hanck, 1996; Doyle et al., 1998). On the other hand, the decay rates of I_{Na} were obviously different between the R1, R2, and R3

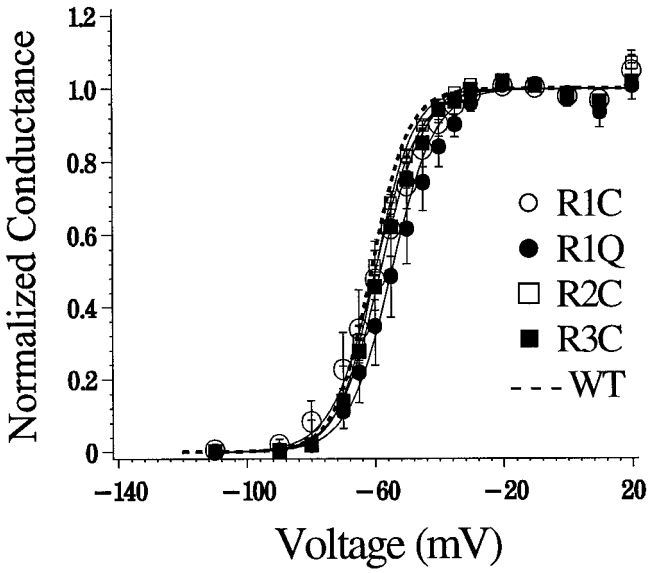

FIGURE 5 The effect of the site-3 toxin, Ap-A, on normalized *G-V* relationships for R1C, R1Q, R2C, and R3C. Also shown is the *G-V* relationship for wild-type hH1 modified by site-3 toxins and recorded under similar control conditions (from Sheets and Hanck, 1999). The lines represent the mean of the best fits of each cell to a Boltzmann distribution (Eq. 1), where $V_{½}$ is the half-point of the relationship and s is the slope factor (in mV). Parameters are given in Table 1. G_{max}, the maximum peak conductance, was normalized to a value of 1 for each cell.

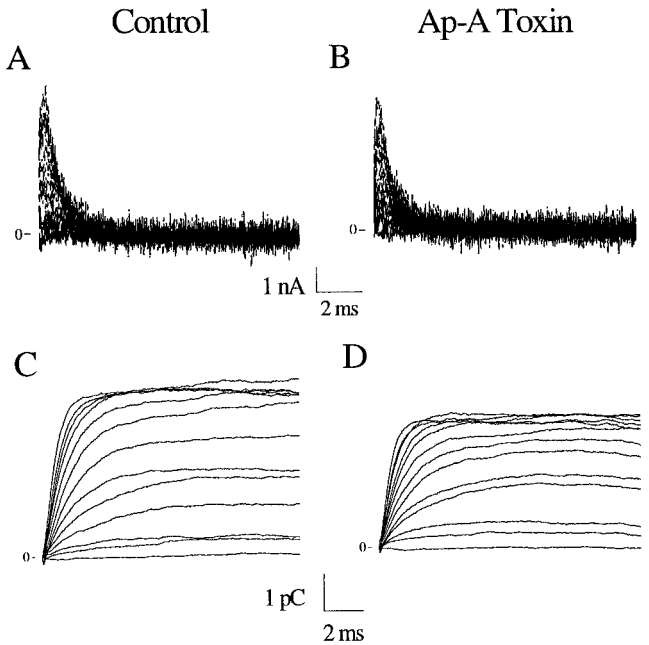

FIGURE 6 Family of gating currents (*top*) and their integrals (*bottom*) from a fused tsA201 cell cotransfected with DNA encoding the R3C Na channel and the β1 subunit. Recordings from cells studied in control solutions (*A* and *C*) and after Ap-A toxin modification (*B* and *D*). The traces were in response to step depolarizations from −120 to 40 mV, with a holding potential of −150 mV. Data shown were capacity and leak corrected and digitally filtered at 15 kHz at every fifth point plotted (cell W4.01).

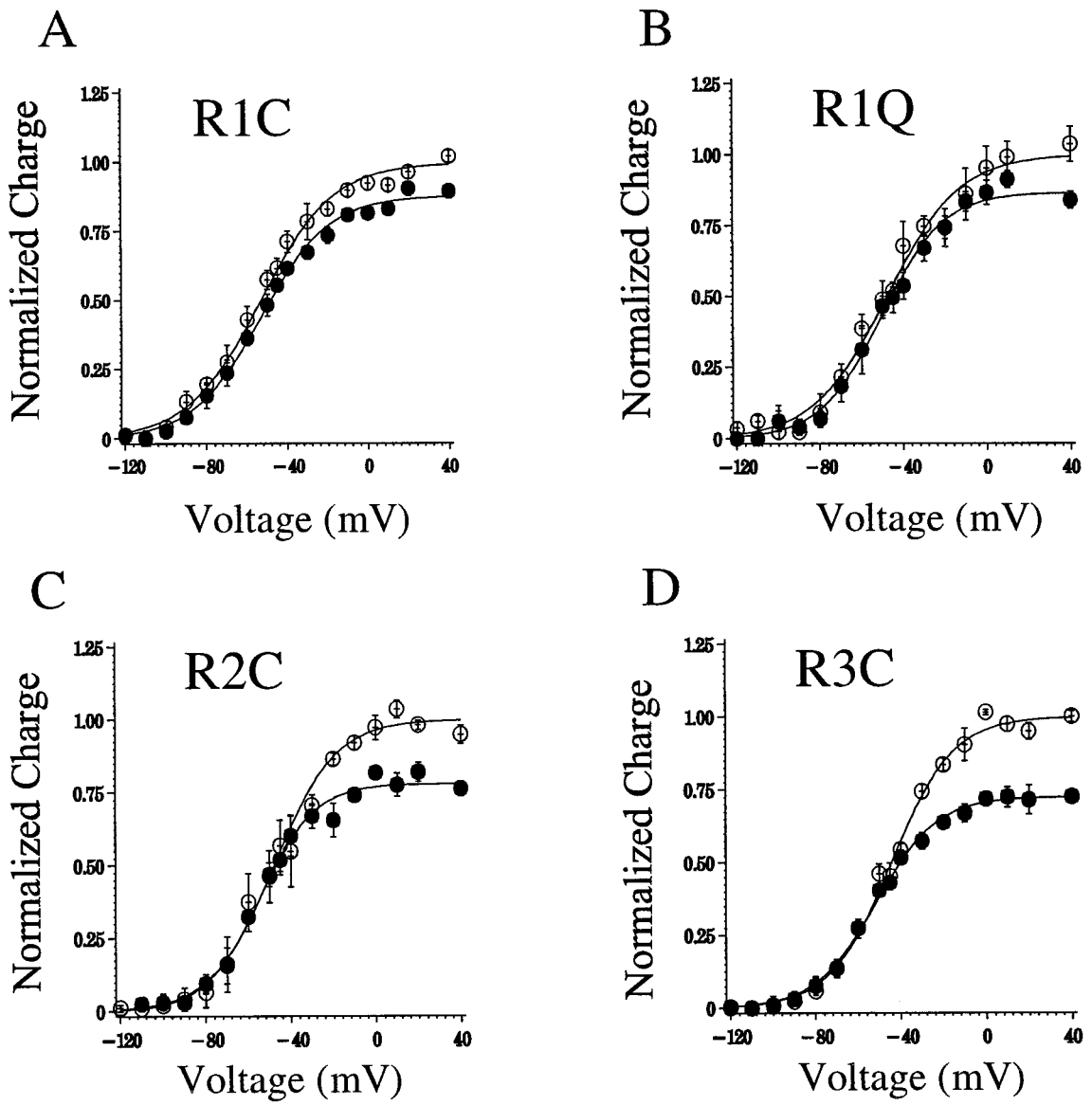

FIGURE 7 Effect of Ap-A toxin on *Q-V* relationships for R1C, R1Q, R2C, and R3C mutant Na channels. Data plotted are means ± SEM for cells in control (○) and after modification by Ap-A toxin (●). The solid lines represent the mean of the best fits to each cell by a Boltzmann distribution:

$$\text{Fractional } Q_{max} = \frac{1}{1 + e^{(V_t - V_{1/2})/s}} \quad (2)$$

where fractional Q_{max} is the charge during depolarizing step, V_t is the test potential, $V_{½}$ is the half-point of the relationship, and s is the slope factor (in mV). Gating charge in toxin was normalized to the Q_{max} determined for each cell in control. See Table 2 for the parameters from the best fits to the data.

mutant channels, which could affect the probability of a channel being open at peak I_{Na}. Changes in the overlap between the time courses of activation and inactivation would be expected to produce both a shift in the half-point of conductance and an increase in current amplitude. The half-point of *G-V* relationships of both wild-type and mutant channels was virtually unchanged by toxin, suggesting that overlap in the time courses of activation and inactivation was minimal. Nonetheless, toxin-modified currents were larger than those in control. We have previously reported small changes in G_{max} after toxin modification when Cs^+ was the intracellular replacement cation, but larger changes in G_{max} with intracellular TMA^+ (Hanck and Sheets, 1995), consistent with the changes in G_{max} reflecting a decrease in voltage-dependent block by intracellular TMA^+ (O'Leary and Horn, 1994). Consequently, the increase in G_{max} after site-3 toxin cannot be taken to reflect solely changes in the overlap of activation and inactivation. With these caveats in mind, G_{max} should be proportional to the number of Na channels, and the nearly identical slopes of Q_{max} and G_{max} for R3C and wild-type hH1 Na channels suggest that the total charge per channel may not be different (see Fig. 8).

TABLE 2 Comparison of Boltzmann parameters (mean ± SD) to fits of *Q-V* relationships for domain IV mutant Na channels and wild-type hH1 channels in control and after Ap-A toxin

Parameter	R1C (n = 10 cells)	R1Q (n = 4 cells)	R2C (n = 4 cells)	R3C (n = 6 cells)	Wild type (n = 5 cells)
$V_{1/2}$ (mV) control	−53 ± 10	−48 ± 9	−48 ± 11	−44 ± 4*	−55 ± 5
Slope (mV) control	−18 ± 3.6*	−18 ± 3*	−15 ± 3*	−14 ± 3*	−11 ± 2
$V_{1/2}$ (mV) toxin	−53 ± 7	−50 ± 4	−54 ± 7	−52 ± 3	−62 ± 9
Slope (mV) toxin	−17 ± 3	−15 ± 3	−13 ± 1	−14 ± 2	−12 ± 2
% reduction in Q_{max} by Ap-A toxin	12 ± 0.6#	13 ± 5#	22 ± 4#	27 ± 5#	31 ± 4#

*Difference in control values of mutant channels to control of wild-type hH1 are significant at $p < 0.05$.
#Reduction of Q_{max} in the presence of site-3 toxin values compared to control for each channel are significant at $p < 0.05$.
Values for wild-type hH1 Na channels are from Sheets and Hanck (1999).

The shallowest slopes were for R1C and R1Q, which would be expected if these channels were to have the smallest total charge per channel. R2C had a slope intermediate between those of R1C and R3C. These results are consistent with the outermost arginine making the greatest contribution to gating charge in hH1 channels while the remainder of the basic residues make a smaller contribution to overall gating charge of the channel.

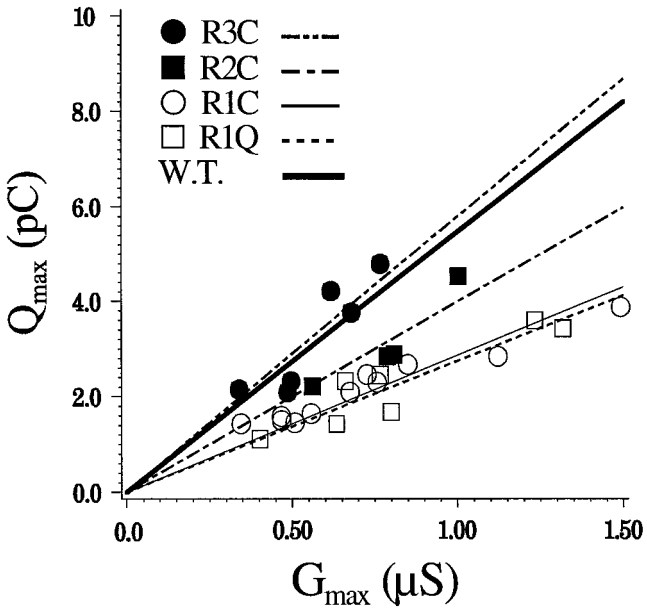

FIGURE 8 Relationship of Q_{max} versus G_{max} for R1C, R1Q, R2C, and R3C mutant Na channels. Q_{max} and G_{max} were obtained from the best fit of Boltzmann distributions to *Q-V* and *G-V* relationships for each of the mutants in control conditions. The lines represent the best fit by a least-squares regression with an intercept set to 0 for the four mutant channels, with the parameters given in the table below. The values for wild-type hH1 Na channels recorded under similar conditions are from Sheets and Hanck (1999).

Channel	Slope (pC nS^{-1})	R^2
R1C (n = 11)	2.9	0.98
R1Q (n = 7 cells)	2.7	0.98
R2C (n = 4)	4.0	0.99
R3C (n = 6)	5.8	0.98
Wild-type hH1 (n = 13)	5.4	0.97

Site-3 toxins inhibit gating charge in segment 4 of domain IV

Site-3 toxins have been shown to reduce Q_{max} by up to 33% in native cardiac Na channels, and we demonstrated that this charge was tightly coupled to the O↔I transition (Sheets and Hanck, 1995). Similar reductions in Q_{max} by site-3 toxins were found for hH1 Na channels (31%) and for rat skeletal muscle (rSkM1) Na channels (33%) (Sheets and Hanck, 1999). We postulated that site-3 toxins exert their effects on the putative voltage sensor formed by S4-DIV because 1) specific antibodies can bind to the extracellular loop between the S5 and S6 segments in domain IV of the α-subunit of the rat brain Na channel and inhibit site-3 toxin binding (Thomsen and Catterall, 1989); 2) chimeric studies of hH1 and rSkM1 Na channels demonstrated that domain IV was primarily responsible for the differing affinities of Ap-A toxin between the two Na channel isoforms (Benzinger et al., 1997); 3) single-channel studies of a mutation in the human skeletal muscle Na channel (hSkM1) where a cysteine is substituted for an arginine at position 1448 in DIV-S4 (i.e., the outermost arginine) demonstrated that inactivation was slowed from the open state, with little or no change in activation (Chahine et al., 1994).

The gating current studies reported here on Ap-A toxin modification of Na channels with mutations in one of three outermost arginines of DIV-S4 strongly support the prediction that site-3 toxins inhibit gating charge associated with the movement of DIV-S4. If the three outermost arginines were responsible for most of the gating charge that could be inhibited by site-3 toxins, it would be expected that Na channels with one of those arginines neutralized would have a smaller reduction in Q_{max} after toxin modification. These studies confirm that expectation and further suggest that the three outermost arginines do not contribute an equal amount to the voltage sensor in DIV-S4. Assuming that all of the reduction (31%) in Q_{max} by site-3 toxins in wild-type hH1 resulted from inhibition of movement by the putative voltage sensor in DIV-S4, the R1C mutation itself should account for the difference between the 31% reduction in wild-type hH1 and the 12% reduction in R1C mutant Na channels and would equal 19%. Table 3 shows the amount of reduction in Q_{max} that can be attributed to the neutralization of each of the outermost arginines (row 2) as well as

TABLE 3 Contribution of each of the three outermost arginine amino acids in segment 4 of domain IV to gating charge in hH1 Na channels

	R1C (n = 10)	R1Q (n = 4)	R2C (n = 4)	R3C (n = 6)	
Measured reduction in Q_{max} by Ap-A toxin (%)	12 ± 0.6*	13 ± 5*	22 ± 4*	27 ± 5*	31% reduction in wild-type
Calculated reduction in Q_{max} resulting from neutralization of arginine (%)[#]	19	18	9	4	Total of R1C + R2C + R3C = 32%
Fraction of Ap-A-sensitive charge caused by mutation*	0.61	0.58	0.29	0.13	Total of R1C + R2C + R3C = 1.03
Amount of charge from arginine based upon Ap-A toxin inhibiting 1.7e[§]	1 e	0.98 e	0.49 e	0.22 e	Total of R1C + R2C + R3C = 1.70e

*Calculated by dividing percentage reduction in Q_{max} by mutation in row 2 by 31% (amount of reduction in Q_{max} in wild-type hH1).
[#]Calculated by subtracting the percentage reduction in Q_{max} in toxin for each of the mutant Na channels from the percentage decrease for wild-type hH1 (i.e., 31%) (Sheets and Hanck, 1999).
[§]Value of total gating charge associated with the O↔I kinetic transition (Sheets and Hanck, 1999).

the relative contribution of each of the three outermost arginines to the total amount of gating charge that can be inhibited by Ap-A toxin (row 3). Note that the sum of relative fraction of gating charge for each of the three mutants has a value of almost 1, suggesting that the arginines from R1 to R3 account for all or almost all of the gating charge that can be inhibited by site-3 toxins.

From our studies on native heart Na channels, we estimated the magnitude of the total gating charge that was tightly coupled to O↔I transitions to be 1.7e (Sheets and Hanck, 1995). Using this value, the absolute amount of gating charge associated with each mutation can be estimated (Table 3) and predicts that R1 contributes a full e itself, whereas R2 and R3 contribute 0.49e and 0.22e, respectively. In addition, one can obtain an estimate of the total electronic charge per hH1 Na channel from the fact that 1e represents 19% of the total gating charge of hH1. This gives an estimate of 5.3e per channel, which is similar to our estimate of 5e obtained for native cardiac Na channels (Sheets and Hanck, 1995). This value is similar to estimates originally made for squid giant axon (Armstrong, 1981; Hille, 1992) but is much less than that predicted for skeletal muscle Na channels based on analysis of single-channel data at very negative potentials, where the probability of reaching the open state is extremely low (Hirschberg et al., 1995), and that predicted for *Shaker* K channels (Schoppa et al., 1992; Bezanilla et al., 1994; Zagotta et al., 1994). The calculation of total charge from the individual mutations is straightforward and reasonable, but it does make several important assumptions. For instance, it assumes that after neutralization of a charged residue the protein does not compensate for the missing charge by altering the electrostatic interactions between charged residues that remain. Studies of neutralization of charged S4 residues in *Shaker* K channels produced a total sum of effects on charge per channel greater than the individual charge perturbations (Aggarwal and MacKinnon, 1996; Seoh et al., 1996). Those findings suggested that small structural rearrangements can occur in mutant channels that are not large enough to produce gross changes in channel assembly or function, but they might affect the movement of residues that participate in channel gating.

Even though neutralization of the outermost arginines in DIV-S4 slowed decay of I_{Na} as did Ap-A toxin, charge neutralization did not appear to be equivalent to toxin binding. For instance, the slope factor of the Q-V relationship for each of the mutations was shallower than that for wild-type channels. The effect was graded, with the outermost arginine having the shallowest slope factor and R3C having the steepest. In addition, as in wild-type channels, toxin modification did not alter the slope factors of any of the mutant channels, suggesting a similar effect of Ap-A toxin on all channels. However, the different slope factors do suggest that DIV-S4 movement is not totally independent of other voltage sensors. It is possible that neutralization of the outer charged residues may unmask underlying cooperative effects that were not previously appreciated or may modify existing cooperativity between subunits.

Comparison with studies of other voltage-gated channels

Our results with site-3 toxins on the gating current of Na channel mutations in the S4 of domain IV are consistent with the three outermost arginines accounting for nearly all of the gating charge that can be inhibited by Ap-A toxin, although the charged residues do not contribute an equal amount to that charge. R1C contributes almost a full e, whereas R2C contributed only 0.5e and R3C contributed even less, 0.25 e. By extrapolation the remaining five basic residues in S4 DIV should make minimal contributions to the gating charge of Na channels. The greater contribution from residues on the NH_2-terminal end of the protein to gating charge compared to the contribution from residues on the CO_2H-terminal end has also been demonstrated for the *Shaker* K channel, which is formed from four identical subunits with each S4 containing seven basic residues (Aggarwal and MacKinnon, 1996). They found that the four outermost basic residues each contributed almost a full e each, the neutralization of the fifth outermost basic residue contributed 0.5 e, and the mutation of the innermost basic residue had no effect on the magnitude of gating charge. Similar results were also found for mutations of the second

to fourth outermost basic residues in N-terminus truncated *Shaker* K channels, except that each mutation resulted in a larger than expected decrease in gating charge, ranging between 4.9 to 6.8*e* instead of the anticipated decrease of 4*e* (Seoh et al., 1996). However, we found that mutations of the outer three arginines of DIV-S4 did not appear to sum to a greater amount of charge than anticipated. This may result from the fact that only one charged residue was mutated at a time in these studies, in contrast to the four charged residues mutated in studies of K channels.

Our results of site-3 toxins on mutations of the S4 in domain IV are consistent with many of the conclusions of studies based upon accessibility of cysteine mutations in the S4 of domain IV in human skeletal muscle (hSkM1) Na channels to methanethiosulfonate reagents (Yang and Horn, 1995; Yang et al., 1996). They found that the three outermost arginines could account for as much as 2.5*e*, although their studies suggested that R3 contributed more charge than R1, whereas our data suggest the opposite. Such a difference may result from a difference between Na channel isoforms, or may result from quantitative measurements based upon cysteine accessability to methanethiosulfonate reagents (Yang and Horn, 1995; Yang et al., 1996). This may reflect, in part, the presence of intrinsic dynamic molecular motions of the voltage sensors in the channel protein similar to the large molecular motions demonstrated by the ability of cysteine residues to form disulfide bonds in the putative Na channel pore (Benitah et al., 1997). In addition, recent studies have suggested that amino acid residues that are thought to be buried away from the pore in the selectivity filter of potassium channels (Doyle et al., 1998) may still be reactive with sulfhydryl-specific reagents when the residue is mutated to a cysteine (Dart et al., 1998).

Because the Na channel has four different domains compared to the four identical subunits of many voltage-gated K channels, it is not unexpected that one or more of the four domains in Na channels may have evolved such that each of the domains contributes uniquely to overall channel behavior. In contrast to *Shaker* K channels, in which most if not all of the gating charge results from channel activation transitions leading to channel opening (Schoppa et al., 1992; Bezanilla et al., 1994; Zagotta et al., 1994; Seoh et al., 1996), voltage-gated Na channels appear to move about one-third of their total gating charge after channels have opened (Sheets and Hanck, 1995, 1999). From our studies it is likely that the "late" movement of gating charge arises, in large part, from the S4 of domain IV and contributes to the coupling of inactivation to activation (French and Horn, 1983; Chahine et al., 1994). Additional evidence for the delayed movement of S4 of domain IV after channel activation has been obtained in hSkM1 Na channels where the outermost arginine in S4 of domain IV was mutated to a cysteine (R1448C) and labeled with a fluorescent probe (Cha et al., 1999 (see comments)). In that study a large component of the fluorescence signal from labeled R1448C was shown to correlate with channel inactivation and not with channel activation. Such a distinct role for the S4 of domain IV in Na channels should be distinguished from cooperativity between channel segments, where nonbasic residue mutations may affect channel activation transitions (Bezanilla et al., 1991; Aggarwal and MacKinnon, 1996; Smith-Maxwell et al., 1998a,b). In addition, evidence for a role of the S4 in domain II in Na channel activation (Mitrovic et al., 1998) suggests that the voltage sensors in domains I and III will also have discrete roles in channel kinetic transitions.

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