Arrangement and Mobility of the Voltage Sensor Domain in Prokaryotic Voltage-gated Sodium Channels

Prokaryotic voltage-gated sodium channels (NaVs) form homotetramers with each subunit contributing six transmembrane α-helices (S1–S6). Helices S5 and S6 form the ion-conducting pore, and helices S1–S4 function as the voltage sensor with the structural and functional information on NaVs remains limited. Here, we show that the domain arrangement in NaChBac, a firstly cloned prokaryotic NaV, is similar to that in KVs. Cysteine substitutions of three residues in helix S4, Q107C, T110C, and R113C, effectively induced intersubunit disulfide bond formation with a cysteine introduced in helix S5, M164C, of the adjacent subunit. In addition, substituting two acidic residues with lysine, E43K and D60K, shifted the activation of the channel to more positive membrane potentials and consistently shifted the preferentially formed disulfide bond from T110C/M164C to Q107C/M164C. Because Gln-107 is located closer to the extracellular side of helix S4 than Thr-110, this finding suggests that the functional shift in the voltage dependence of activation is related to a restriction of the position of helix S4 in the lipid bilayer. The domain arrangement and vertical mobility of helix S4 in NaChBac indicate that the structure and the mechanism of voltage-dependent activation in prokaryotic NaVs are similar to those in canonical KVs.

Voltage-gated ion channels play essential roles in electric signaling, muscle contraction, and other important physiological processes. Mammalian voltage-gated sodium channels (NaVs) are formed by a single, long polypeptide (~300 amino acids) that contains four homologous domains.

Prokaryotic NaVs are simpler than mammalian NaVs, comprising shorter polypeptides of ~300 amino acids that form homotetramers (3–6). Each subunit, corresponding to one homologous domain in mammalian NaVs, contains six transmembrane α-helices (S1–S6). Helices S5 and S6 form the ion-conducting pore in the center of the tetrameric channel, and helices S1–S4 form voltage sensors that surround the pore domain and detect the membrane potential. Helix S4 features a series of positively charged residues that are essential for voltage-dependent gating (7, 8). It is thought that changes in the membrane potential cause some of these charges to move vertically in the lipid bilayer (9).

NaChBac is a prokaryotic NaV cloned from Bacillus halodurans. Its function has been studied by expression in mammalian cells and confirmed to be a Na+-selective channel (3), providing insight into gating charge movements related to voltage-dependent gating (10), and C-type inactivation (6, 11). Different prokaryotic NaVs differ in their voltage dependence and ion conduction kinetics (5, 6). The structural simplicity and functional diversity of prokaryotic NaVs make them an ideal model for studying the structure and function of other NaVs.

The best studied voltage-gated ion channels are voltage-gated K channels (KVs). The arrangement of subunits in Kv tetramers was initially investigated by introducing double cysteine mutations at the extracellular side of helices S4 and S5 in the Shaker channel from Drosophila melanogaster (12–14). Some double cysteine mutation pairs result in the formation of intersubunit disulfide bonds, showing that helix S4 of one subunit is in close proximity to helix S5 of an adjacent subunit. The proximity of the residues identified in these studies was subsequently verified with the crystal structures of the rat Kv1.2 and Kv1.2/2.1 chimera channels (15, 16), which showed that helix S4 of the voltage sensor domain indeed faces helix S5 of the pore domain of an adjacent subunit in the Kv tetramer.

The domains in tetramers formed by prokaryotic NaVs are thought to have an arrangement similar to that of tetrameric Kv. Confirming that Kv and prokaryotic NaVs have similar domain arrangements would allow findings obtained from analyses of prokaryotic NaVs to be generalized to all tetrameric voltage-gated ion channels. Although recent distance measurements by luminescence resonance energy transfer suggested that NaChBac and KvAP, a prokaryotic Kv, share a similar subunit organization (17), direct evidence for a similar domain arrangement in Kv and prokaryotic NaVs is still missing.

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Domain Arrangement of Prokaryotic Na\textsubscript{v}s

Using the same double cysteine mutagenesis approach previously used for the Shaker K\textsubscript{v}, we confirmed the proximity between helix S4 and helix S5 of an adjacent subunit in NaChBac. The double mutants that formed disulfide-bonded tetramers were consistent with previous results obtained for K\textsubscript{v}s. These results suggest that NaChBac has the same domain arrangement as K\textsubscript{v}s. We also show that substituting Gln-107, Thr-110, and Arg-113 in helix S4 with cysteine is the most efficient method of forming a disulfide bond with M164C in helix S5. Helix S4 is thought to move vertically during voltage-dependent activation (18–20). The finding that multiple residues in helix S4 can form disulfide bonds with the same residue in helix S5 indicates that helix S4 is very mobile in the vertical direction. To examine the relationship between the mobility of helix S4 and voltage-dependent activation, we assessed the effect of mutations in helices S1 and S2, which shifted the activation of NaChBac to a more positive membrane potential. The additional mutations resulted in M164C forming disulfide bonds preferentially with residues in helix S4 closer to the extracellular surface. These results demonstrate that the vertical position of helix S4 depends on the charges surrounding the voltage sensor domain and that changes in the electrostatic environment shift the voltage dependence of activation of Na\textsubscript{v}s.

EXPERIMENTAL PROCEDURES

Molecular Biology, Protein Expression, and Western Blot Analysis—Construction of vectors carrying each NaChBac mutant and expression of the wild-type and mutant NaChBac vectors were performed as previously reported with slight modification (6). For expression in *Escherichia coli*, cDNA encoding wild-type and mutant NaChBac was inserted into the pQE-80L vector (Qiagen) using the BamHI and HindIII sites or into the pET-21b vector (Novagen) using the NdeI and SalI sites.

NaChBac proteins were expressed using the pQE-80L vector in *E. coli* BL21 (Invitrogen). Cell cultures (15 ml) were grown for 72 h at 37 °C after induction with 0.5 mM isopropyl-β-D-galactopyranoside (Wako) to allow for disulfide bond formation. The cells were pelleted and resuspended in 1 ml of TBS buffer (20 mM Tris–HCl, pH 7.4, 300 mM NaCl). The cell samples were mixed with electrophoresis sample buffer containing 20 mM iodoacetamide for nonreducing conditions or 2% -mercaptoethanol for reducing conditions. After boiling for 5 min at 70 °C, the samples were analyzed by SDS-PAGE using 10 to 20% gradient gels (Wako). For Western blotting, the proteins were transferred onto nitrocellulose membranes that were treated with blocking buffer (PBS buffer containing 0.25% gelatin, 2.5% BSA, and 0.01% NaN\textsubscript{3}) for 30 min. The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were treated with Blocking One solution (Nacalai Tesque) at room temperature for 30 min and incubated with a monoclonal antibody against NaChBac at 4 °C for ~12 h. The antibody was detected by horseradish peroxidase-conjugated secondary antibody (Promega) and visualized using ECL Plus (GE Healthcare). The bands were scanned and quantified using an LAS-3000 image analyzer (Fuji Film). Disulfide bond formation efficiency was calculated as the intensity of the band representing the disulfide-bonded tetramer divided by the sum of the intensity of all bands. The efficiency of disulfide bond formation of each mutant was compared using Tukey’s test.

Electrophysiology—CHO-K1 or HEK-293 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Biowhitaker), 100 units/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen) at 37 °C under a 5% CO\textsubscript{2} atmosphere. The cells were transfected with NaChBac and pEGFP DNA using a calcium phosphate transfection kit (Invitrogen) and plated on coverslips. HEK-293 cells were used to confirm the sodium currents of mutant channels under reducing and nonreducing conditions, and CHO-K1 cells were used to analyze the voltage dependence of activation. The cells were voltage-clamped with an EPC10 amplifier (HEKA Elektronik, Lambrecht, Germany). Whole cell currents were recorded from CHO-K1 or HEK-293 cells using the pET-21b vector (Novagen) using the NdeI and SalI sites.

NaChBac proteins were expressed using the pQE-80L vector in *E. coli* BL21 (Invitrogen). Cell cultures (15 ml) were grown for 72 h at 37 °C after induction with 0.5 mM isopropyl-β-D-galactopyranoside (Wako) to allow for disulfide bond formation. The cells were pelleted and resuspended in 1 ml of TBS buffer (20 mM Tris–HCl, pH 7.4, 300 mM NaCl). The cell samples were mixed with electrophoresis sample buffer containing 20 mM iodoacetamide for nonreducing conditions or 2% -mercaptoethanol for reducing conditions. After boiling for 5 min at 70 °C, the samples were analyzed by SDS-PAGE using 10 to 20% gradient gels (Wako). For Western blotting, the proteins were transferred onto nitrocellulose membranes that were treated with blocking buffer (PBS buffer containing 0.25% gelatin, 2.5% BSA, and 0.01% NaN\textsubscript{3}) for 30 min. The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were treated with Blocking One solution (Nacalai Tesque) at room temperature for 30 min and incubated with a monoclonal antibody against NaChBac at 4 °C for ~12 h. The antibody was detected by horseradish peroxidase-conjugated secondary antibody (Promega) and visualized using ECL Plus (GE Healthcare). The bands were scanned and quantified using an LAS-3000 image analyzer (Fuji Film). Disulfide bond formation efficiency was calculated as the intensity of the band representing the disulfide-bonded tetramer divided by the sum of the intensity of all bands. The efficiency of disulfide bond formation of each mutant was compared using Tukey’s test.

RESULTS

Identification of Paired Cysteine Mutations in Helices S4 and S5 That Lead to the Formation of Intersubunit Disulfide Bonds—To investigate whether the domain arrangements in prokaryotic Na\textsubscript{v}s and K\textsubscript{v}s are similar, we systematically introduced double cysteine mutations in NaChBac to identify pairs
that would allow the formation of intersubunit disulfide bonds. A previous study of Shaker KV showed that the cysteine substitutions of both Arg-362 in helix S4 and Ala-419 in helix S5 resulted in disulfide-bonded channel tetramers (12), indicating that residues Arg-362 and Ala-419 of the adjacent subunit are sufficiently close to each other to allow for the formation of a disulfide bond. If NaChBac has a similar domain arrangement as the Shaker channel, double cysteine substitution of the corresponding residues in helices S4 and S5 should allow for the formation of a disulfide-bonded NaChBac tetramer (Fig. 1).

To determine what double cysteine mutants would form disulfide-bonded tetramers, we expressed them in E. coli, because it is an easy and convenient expression system that makes it feasible to screen a large number of mutants. Of note, NaChBac contains no cysteine residues. We first introduced a cysteine substitution for residue Arg-113 in helix S4 of NaChBac, the residue that corresponds to residue Arg-362 in the Shaker channel. We then generated 13 mutants with an additional cysteine substitution at positions 106–117 in helix S4 and 160–172 in helix S5 (Fig. 1). Western blot analysis of the various NaChBac mutants showed that most mutants showed a band consistent with the molecular mass of a monomer (29 kDa; arrow 1 in Fig. 2A), the R113C/M164C double mutant migrated as a band of higher molecular mass (arrow 4 in Fig. 2A). With a molecular mass of ~120 kDa, approximately four times the molecular mass of a NaChBac monomer, the band should represent a disulfide-bonded tetramer. Thus, among the 13 residues tested, Met-164 was the residue in helix S5 closest to Arg-113.

After identifying Met-164 in helix S5, we combined the M164C mutation with 12 cysteine mutations at positions 106–117 in helix S4 (Fig. 1). Western blot analysis of the double mutants showed that the T110C/M164C mutant was the most efficient in forming disulfide-bonded tetramers migrating at a molecular mass of ~120 kDa, whereas the R113C/M164C mutant was the second most efficient in forming disulfide-bonded tetramers (Fig. 2B). Of the 12 mutants tested, eight showed a band corresponding to the disulfide-bonded tetramer (cysteine residues introduced from positions 107 to 114). The Q107C/M164C mutant was slightly more efficient in forming disulfide-bonded tetramers than the other mutants but less efficient than the T110C/M164C and R113C/M164C mutants. By combining the T110C substitution in helix S4 with the 13 cysteine substitutions in helix S5, we confirmed that M164C most efficiently formed a disulfide bond with T110C (supplemental Fig. S1). M164C also most efficiently formed disulfide bonds with V111C and L112C (supplemental Fig. S1). Thr-110 and Met-164 thus appear to be the closest pair of residues between helices S4 and S5.

SDS-PAGE analysis of wild-type NaChBac; the Q107C, T110C, R113C, and M164C single mutants; and the Q107C/M164C, T110C/M164C, and R113C/M164C double mutants showed that all variants migrated mainly as monomers under reducing conditions, and only three double mutants migrated as tetramers under nonreducing conditions (supplemental Fig. S2).
results confirmed that the Q107C/M164C, T110C/M164C, and R113C/M164C tetramers are stabilized by intersubunit disulfide bonds between the substituted cysteines in helix S4 and M164C in helix S5. Inter-subunit Disulfide Bonds Formed in NaChBac T110C/M164C, and R113C/M164C Mutants Lock the Channels in a Non-conductive State—To assess the effect of intersubunit disulfide bonds, we used whole cell patch clamp analysis to compare the channel activity of the R113C/M164C double mutants with that of wild-type NaChBac. When transfected into mammalian cell lines, wild-type NaChBac generated a sodium current upon application of a series of test pulses from a holding potential of −120 mV (Fig. 3A). Using the same regimen, R113C and M164C single mutants generated the same sodium currents under reducing and nonreducing conditions (supplemental Fig. S3). In contrast, the R113C/M164C double mutant failed to generate a sodium current under nonreducing conditions, but treatment with 5 mM DTT restored the sodium currents of R113C/M164C (Fig. 3B). Although the mean amplitude of the R113C/M164C mutant with DTT pretreatment was smaller and the rate of current inactivation was slower than wild type (Table 1), the sodium currents were generated normally, as in the wild type. The same result was previously obtained for the T110C/M164C double mutant (21). These results indicate that positions 110 and 113 in helix S4 and position 164 in helix S5 are close enough to allow for the formation of intersubunit disulfide bonds in the T110C/M164C and R113C/M164C double mutants. Thus, the intersubunit disulfide bonds appear to lock the voltage sensor domain in a nonconductive but returnable state.

The D60K and E43K Mutations Shift the Activation of NaChBac to a More Positive Membrane Potential—Cysteines introduced at multiple positions in helix S4 resulted in disulfide bond formation with M164C. This result may reflect the mobility of the voltage sensor domain. Residues Gln-107, Thr-110, and Arg-113 would be positioned in a line along the axis of helix S4 with all of the side chains projecting from the helix in approximately the same direction but a turn of the helix apart, and the fact that double mutants Q107C/M164C, T110C/M164C, and R113C/M164C all formed disulfide-bonded tetramers indicates that helix S4 has substantial vertical mobility in the lipid bilayer. To examine the relationship between the vertical mobility of helix S4 and the voltage-dependent activation of the channel, we investigated the effect of other mutations that affect the voltage dependence of NaChBac activation on the efficiency of disulfide bond formation in the three double mutants.

Residues Glu-43 and Asp-60 are located close to the extracellular surface in helices S1 and S2, respectively (Fig. 1B). The D60K mutation is known to shift the voltage dependence of NaChBac activation toward a more positive membrane potential (22, 23), and the crystal structure of the rat Kv1.2/2.1 chimeric channel shows that the residues corresponding to Glu-43 and Asp-60 interact with one of the positively charged residues in helix S4 (16). To determine the voltage dependence of the mutant channels, we used whole cell patch clamp to measure deactivation tail currents. The mean amplitude of each mutant was generally smaller than that of the wild type but normally generated (Table 2 and supplemental Fig. S4). A Boltzmann fit of the mean activation curve for the D60K mutant showed that the potential for 50% activation ($V_{1/2}$) was $42.0 \pm 2.0$ mV, a shift of $+79$ mV compared with that of wild-type NaChBac ($V_{1/2}$ of $-36.6 \pm 2.0$ mV) (Fig. 4A). Similarly, $V_{1/2}$ for the E43K mutant was $18.0 \pm 1.3$ mV, a shift of $+55$ mV compared with the wild-type channel. The E43K mutation thus has a similar effect on the voltage dependence as the D60K mutation. The R113C mutation is known to cause a substantial shift in the voltage-dependent activation of NaChBac (24), and in the R113C/M164C double mutant, this mutation is involved in the formation of a disulfide bridge. We therefore also analyzed the change in the voltage dependence of activation caused by the E43K or D60K mutations in the presence of an additional R113A muta-
Bonds formed in NaChBac T110C/M164C and R113C/M164C mutants lock the channels in a nonconductive state. The representative current traces for wild type (A) and R113C/M164C mutant (B) were generated by a series of test pulses in HEK-293 cells. The currents were recorded without (top panels) or with (bottom panels) a 30-min preincubation with 5 mM DTT. The panels below each current trace are averaged I-V curves derived from each experimental condition. The error bars correspond to the S.E. of the mean.

V_{1/2} values for the R113A and D60K/R113A mutants were 2.59 ± 3.6 and 80.3 ± 0.9 mV, respectively, a difference between them of +78 mV (Fig. 4B). The V_{1/2} for the E43K/R113A mutant was 24.5 ± 2.5 mV, a shift of +22 mV compared with the R113A mutant (Fig. 4). Hence, the E43K or D60K mutations shift the voltage dependence of activation toward a more positive membrane potential, with or without the additional R113A mutation.

The D60K and E43K Mutations Change Which Residues in the Double Cysteine Mutants Preferentially Form Disulfide Bonds—We tested whether the D60K and E43K mutations would affect the disulfide bond formation of the three mutants, Q107C/R113A/M164C (QC), T110C/R113A/M164C (TC), and R113C/M164C (RC), in which residue Arg-113 was neutralized by substitution to alanine or cysteine. The mutants were expressed in E. coli, and disulfide bond formation in the proteins was assessed by quantitative Western blot analysis (Fig. 5A). The efficiency of disulfide bond formation was determined as the intensity of the band representing the disulfide-bonded tetramer divided by the sum of the intensity of all bands (Fig. 5B). The QC mutant formed significantly fewer disulfide-bonded tetramers than the TC (p < 0.01) and RC (p < 0.05) mutants. Thus, neutralization of Arg-113 by substitution to alanine or cysteine did not change the order concerning the efficiency with which the three double cysteine mutants formed disulfide bonds. We next quantified the efficiency with which the three double cysteine mutants formed disulfide bonds when we introduced an additional D60K or E43K mutation. The D60K/QC mutant formed disulfide bonds significantly more efficiently than the D60K/TC (p < 0.01) and D60K/RC (p < 0.01) mutants, and the E43K/QC mutant more efficiently than the E43K/RC mutant (p < 0.05). With both the E43K and D60K mutations, the QC mutant was most efficient in forming disulfide bonds, followed by the TC mutant and finally the RC mutant. These results suggest that in the presence of the E43K and D60K mutations, the residue in helix S4 closest to Met-164 in helix S5 is no longer Thr-110 but rather Gln-107, and that the charges surrounding the voltage sensor domain affect the preferred vertical position of helix S4.

### TABLE 1

| V_{1/2} (mV) | mV | mV/e-fold |
|--------------|----|------------|
| WT           | 5.3| 6          |
| E43K         | 5.0| 6          |
| D60K         | 4.7| 6          |
| R113A        | 4.0| 6          |
| E43K/R113A   | 3.6| 6          |
| D60K/R113A   | 3.6| 6          |

### TABLE 2

| V_{1/2} (mV) | mV | mV/e-fold |
|--------------|----|------------|
| WT           | 5.3| 6          |
| E43K         | 5.0| 6          |
| D60K         | 4.7| 6          |
| R113A        | 4.0| 6          |
| E43K/R113A   | 3.6| 6          |
| D60K/R113A   | 3.6| 6          |

### FIGURE 3

Intersubunit disulfide bonds formed in NaChBac T110C/M164C and R113C/M164C mutants lock the channels in a nonconductive state. The representative current traces for wild type (A) and R113C/M164C mutant (B) were generated by a series of test pulses in HEK-293 cells. The currents were recorded without (top panels) or with (bottom panels) a 30-min preincubation with 5 mM DTT. The panels below each current trace are averaged I-V curves derived from each experimental condition. The error bars correspond to the S.E. of the mean.

V_{1/2} values for the R113A and D60K/R113A mutants were 2.59 ± 3.6 and 80.3 ± 0.9 mV, respectively, a difference between them of +78 mV (Fig. 4B). The V_{1/2} for the E43K/R113A mutant was 24.5 ± 2.5 mV, a shift of +22 mV compared with the R113A mutant (Fig. 4). Hence, the E43K or D60K mutations shift the voltage dependence of activation toward a more positive membrane potential, with or without the additional R113A mutation.

The D60K and E43K Mutations Change Which Residues in the Double Cysteine Mutants Preferentially Form Disulfide Bonds—We tested whether the D60K and E43K mutations would affect the disulfide bond formation of the three mutants, Q107C/R113A/M164C (QC), T110C/R113A/M164C (TC), and R113C/M164C (RC), in which residue Arg-113 was neutralized by substitution to alanine or cysteine. The mutants were expressed in E. coli, and disulfide bond formation in the proteins was assessed by quantitative Western blot analysis (Fig. 5A). The efficiency of disulfide bond formation was determined as the intensity of the band representing the disulfide-bonded tetramer divided by the sum of the intensity of all bands (Fig. 5B). The QC mutant formed significantly fewer disulfide-bonded tetramers than the TC (p < 0.01) and RC (p < 0.05) mutants. Thus, neutralization of Arg-113 by substitution to alanine or cysteine did not change the order concerning the efficiency with which the three double cysteine mutants formed disulfide bonds. We next quantified the efficiency with which the three double cysteine mutants formed disulfide bonds when we introduced an additional D60K or E43K mutation. The D60K/QC mutant formed disulfide bonds significantly more efficiently than the D60K/TC (p < 0.01) and D60K/RC (p < 0.01) mutants, and the E43K/QC mutant more efficiently than the E43K/RC mutant (p < 0.05). With both the E43K and D60K mutations, the QC mutant was most efficient in forming disulfide bonds, followed by the TC mutant and finally the RC mutant. These results suggest that in the presence of the E43K and D60K mutations, the residue in helix S4 closest to Met-164 in helix S5 is no longer Thr-110 but rather Gln-107, and that the charges surrounding the voltage sensor domain affect the preferred vertical position of helix S4.
DISCUSSION

The Domain Arrangement in NaChBac Is Similar to that in KVs—By generating double cysteine mutants, we confirmed that in NaChBac, helix S4 is close to helix S5 of an adjacent subunit. The observation of disulfide-bonded tetramers for the T110C/M164C and R113C/M164C mutants indicates that residues Thr-110 and Arg-113 in helix S4 are close to residue Met-164 in helix S5 of the adjacent subunit (Fig. 2), suggesting that the putative domain arrangement shown in Fig. 1 is likely correct for prokaryotic NaVs. Our electrophysiologic analysis shows that the T110C/M164C and R113C/M164C mutants do not generate currents under nonreducing conditions, but activity is recovered under reducing conditions (Fig. 3) (21). The intersubunit disulfide bonds presumably prevent conformational changes of the voltage sensor domains and keep the mutant channels in a nonconductive state. Because alignment of the NaChBac and Shaker channel sequences shows that the T110C/M164C mutations in NaChBac correspond to the R362C/F416C mutations in the Shaker channel, previously shown to result in the formation of an intersubunit disulfide bond (Fig. 1A) (12), these results are strong evidence that the subunit arrangement in NaChBac is similar to that in the Shaker channel (Fig. 1C).

The efficiency of disulfide bond formation of the mutants with the M164C substitution depended on which position in helix S4 was mutated to cysteine (Fig. 2B). The difference in efficiency reflects both the vertical position as well as the rotational orientation of the side chains of the residues. Disulfide bond formation was most efficient when residues Glu-3 and Thr-110 were mutated to cysteine. Because these residues are three positions apart, the approximate distance of a helical turn, they presumably form a line along helix S4 that faces residue Met-164 in helix S5 of the adjacent subunit. The Q107C/M164C mutant was less efficient in forming disulfide bonds than the T110C/M164C and R113C/M164C mutants, suggesting that Thr-110 and Arg-113 are closer to Met-164 than Glu-3.

**FIGURE 4.** The D60K and E43K mutations shift the activation of NaChBac to a more positive membrane potential. Normalized curves of the voltage-dependent activation of NaChBac mutants derived from CHO-K1 cells. The voltage dependence was determined by measuring deactivation tail currents. The error bars correspond to the S.E. of the mean. A, activation curves for wild-type NaChBac (closed circles) and the E43K (open circles) and D60K (closed triangles) mutants. B, activation curves for the R113A single mutant (closed circles) and the E43K/R113A (open circles) and D60K/R113A (closed triangles) double mutants.

**FIGURE 5.** The D60K and E43K mutations change which residues in the double cysteine mutants preferentially form disulfide bonds. Residues Asp-60 or Glu-43 were mutated to lysine in the Q107C/R113A/M164C (QC), T110C/R113A/M164C (TC), and R113C/M164C (RC) mutants. The membranes isolated from E. coli expressing these mutants were analyzed by quantitative Western blotting. A, representative Western blot of NaChBac double cysteine mutants with no additional mutation (left panel), with the D60K mutation (middle panel), and the E43K mutation (right panel). The numbered arrows indicate the bands representing the monomer, dimer, trimer, and tetramer species. B, quantification of the amount of disulfide-bonded tetramer that formed with the various mutants. The presented values are the means of the intensity of all protein bands (no addition, n = 9; D60K, n = 9; E43K, n = 6). The error bars correspond to the S.E. of the mean. The asterisks indicate the differences from control (*, p < 0.05; **, p < 0.01). Comparisons without asterisks were not statistically significant.
107. In the structure of the Kᵥ1.2/2.1 chimera channel, the residues corresponding to Gln-107, Thr-110, and Arg-113 (Arg-287, Gln-290, and Arg-293, respectively) also form a line along helix S4 (16), and residues Gln-290 and Arg-293 in helix S4 are closest to Phe-344 in helix S5, the residue corresponding to Met-164 in NaChBac. In the structure of the Kᵥ1.2/2.1 chimera channel, the distances from the Cₛ carbon of Phe-344 to those of Arg-287, Gln-290, and Arg-293 are 11.5, 8.4, and 8.1 Å, respectively. Thus, the locations of residues Gln-107, Thr-110, and Arg-113 in NaChBac predicted from our disulfide cross-linking analysis are consistent with the positions of the corresponding residues in the crystal structure of Kv1.2/2.1. These results further support a similar domain arrangement in NaChBac and Kᵥs.

The proximity of helices S4 and S5 of an adjacent subunit has also been observed in other six-transmembrane tetrameric ion channels. In KvAP, cysteine substitution of Arg-117 and Tyr-169, the residues corresponding to Arg-113 and Thr-163 in NaChBac, also results in the formation of intersubunit disulfide bonds (25). Furthermore, the crystal structure of MloT1, a prokaryotic cyclic nucleotide-gated ion channel, showed that helix S4 is close to helix S5 of the adjacent subunit (26). The proximity of helix S4 to helix S5 of an adjacent subunit thus appears to be a characteristic feature of six-transmembrane tetrameric ion channels. The role of the interaction between helices S4 and S5 has not yet been firmly established, but some studies suggest that the interaction is required for channel function, particularly for voltage-dependent activation and inactivation (27–30).

Helix S4 Is Highly Mobile—The efficient formation of disulfide bonds between M164C and cysteines introduced at positions 107, 110, and 113 indicates that all three of these residues on helix S4 can come close to Met-164 in helix S5 (Fig. 2B). This result implies that helix S4 has substantial mobility, as much as 7.2 Å, the approximate distances of two helical turns, in the vertical direction. Of the 12 cysteine substitutions in helix S4 of NaChBac, eight formed some disulfide-bonded tetramers with M164C (from Q107C to N114C) (Fig. 2B). Disulfide bond formation between substituted cysteine residues is a well established method for analyzing protein structure (31). Because of its helical structure, a rotational movement of helix S4 would allow residues in helix S4 other than Gln-107, Thr-110, and Arg-113 to form disulfide bonds with Met-164, but this appears to occur rarely. In contrast, of the 13 cysteine substitutions in helix S5 (from Val-160 to Glu-172), only M164C fully formed disulfide bonds with residues in helix S4 (Fig. 2A and supplemental Fig. S1). This result indicates that helix S4 is highly mobile, appearing to move both vertically and to some degree rotationally, in the lipid bilayer, whereas helix S5 was more static.

The Vertical Position of Helix S4 Is Related to the Voltage Dependence of Activation—Introducing the additional D60K mutation changed the preferentially formed disulfide bond in NaChBac from TC to QC (Fig. 5). Because the D60K mutation shifts the voltage dependence of activation toward a more positive membrane potential (Fig. 4), the change in the closest residues between helices S4 and S5 may be caused by the shift in activation potential. In the Shaker channel, the negative charge of the residue corresponding to Asp-60 in NaChBac assists the charge transfer during voltage-dependent activation by forming a salt bridge with a positively charged residue in helix S4 (32, 33). Substitution of Asp-60 with lysine prevents the formation of this salt bridge, thus suppressing the vertical movement of helix S4. As a result, Gln-107, which is closer to the extracellular surface than Thr-110, would become more accessible for disulfide bond formation with Met-164 and could be the cause of the shift in the voltage dependence of activation to a more positive membrane potential.

The effect of the E43K mutation on the voltage dependence of activation and the preferentially formed disulfide bridge was similar to those of the D60K mutation (Figs. 4 and 5). In the structure of the rat Kᵥ1.2/2.1 chimera, the residue corresponding to Glu-43 forms a salt bridge with the third positively charged residue in helix S4, whereas the residue corresponding to Asp-60 interacts with the fourth positively charged residue (16). The role of residue Glu-43 in voltage-dependent activation may thus be similar to that of Asp-60, and the E43K mutation may thus also interfere with the vertical movement of helix S4. The role of negative charges in the vertical movement of helix S4 was recently demonstrated in NaChBac, because residues Asp-60 and Glu-70 were shown to interact sequentially with positive charges in S4 during voltage-dependent activation (34). E43 also interacts with Arg-113 and Arg-116 during channel activation in NaChBac (35). The involved negative charges, including Glu-43, might work cooperatively to assist the vertical movement of helix S4. These results imply that the range of the vertical mobility of helix S4 should be more suppressed in ion channels that are activated at higher membrane potentials (Fig. 6). Although the charged residues in the voltage sensor domain are generally well conserved, the residues at the positions corresponding to those of Glu-43 and Asp-60 in NaChBac are less conserved (36). The diversity in amino acids at these positions might thus contribute to differences in the voltage dependence of activation among voltage-gated ion channels.

Indications for the Voltage-dependent Movement of Helix S4—Helix S4 has been proposed to underlie voltage-dependent activation by moving vertically in the lipid bilayer upon changes in membrane potential (18–20, 37). Movement of helix S4 is thought to result in a conformational change of the voltage sensor domain and eventually the opening of the pore gate (9). Double cysteine mutagenesis and structural studies of Kᵥs showed that in the activated state the residues corresponding to Thr-110 or Arg-113 are close to helix S5 (12, 16), whereas in the resting state residues in the more extracellular part of helix S4 are close to residues in helix S5 (13). The D60K and E43K mutations increase the energy needed to activate the channels so that these two mutations keep the channel in the resting state as the disulfide bonds are formed. Therefore, the change caused by the E43K or D60K mutations in the efficiency with which disulfide bonds are formed in QC, TC, and RC might indicate a shift in the residues that are close to each
other during voltage-dependent gating (Fig. 5). Residue Gln-107, which is closer to the extracellular surface than residues Thr-110 or Arg-113, might be close to Met-164 in the resting state or in the early phase of voltage-dependent activation. These results imply that helix S4 moves vertically with Gln-107, Thr-110, and Arg-113 facing Met-164 and that the positive charges move from the intracellular toward the extracellular side during voltage-dependent gating.

The voltage-dependent movement of helix S4 in NaChBac suggested by our double cysteine mutagenesis and additional lysine substitution of residues Glu-43 and Asp-60 is consistent with previously proposed models for voltage-dependent gating. For example, the helical screw and paddle models both require a large movement of helix S4 along its axis (20, 37). It was recently shown that the voltage sensor of NaChBac has an overall structure similar to Kvs and changes its conformation during activation in the same manner as well (38). The rotational and vertical movement of helix S4 in NaChBac indicated by our cysteine mutagenesis agrees well with the helical screw model, which requires that helix S4 moves both vertically and rotationally. Our results thus indicate that the movement of helix S4 in NaChBac is similar to that of canonical voltage-gated ion channels. Our study only shows, however, that helix S4 is mobile. Structural studies are needed to reveal its detailed movement during voltage-dependent activation of NaChBac.

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