The Outermost Lysine in the S4 of Domain III Contributes Little to the Gating Charge in Sodium Channels

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ABSTRACT We investigated the contribution the four outermost basic residues (K1, R2, R3, R4) in segment 4 of domain III in the human cardiac Na channel (hH1a, $Na_V1.5$) to the total gating charge (Q_{max}). Each of the four basic residues were mutated individually to a cysteine. In addition, R2 was also mutated to a glutamate. All mutant channels were transiently expressed with the α1 subunit in fused tsA201 cells. We used the relative reduction in Q_{max} caused by anthopleurin-A (ApA) toxin, a site-3 toxin known to inhibit the movement of gating charge associated with domain IV, to estimate the size of the contribution from each basic residue. Studies of the toxin's ability to inhibit gating charge in mutant channels showed that R2 contributed 19–20% to the Q_{max}, R3 contributed 10%, and K1 and R4 made almost no contribution. In contrast to the outermost basic residue in the S4 of *Shaker* K channels and in the S4 of domain IV in hH1a, the outermost charge (K1) in domain III of Na channels is outside the voltage field.

INTRODUCTION

Voltage-gated ion channels have specialized structures called voltage sensors that respond to changes in the potential field resulting in an ordered voltage-dependent transition between non-conducting and conducting channel states. Voltage sensors were first proposed by Hodgkin and Huxley (1952), and were first recorded in 1973 (Schneider and Chandler, 1973; Armstrong and Bezanilla, 1973). Voltage sensors have been shown to be formed, in large part, by the fourth segment (S4) within a six transmembrane spanning segment motif in voltage dependent channels. S4 segments are thought to form α helices that each contain up to eight basic amino acid residues separated by two neutral residues (Bezanilla, 2000). Each of the six segments form a single subunit for channels such as the *Shaker* K channel or a single domain for channels such as the Na channel with a channel consisting of either four subunits or four domains. As a consequence, the *Shaker* K channel has four identical subunits each with a S4 containing seven basic residues while Na channels have four different domains each with S4's that contain different numbers basic residues (4 in I, 5 in II, 6 in III, and 8 in IV). Consequently, it would be expected that the relative magnitudes of gating charge contributed by each of the S4's may vary for Na channels but not for *Shaker* K channels.

Gating current (I_g) experiments from this laboratory have estimated the charge in domain IV charge to account for approximately 31% of total charge of the channel (Sheets et al., 1999), and we have shown that it moves slowly, largely after channel opening (Sheets and Hanck, 1995). FRET experiments have demonstrated that the S4's in domains I and II move rapidly while those in domains III and IV moves more slowly (Cha et al., 1999). These data suggest a critical role for domain III gating charge in channel opening, and we, therefore, sought to quantify the contribution of S4 charges in domain III to channel gating.

According to the alignments given for Na channel sequences (Goldin, 1995), the S4 in domain III of the human heart Na channel (hH1a, $Na_V1.5$) contains a total of six basic residues. In order to access their contribution to channel gating we individually neutralized the four outermost basic residues in the human heart Na channel. We took advantage of the fact that site-3 toxins selectively inhibit the movement of domain IV, S4 charge (Sheets et al., 1999) by binding to extracellular amino acid residues in that domain and perhaps to residues in domain I (Tejedor and Catterall, 1988; Thomsen and Catterall, 1989; Benzinger et al., 1998) but not to domain III. Comparison of gating charge recorded in the absence and presence of anthopleurin A toxin, therefore, allowed us to estimate the contribution of each residue to maximal gating charge (Q_{max}). Similar to previous studies of domain IV of the Na channel (Yang et al., 1996; Sheets et al., 1999) and for *Shaker* K channels (Aggarwal and MacKinnon, 1996; Seoh et al., 1996), residues in the S4 of domain III that were closer to the intracellular side of the channel contributed sequentially less to charge than those located more extracellularly. However, the second outermost basic residue, an arginine, made the greatest contribution to gating charge while the outermost basic residue, a lysine, made no significant contribution, suggesting that K1 was outside the electric field.

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MATERIALS AND METHODS

cDNA clones

In hH1a Na ($Na_V1.5$) channels (kindly provided by H. Hartmann and A. Brown (Hartmann et al., 1994) the basic amino acid residues at positions

1299, 1302, 1305, and 1308 (referred to K1, R2, R3, and R4, respectively) were individually mutated to cysteines by 4-primer PCR (Benzinger et al., 1998). In addition, R2 was also mutated to a glutamine (R2Q). The equivalent positions in the hH1 Na channel (Gellens et al., 1991) are 1300, 1303, 1306, and 1309. All cDNA inserts were confirmed by sequencing. Because our anecdotal experience has suggested that block of I_{Na} by tetrodotoxin increased the survival of cultured cells transiently transfected with Na channels, the sensitivity of mutant channels and wild-type channel to block by tetrodotoxin was increased by mutating the cysteine at position 373 to a tyrosine (C373Y) (Satin et al., 1993; Chen et al., 1996). The cDNA's were subcloned directionally into the mammalian expression vector pRcCMV (Invitrogen, Carlsbad, CA) as was the cDNA for the rat α1 subunit (Satin et al., 1994). For all studies, both α and α1 subunits were cotransfected with a mole ratio of α to α1 of about 1:2.

Cell preparation

Multiple tsA201 cells (SV40 transformed HEK293 cells) were fused together using polyethylene glycol as previously described (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, Grand Island, NY). Tetrodotoxin (300 nM) was added to the culture media one day after transfection. 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). Currents were recorded 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 kHz. A fraction of the current was fed back to compensate for series resistance. Temperature was controlled using a Sensortek (Physiotemp Instruments, Inc., Clifton, NJ) 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 sodium channel availability, the holding membrane potential was set between -150 and -180 mV. I_g protocols contained four repetitions at each test voltage that were ¼ of a 60 Hz cycle out of phase to improve the signal to noise ratio.

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 measurement of I_g the extracellular Na^+ was removed and replaced with TMA^+, and 10 μM saxitoxin (Calbiochem Corp., San Diego, CA) was added to the extracellular solution. Anthopleurin-A toxin (Sigma Chemical Co, St. Louis, MO) was used at a concentration of 1 μM, which is three orders of magnitude greater than the K_D (Hanck and Sheets, 1995; Khera et al., 1995).

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. Leak currents were calculated from the linear conductance measurements obtained between -190 and -110 mV. Data were capacity corrected using 4 to 16 scaled current responses recorded from voltage steps of 40 mV negative to the holding potential. Normalized peak G-V relationships were fit with a Boltzmann distribution:

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

where I_{Na} is the peak current in response to a step depolarization, V_t is the test potential and the fitted parameters were $V_{1/2}$, the half-point of the relationship, s, the slope factor in millivolts, G_{max}, the maximum peak conductance, and V_{rev}, the reversal potential. For comparison between cells, data were normalized to G_{max} in control solutions before exposure to ApA toxin. Time-to-peak I_{Na} relationships were fit with a single exponential equation:

$$\text{Time to peak } I_{Na} = A*e^{-V_t/s} + K \quad (2)$$

where A is the time to peak I_{Na} at a test voltage of -65 mV, V_t is the test potential, s is the slope factor in milliseconds, and K is a constant. An equation similar to Eq. 2 was used to determine the voltage shift required to relocate the curve for R3C onto the mean time-to-peak values of the other three cysteine-mutant channels by fixing the parameters for A, s, and K to those for R3C, and introducing an additional variable, V, in the exponential, i.e., $(-V_t + V)$, before refitting the data.

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

$$Q = Q_{max}/(1 + e^{(V_t - V_{1/2})/s}) \quad (3)$$

where Q is the charge during depolarizing step, Q_{max} is the maximum charge, V_t is the test potential, $V_{1/2}$ is the half-point of the relationship, and s is the slope factor in millivolts. For comparison between cells fractional Q was calculated as Q/Q_{max} for each cell in control solution.

Data were analyzed and graphed on a Sun Sparcstation using SAS (Statistical Analysis System, Cary, NC). Unless otherwise specified summary statistics are expressed as means $\pm$ 1 S.D., and figures show means $\pm$ S.E. Experimental parameters for mutant channels were compared using paired t-tests, and were considered significantly different at $p < 0.05$.

RESULTS

Ionic current studies

All of the four mutant Na channels, K1C, R2C, R3C, and R4C in the S4 of domain III, expressed well, and families of ionic currents in response to step depolarizations are shown in Fig. 2 (*left*). Both the onset and decay of I_{Na} for the four mutant hH1a channels were similar to wild-type channels in control solutions, as has previously been observed for mutations of R2 and R4 in rat brain IIA (Kontis and Goldin, 1997) and in K1 and R3 in hH1 (Chen et al., 1996). All of the mutant channels could be fully modified by ApA toxin, i.e., currents exhibited the expected prominent slowing of decay while the onset of I_{Na} appeared unchanged (Fig. 2, *right*). This was consistent with previous findings that site-3 toxins exert their most prominent effect on channel inacti-

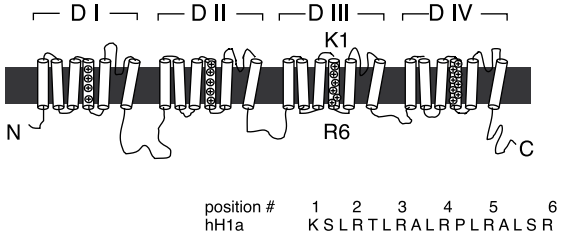

FIGURE 1 Predicted membrane topology of the sodium channel showing the four domains each with six transmembrane segments, the basic residues in the fourth membrane spanning regions (S4), and the intracellular locations of both the N and C termini (*top*). The amino acid sequence for the S4 of domain III with numbering of the six basic residues from the extracellular surface to the intracellular surface is shown at the *bottom*. The sequence corresponds to amino acids at positions 1299 to 1315 in the hH1a Na channel (Hartmann et al., 1994.)

vation with little or no change in channel activation both in wild-type hH1a channels (Sheets and Hanck, 1999) and for hH1a channels with mutations of in the S4 of domain IV (Sheets et al., 1999).

To allow better comparison of the four mutations, normalized peak conductance-voltage (*G-V*) relationships were constructed and fit with Boltzmann relationships (Fig. 3 *A*).

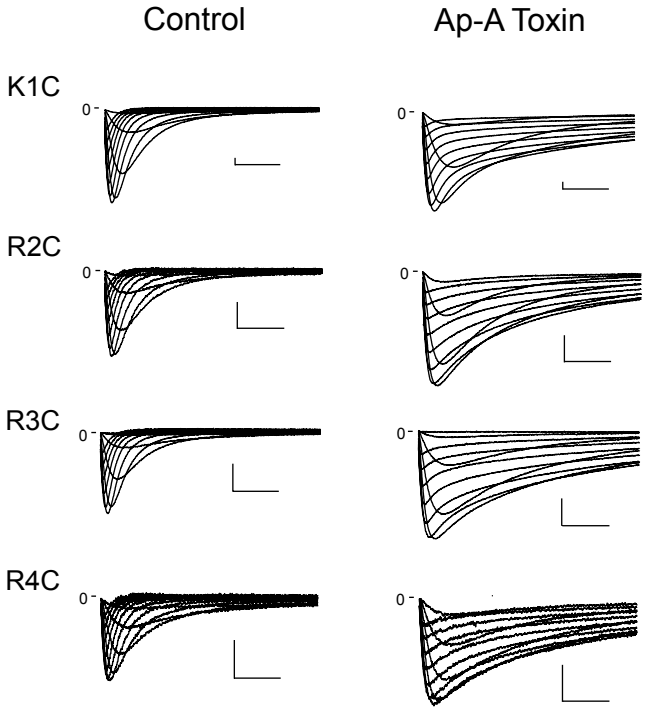

FIGURE 2 Families of leak-and capacity-corrected I_{Na} during step depolarizations to potentials between −80 and +20 mV in increments of 10 mV for each of the four mutations studied. Traces are shown in control solutions (*left*) and after modification by ApA toxin (*right*). Scale bars are 10 nA and 10 ms in each panel. Cells b3.20, b4.22, b5.02, b6.32.

Also shown is the *G-V* relationship for wild-type hH1a with the pore mutation alone, C373Y, studied under identical conditions. Because Na channel kinetic indices can vary as a function of time after the start of internal perfusion (Hanck and Sheets, 1992; Sheets and Hanck, 1999), the *G-V* data were selected so that the mean durations of time from the start of internal perfusion for each cell were similar and varied by only 5 min (range 16–21 min). As might be expected for channels in which basic residues that were putatively involved in channel activation had been neutralized, the slope factors were modestly reduced for most of the mutants (K1C, R2C, and R4C), although this was not the case for R3C (Table 1). The most obvious differences between the mutations were the conductance half-points ($V_{1/2}$) (Table 1), i.e., the voltage range over which channels activated. $V_{1/2}$ varied 13 mV from −49 mV for R3C to −62 mV for R4C. Time-to-peak I_{Na}, a measurement influenced by both channel activation and inactivation, is also shown in Fig. 3 *B* for each of mutations. To allow for comparison between the mutant channels, the time-to-peak I_{Na} were fit with a single exponential function (Eq. 2), and fitted values are reported in Table 2. The similar slope factors for the time-to-peak I_{Na} relationships between the five Na channels permitted the determination of the voltage shift (V) required to relocate the most positive curve (R3C) onto the other curves (see Methods). The voltage shifts for the time-to-peak curves were comparable to the differences in $V_{1/2}$ of the *G-V* relationships for each of the Na channels with those for K1C and R4C showing the greatest negative shift by 13 and 10 mV, respectively (Table 2).

Gating current studies

We have previously compared the maximum ionic conductance to maximum gating charge for neutralizations in domain IV (Sheets et al., 1999), but this measurement requires that both single channel conductance and the maximum probability of channel opening at the peak I_{Na} remain similar for all channels. Although charge neutralization in domain III is not expected to affect single channel current magnitude, Na channel mutations with neutralizations in the voltage sensors putatively involved in activation are likely to affect the probability of channel's being open at peak I_{Na}. To avoid this concern, we took advantage of the fact that site-3 toxins inhibit movement of the voltage sensor associated with the S4 in domain IV (Sheets et al., 1999) producing a decrease in Q_{max} of 31% in wild-type channels (Sheets and Hanck, 1999). Because the actions of ApA toxin are confined to the S4 of domain IV, the relative contribution of basic residues to gating charge in the S4's from other domains to overall Q_{max} could be measured by comparing the fractional reduction in Q_{max} by ApA toxin in the mutated channel to that in the wild-type channel. For example, if a basic residue in domain III were to make a large contribution to the overall Q_{max}, and that residue were

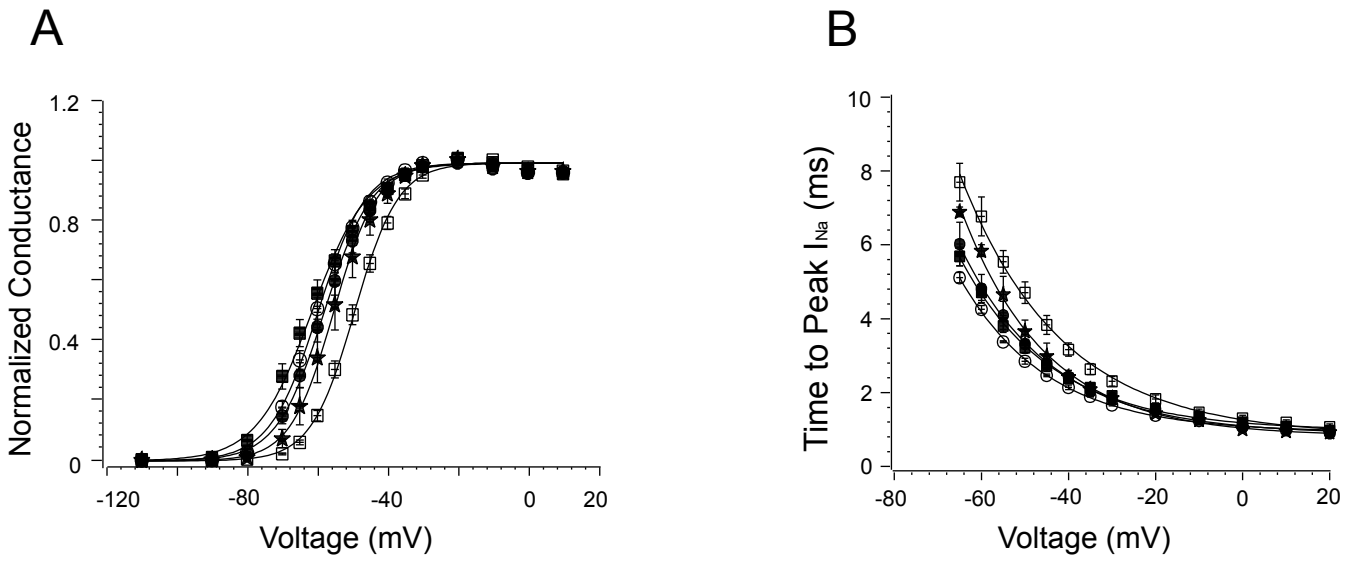

FIGURE 3 Normalized peak *G-V* relationships for R1C (○), R2C (●), R3C (□) , R4C (■) and wild-type hH1a (★) in control solutions (*A*) and their time-to-peak I_{Na} curves (*B*). The lines in *A* are the best fits by Boltzmann relationship (Eq. 1) with parameters given in Table 1. Data were normalized to maximum peak conductance for each cell. The lines in *B* are single exponential fits (Eq. 2) to the data; best fit parameters are given in Table 2.

neutralized, then the relative proportion of gating charge contributed from the S4 of domain IV in that mutant channel would increase, i.e., ApA toxin would cause a larger reduction in the Q_{max}. Conversely, if a basic residue made no contribution to Q_{max} in wild-type Na channels, then neutralization of that charge residue would have no effect on the magnitude of reduction of Q_{max} by toxin. Eq. 4 describes the relationship between the contribution to total gating charge by a residue in domain III and the reduction in Q_{max} by site-3 toxin:

$$\text{Fraction contributed by residue} = 1 - (0.31/\text{reduction of } Q_{max} \text{ in mutant channel by toxin}) \quad (4)$$

where 0.31 represents the decrease in Q_{max} by ApA toxin in wild-type channels.

Gating currents were recorded in all mutant Na channels by removing extracellular Na^+ and adding 10 μM STX. Fig. 4 shows an example of a family of capacity and leak-corrected I_g traces and their corresponding integrals both in control solutions and after modification by ApA toxin for a cell expressing Na channels with the R2C mutation. In this cell, toxin modification reduced Q_{max} from 12.6 pC to 8.0 pC, a reduction of 37% that is 6% greater than the 31% found for wild-type hH1a (Sheets et al., 1999).

The mean *Q-V* relationships for R2C and the other mutant Na channels are shown in Fig. 5, and the values from the fits of Boltzmann distributions (Eq. 3) are summarized in Table 3. Also included in Table 3 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). Similar to the *G-V* relationships, the half-point of the *Q-V* relationship for R3C was the most positive (−48 mV) and the most negative for R4C (−63mV). As is the case for both native heart Na channels (Hanck et al., 1990) and wild-type hH1a Na channels (Sheets and Hanck, 1999), the half-points of the *Q-V* relationships for the mutant channels were similar to those of the *G-V* relationships. Based on the fits of the *Q-V* relationships the voltage dependence of gating charge (i.e., slope factor) for each mutant was modestly decreased com-

TABLE 1 Comparison of Boltzmann parameters (mean ± S.D.) from fits to *G-V* relationships for K1C, R2C, R3C, R4C (all in domain 3), and wild-type hH1a in control solutions

Parameter	K1C (n = 4)	R2C (n = 7)	R3C (n = 6)	R4C (n = 10)	Wild type (N = 3)
$V_{1/2}$ (mV) control	−60 ± 2	−58 ± 4	−49 ± 2*	−62 ± 5	−55 ± 4
Difference in $V_{1/2}$ compared to R3C (mV)	11	9	0	13	6
s (mV) control	−6.9 ± 0.3*	−6.8 ± 0.4*	−6.2 ± 0.3	−7.9 ± 0.7*	−6.2 ± 0.3

*Difference in values compared to control for each channel is significant at $p < 0.05$

TABLE 2 Comparison of single exponential fits (mean ± S.D.) to time-to-peak I_{Na} curves for K1C, R2C, R3C, R4C (all in domain 3), and wild-type hH1a in control solutions

Parameter	K1C (n = 4)	R2C (n = 7)	R3C (n = 6)	R4C (n = 10)	Wild type (N = 3)
s (ms)	20 ± 2	21 ± 3	24 ± 5	21 ± 1	19 ± 3
Shift to align to R3C (mV)	13	9	0	10	5

parable to the findings for the *G-V* relationships. In addition, the slope factors and half-points of the *Q-V* relationships were not affected by the site-3 toxin similar to previous findings for mutations in domain IV (Sheets et al., 1999).

As anticipated, the magnitude of reduction in Q_{max} by toxin differed between the mutant channels. Surprisingly, for K1C, a mutation of the outermost basic residue, the reduction in Q_{max} of was 32%, a value comparable to wild-type channels, which predicts that this charge made little or no contribution to the gating charge of hH1a channels. In contrast, ApA toxin caused a 38% reduction in the Q_{max} of R2C, the largest reduction by toxin in all four of the Na channel mutations. Its calculated fractional contribution to total Q_{max} was 19%. In addition, we studied the neutralization of R2 to a glutamine, a residue that is similar in size to arginine and less affected by the surrounding pH compared to cysteine. As a consequence, R2Q may be less disruptive of secondary structure. The reduction in the Q_{max} of R2Q by toxin (39%) was nearly identical to that for R2C, confirming that the results were not specific to the choice of a cysteine as a substitute amino acid residue. The mutation, R3C, had a reduction of 34%, an intermediate value, and its relative contribution to Q_{max} was about 10%. The 32% reduction in Q_{max} in R4C by toxin was similar to that for both K1C and wild-type, predicting that it made only a negligible contribution to total Q_{max}.

DISCUSSION

We studied the contributions of the basic residues in the S4 of domain III in hH1a to Q_{max} by mutating, one-by-one, the four outermost basic residues to neutral residues. All mutant Na channels expressed well in a mammalian expression system that allowed for measurement of both ionic currents and gating currents. The S4 of domain III has been shown to translocate after movement of the S4's in domains I and II, but before movement of the S4 in domain IV (Cha et al., 1999). In contrast to neutralization of the three outermost basic residues of the S4 in domain IV of hH1a where the half-points of the *G-V* relationships varied less than 5 mV (−55 mV to −60 mV) (Sheets et al., 1999), the half-points of the *G-V* relationships for the neutralization of charged residues by cysteines in the S4 of domain III showed a greater variation (Fig. 3) ranging from −49 mV in R3C to −62 mV in R4C (13 mV). Similar shifts were also present in the voltage dependence of the time-to-peak I_{Na} (Table 2). Because peak I_{Na}, in large part, is dependent upon channel

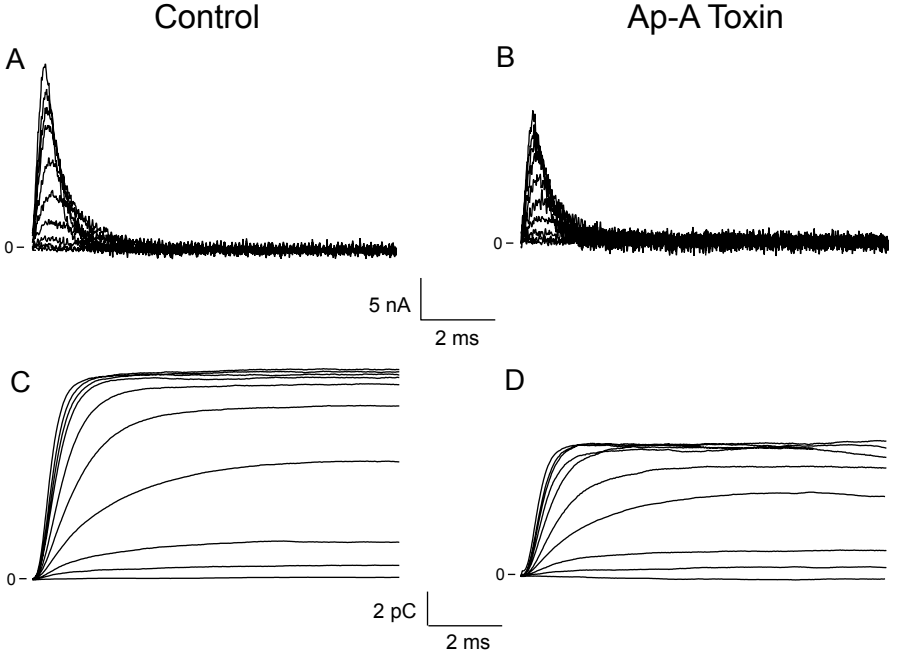

FIGURE 4 An example of a family of gating currents (*top*) and their integrals (*bottom*) from a fused tsA201 cell expressing the R2C mutant Na channel. Recordings from a cell studied in control solutions (*A* and *C*) and after ApA toxin modification (*B* and *D*). The traces were in response to step depolarizations from −120 to 40 mV with a holding potential of −160 mV. The data are shown capacity and leak-corrected and digitally filtered at 15 kHz with every fourth point plotted. (Cell b4.11).

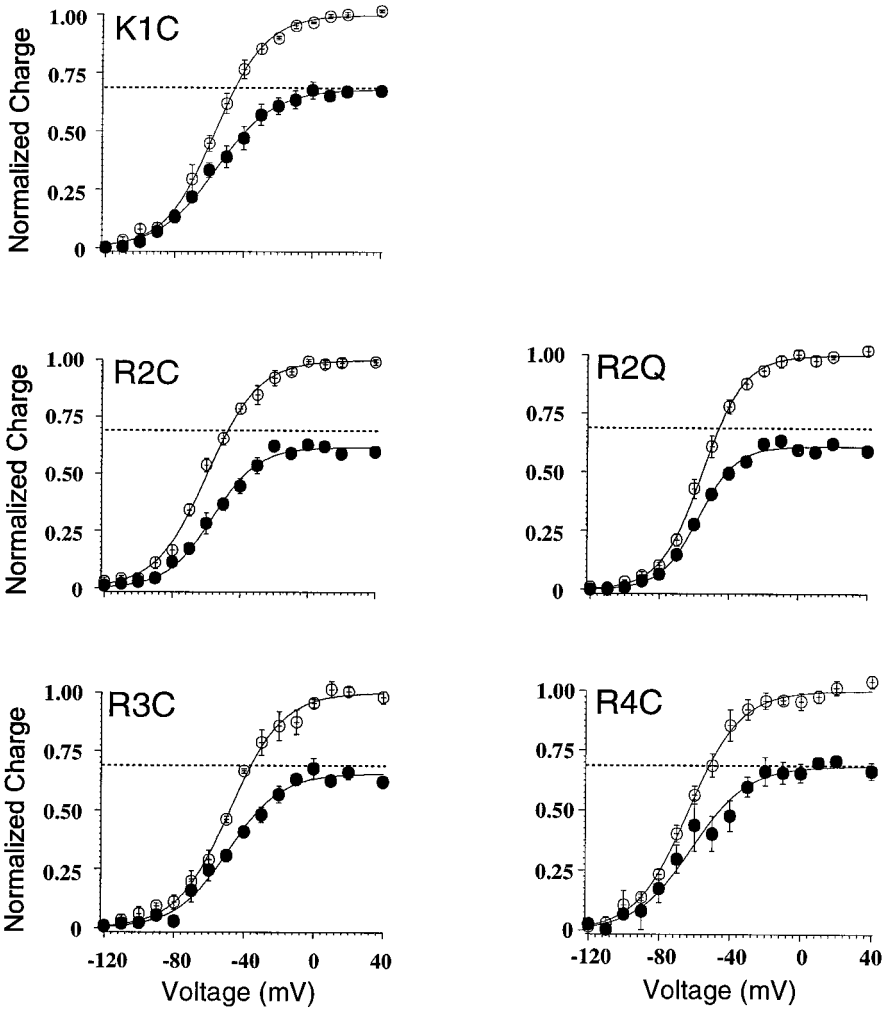

FIGURE 5 Effect of ApA toxin on *Q-V* relationships for K1C, R2C, R2Q, R3C, and R4C mutant Na channels. Data plotted are means ± S.E. for cells in control (○) and after modification by ApA toxin (●). The solid lines represent the mean of the best fits to each cell by a Boltzmann distribution (Eq. 3) while the dashed line in each panel represents a 31% decrease in Q_{max} seen in wild-type hH1a (see text). Gating charge in toxin was normalized to the Q_{max} determined for each cell in control. The number of cells and the parameters from the best fits to the data are given in Table 3.

activation leading to the open state(s), it is not surprising that the half-point of *G-V* relationships of mutations in domain III were more affected by neutralizations than those mutations of the S4 in domain IV, a domain that has been shown to move largely after channel opening (Hanck and Sheets, 1995; Sheets and Hanck, 1995). Similar variations in the voltage range of Na channel activation have been report for charge neutralizations of R2Q and R4Q in the S4 of domain III in the rat brain IIA Na channel (Kontis et al., 1997). Furthermore, neutralization of K1 to glutamine in

TABLE 3 Comparison of Boltzmann parameters (mean ± S.D.) from fits of *Q-V* relationships for K1C, R2C, R3C, R4C (all in domain 3) in control and after ApA toxin

Parameter	K1C (n = 4 cells)	R2C (n = 5 cells)	R2Q (n = 4 cells)	R3C (n = 3 cells)	R4C (n = 3 cells)	Wild type* (n = 5 cells)
$V_{1/2}$ (mV) control	−57 ± 4	−59 ± 2	−55 ± 4	−48 ± 1	−63 ± 4	−55 ± 5
s (mV) control	−14 ± 1	−15 ± 3	−12 ± 2	−15 ± 3	−15 ± 3	−11 ± 2
$V_{1/2}$ (mV) toxin	−56 ± 6	−56 ± 5	−57 ± 3	−49 ± 4	−62 ± 10	−62 ± 9
s (mV) toxin	−16 ± 3	−13 ± 1	−11 ± 1	−15 ± 3	−16 ± 3	−12 ± 2
% reduction in Q_{max} by ApA toxin	32 ± 2†	38 ± 2†	39 ± 2†	34 ± 3†	32 ± 4†	31 ± 4
% relative contribution to Q_{max}‡	2 ± 7	19 ± 4	20 ± 4	10 ± 7	1 ± 11	Not applicable

*Values for wild-type hH1a Na channels are from Sheets and Hanck (1999).
†Reduction of Q_{max} in the presence of site-3 toxin compared to Q_{max} in control solution for each channel are significant at $p < 0.05$.
‡Calculated using Eq. 4.

hH1 expressed in *Xenopus* oocytes also produced channels with a large negative shift of the half-point of conductance while the mutation R3Q showed a large positive shift (Chen et al., 1996), both findings consistent with the results in this study. The concordance of findings in the three studies indicates that shifts in the voltage range over which channels activate were not specific to the selection of the neutral residue (i.e., to the size of the replacement residue), to the channel isoform, or to the background in which channels were expressed, but suggest a direct role of charge within the voltage field. Consistent with a field effect of charge neutralization, the half-points of the *Q-V* relationships of the mutant channels were similar to those for their *G-V* relationships as previously observed for wild-type hH1a Na channels (Sheets and Hanck, 1999) and for cardiac Na channels in native hearts cells (Hanck et al., 1990).

Calculation of charge per channel

The sum of the individual contributions by the four basic residues from K1 to R4 predict that domain III contributed approximately 30% to the overall channel's gating charge (Table 3). Together with domain IV, previously estimated to contribute 31% to Q_{max} (Sheets et al., 1999), the S4's of domains III and IV contribute about 61% of the total gating charge. This value is comparable to the approximate 60% of gating charge that could be "immobilized" by fast inactivation (Armstrong and Benzanilla, 1977; Kuhn and Greeff, 1999; Sheets et al., 2000) that has been shown to result from the slow movement of the S4's in domains III and IV during repolarization (Cha et al., 1999). Consequently, it follows that the S4's in domains I and II should contribute the remaining 40% of gating charge.

Additionally, an estimate of the total charge per hH1a Na channel can be obtained if it is assumed that the most charge that any charged residue can contribute is 1 electronic charge (*e*) and assign that value to the residue making the largest fractional contribution. This would specify 1 *e* to R2 that was shown to contribute 19–20% of the total gating charge, and would predict that the total gating charge for the channel to be around 5 *e*. This is similar to our previous estimate of 5 *e* predicted for native cardiac Na channels (Sheets and Hanck, 1995). However, this value is much less than the 12 *e* predicted for skeletal muscle Na channels based on analysis of single channel data (Hirschberg et al., 1995) and the 12–14 *e* found for *Shaker* K channels (Schoppa et al., 1992; Bezanilla et al., 1994; Zagotta et al., 1994; Aggarwal and MacKinnon, 1996; Seoh et al., 1996; Noceti et al., 1996). Although the estimation of total charge using this method is straightforward, it does make several important assumptions that are difficult to experimentally verify. Most importantly, it assumes minimal secondary changes in channel behavior such as redistribution of adjacent charge, i.e., it assumes the effects of a neutralizing mutation is only the loss of that charge. This has not always been the case. For example, a single neutralization of a basic residue in the S4 of *Shaker* K channels reduced the charge per channel by as much as 7 *e* (Seoh et al., 1996) in contrast to an anticipated maximum of 4 *e* (up to one *e* per subunit of a tetrameric channel). It is possible that neutralization resulted in a greater reduction in Q_{max} than anticipated by the removal of one charged residue (and 1 *e*), thus causing the estimate of total charge per channel to be underestimated.

The *Q-V* relationships for all the mutations reported in this study were less voltage dependent than wild type, i.e., all showed a more shallow slope factor when fit with a Boltzmann relationship (Table 3), regardless of the contribution to overall gating charge estimated by the relative reduction in charge by toxin. Similar results were found for the *Shaker* K channel where R371 (the fourth outermost arginine) was found to make a large contribution to Q_{max} even though there was little change in the slope factor of either the *G-V* or *Q-V* relationship (Seoh et al., 1996). Our findings as well as those of others suggest that small structural rearrangements can occur in mutant channels, which are not large enough to produce gross changes in channel assembly or function, but which can affect the movement of residues that participate in channel gating, the interaction with, or the form of the voltage field.

Relative contribution by basic residues in the S4 of domain III to Q_{max}

Although the gating charge associated with a single Na channel has not yet been directly measured, it is possible to determine the relative contribution of basic amino acid residues to the total gating charge of a Na channel using inhibition of charge by site-3 toxins as a caliper. Our results predict that the outermost basic residue, K1, makes little contribution to the gating charge of Na channels, while the second-outermost residue, R2, makes the greatest contribution (about 19%). R3 makes an intermediate contribution (about 10%) and R4 makes almost no contribution to Q_{max}. Furthermore, in no mutation in the S4 of domain III was the toxin-induced decrease in Q_{max} significantly less than 31% consistent with available data showing that site-3 toxins do not bind to domain III (Tejedor and Catterall, 1988; Thomsen and Catterall, 1989; Benzinger et al., 1998), and they only inhibit movement of charge of domain IV (Sheets et al., 1999).

The finding that the outermost lysine makes little contribution to overall gating charge was an unexpected finding. In *Shaker* K channels, the four to five outermost basic residues have been shown to contribute the most to gating charge while the innermost basic residues contribute the least (Aggarwal and MacKinnon, 1996; Seoh et al., 1996). Aggarwal et al. (1996) found that R1 to R4 contributed the largest magnitude of charge, while R5 contributed an intermediate amount and R7 made no contribution (the R6 mutation did not express). We found similar results for the S4 in domain IV of the hH1a channel where R1 contributed the

most charge, R2 made an intermediate contribution while R3 made almost no contribution (Sheets et al., 1999). Although our results regarding the outermost basic residue apply directly to the hH1a channel, it is likely to be a general characteristic of mammalian voltage-gated Na channels because the S4 segments are highly conserved between channels and a lysine in the outermost position is common (for review see Goldin, 1995). As a consequence of the lack of gating charge contributed by K1 it is likely the basic residue of lysine is surrounded by solvent perhaps in aqueous crevices that may be surrounding portions of the S4's (Bezanilla, 2000; Horn, 2000; Sato et al., 2001; Catterall, 2001).

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