Functional Roles of the Extracellular Segments of the Sodium Channel α Subunit in Voltage-dependent Gating and Modulation by β1 Subunits*

Voltage-gated sodium channels consist of a pore-forming α subunit associated with β1 subunits and, for brain sodium channels, β2 subunits. Although much is known about the structure and function of the α subunit, there is little information on the functional role of the 16 extracellular loops. To search for potential functional activities of these extracellular segments, chimeras were studied in which an individual extracellular loop of the rat heart (rH1) α subunit was substituted for the corresponding segment of the rat brain type IIA (rIIA) α subunit. In comparison with rH1, wild-type rIIA α subunits are characterized by more positive voltage-dependent activation and inactivation, a more prominent slow gating mode upon coexpression of β1 subunits in Xenopus oocytes. When α subunits were expressed alone, chimeras with substitutions from rH1 in five extracellular loops (IIB5-S1, IIS2-S6, IIS1-S2, IIVS2-S6, and IIVS3-S4) had negatively shifted activation, and chimeras with substitutions in three of these (IIS2-S6, IIS1-S2, and IIVS3-S4) also had negatively shifted steady-state inactivation. rIIA α subunit chimeras with substitutions from rH1 in five extracellular loops (IIS5-S1, IIS2-S6, IIS2-S6, IIS1-S2, and IIS3-S4) had negatively shifted activation, and chimeras with substitutions in three of these (IIS2-S6, IIS1-S2, and IIVS3-S4) also had negatively shifted steady-state inactivation. rIIA α subunit chimeras with substitutions from rH1 in five extracellular loops (IIB5-S1, IIS2-S6, IIS2-S6, IIS1-S2, and IIVS3-S4) favored the fast gating mode. Like wild-type rIIA α subunits, all of the chimeric rIIA α subunits except chimera IVS2-S6 were shifted almost entirely to the fast gating mode when coexpressed with β1 subunits. In contrast, substitution of extracellular loop IVS2-S6 substantially reduced the effectiveness of β1 subunits in shifting rIIA α subunits to the fast gating mode. Our results show that multiple extracellular loops influence voltage-dependent activation and inactivation and gating mode of sodium channels, whereas segment IVS2-S6 plays a dominant role in modulation of gating by β1 subunits. Evidently, several extracellular loops are important determinants of sodium channel gating and modulation.

Voltage-gated sodium channels mediate the sodium conductance responsible for the rapidly rising phase of the action potential in nerve and muscle cells. The major form of the sodium channel in rat brain is a heterotrimERIC complex of an α subunit (260 kDa), a noncovalently bound β1 subunit (36 kDa), and a disulfide-linked β2 subunit (33 kDa) (1, 2). α subunits can function as voltage-gated ion channels by themselves (e.g. rat brain type II/IIA; Refs. 3 and 4). They are composed of four homologous domains (I–IV), which each contain six probable α-helical transmembrane segments (S1–S6) and an additional membrane-associated pore loop (e.g. rat brain type II/IIA; Refs. 5–7), whereas the β1 and β2 subunits are single membrane-spanning glycoproteins with a large extracellular domain and a small intracellular domain (8, 9). Extensive structure-function analyses of α subunits have shown that the S4 transmembrane segments in each domain serve as voltage sensors for channel activation; the S5 and S6 segments and the pore loop between them form the transmembrane pore; and the short, highly conserved intracellular loop between domains III and IV forms the inactivation gate (reviewed in Refs. 10 and 11). The large intracellular domains are targets for channel modulation by protein phosphorylation and G protein binding (reviewed in Ref. 12). In contrast to the well established functional roles of the transmembrane and intracellular domains of the channel, the functional roles of the extracellular loops of the sodium channel α subunit have not been defined. Peptide neurotoxins from scorpions and sea anemones modulate gating by binding to receptor sites in the extracellular domains (13, 14), and the extracellular domain of the β1 subunit is primarily responsible for its modulation of α subunit function (15–17). These results suggest that the extracellular loops of α subunits might also be important determinants of sodium channel gating. Cardiac sodium channel α subunits (type H1) (18, 19) differ substantially from brain type IIA in their voltage dependence and kinetics of activation and inactivation and in their response to association with β1 subunits (11). In this study, we have analyzed the functional properties and the modulation by the β1 subunit of chimeras constructed between rat brain type IIA (rIIA) and rat heart (rH1) sodium channel α subunits to reveal functional activities of the extracellular loops of the α subunit and to identify specific extracellular segments that are important determinants of voltage-dependent gating and interaction with the β1 subunit.

EXPERIMENTAL PROCEDURES

Construction of Chimeric rIIA Sodium Channels—Five M13 constructs collectively spanning nearly the entire rIIA sequence were used as templates for site-directed mutagenesis. The template mpISSSNC (nucleotides 1898–2700) contains the SmaISph1 rIIA fragment. The template mp19BatNC was created by first introducing a BstEII site in the mp19 vector and then subcloning the SphI/BstEII rIIA fragment

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The abbreviations used are: rIIA, rat IIA; rH1, rat H1; WT, wild-type.
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(2701–4636). The remaining three templates, mp15KXNC (nucleotides 23–540), mp19Xa1 (nucleotides 541–1897), and mp18RVNC (nucleotides 4279–5997) have been described (13).

Of the 16 extracellular loops, one loop is identical between the rIIA and rH1 isoforms (IIS3-S4), and 12 of the remaining loops were short enough to use oligonucleotide-directed mutagenesis to directly introduce the rIIA-to-rH1 amino acid changes, using uracil-containing templates and the dUT ung selection procedure (20). The three remaining large loop chimeras (I55-S11, IHS-S11, and IVS3-S6) were created by using oligonucleotide-directed mutagenesis to delete the sequence encoding each loop while simultaneously introducing silent restriction sites flanking these individual regions. Cardiac-specific primers containing the flanking restriction sites were then used to amplify the sequences encoding each corresponding loop from rIIA cDNA for cloning into the appropriate region of rIIA, as described previously (13). Fragments containing mutations were then excised from these mutagenesis templates and cloned into pCDMSal-NC or pCDM8sal-NC. All mutations were confirmed in the final constructs by DNA sequencing and extensive restriction mapping.

Sodium Channel Expression—pCDMS plasmids encoding WT and chimeric sodium channel α subunits were linearized with ClaI, and plasmids encoding β1 subunits were linearized with HindIII. Transformation was performed with T7 RNA polymerase (Ambion Inc., Austin, TX). Isolation, preparation, and maintenance of Xenopus oocytes were carried out as described previously (21). Healthy oocytes selected manually were pressure-injected with 50 nl of a solution containing either a 1:4 or 1:1 molar ratio of α to β1 subunit RNA. Electrophysiological recordings were carried out 2–5 days after injection.

Electrophysiological Recording—Two-electrode voltage-clamp experiments were performed as described previously (22). The amplitude of expressed sodium currents was typically 0.5–5 μA. The voltage-clamp protocols are described in the figure legends. Conductance-voltage (g-V) relationships were derived from current-voltage (I-V) relationships according to $g = I(V - V_r)$, where I is the peak current amplitude measured at voltage V and the reversal potential $V_r$ is assumed to be +55 mV under our recording conditions. Normalized g-V relationships and inactivation curves were fit with a Boltzmann distribution, 1/(1 + $\exp(-V_b - V_r))/k$, where $V_b$ is the voltage at which half-activation or half-inactivation occurred and $k$ is a slope factor. The time courses of current decay and recovery from inactivation were described with two exponentials, $a_1(1 - \exp(-\tau_1)) + (1 - a_1)(1 - \exp(-\tau_2))$, where $a_1$ is the fraction of the fast component, $\tau_1$ is the time constant of the fast component, and $\tau_2$ is the time constant of the slow component. Pooled data are reported as means ± S.D. Statistical comparisons were done using Student’s t test, with p < 0.05 as the criterion for significance.

RESULTS

Effects of the Extracellular Loops of the Sodium Channel α Subunit on Gating

Chimeric Brain/Heart Sodium Channels—Compared with rat brain sodium channels, rat cardiac sodium channels containing the rH1 α subunit (18, 19) activate and inactivate at more negative membrane potentials, and the kinetics of inactivation and recovery from inactivation are faster when expressed in Xenopus oocytes (11, 23, 24). The electrophysiological properties of the two wild-type sodium channels and each chimeric channel were analyzed in two-microelectrode whole-cell voltage-clamp experiments in Xenopus oocytes. Fig. 1 shows typical sodium currents elicited from WT rIIA and rH1 α subunits expressed alone in Xenopus oocytes. The resolution of the two-microelectrode voltage-clamp small of oocytes (25) was adequate for detailed analysis of the kinetics of inactivation, but the rate of activation was not resolved with sufficient precision for accurate analysis. As reported previously (23, 24), the rate of inactivation of the rH1 α subunit was faster than that of the rIIA α subunit expressed alone in Xenopus oocytes (Fig. 1, A and B). Coexpression of β1 subunits (Fig. 1A, B) accelerated inactivation of rIIA α subunits (Fig. 1A), but had little effect on the rH1 α subunit (Fig. 1B). Modulation by the β1 subunits therefore results in faster activation and inactivation of wild-type rIIA than rH1 α subunits.

To examine the functional role of the extracellular loops in the sodium channel α subunit, we substituted each predicted extracellular loop in the brain rIIA α subunit individually with the corresponding sequence of the heart sodium channel rH1 α subunit (Table I), except for the loop between S3 and S4 in domain I, which is identical between rIIA and rH1. No sodium current was detected when oocytes were injected with mRNAs from two chimeras, one having substitutions in loop IIS1-S2 and the other in IIS3-S4. Although these two extracellular loop chimeras were not analyzed in this study, a set of single amino acid chimeras containing each of the amino acid differences in these two extracellular loops was analyzed previously, and no changes in sodium channel gating or response to β1 subunits were observed (14). Each of the other 13 extracellular loop chimeras gave sodium currents sufficient for detailed analysis (0.5–5 μA). For most chimeras, the expression level was comparable to that of WT when 20 ng of each mRNA was injected. For chimeras with lower expression levels than WT, up to 160 ng of α subunit mRNA was injected to obtain sodium currents of similar amplitude to those observed for 20 ng of WT α subunit mRNA.

Voltage Dependence of Activation and Inactivation—The voltage dependence of activation of WT rIIA and rH1 α subunits and selected α subunit chimeras expressed alone in Xenopus oocytes is illustrated as conductance-voltage curves in Fig. 2A, and the mean values for $V_{1/2}$, the voltage for half-maximal activation, are presented in Fig. 2C. $V_{1/2}$ for rH1 is 18 mV more negative than for rIIA (Fig. 2, A and C, open symbols). Five chimeras (IIS5-S11, IIS2-S6, IIS1-S2, IIS2-S6, and IVS3-S4) had a more negative voltage dependence of activation than WT rIIA (Fig. 2, A and C). Only chimera IVS2-S6 was observed to have a slightly more positive voltage dependence of activation than WT rIIA. Thus, these extracellular loops are important determinants of the voltage dependence of activation and contribute to the more negative voltage dependence observed for cardiac rH1 channels. If their effects were additive, they would more than account for the difference in activation gating between the two channels.

The voltage dependence of steady-state inactivation was studied with 100-ms conditioning prepulses. With this protocol, the voltage for half-inactivation of rH1 was 17 mV more negative than that of rIIA (Fig. 2B). Three chimeras (IIS2-S6, IIS1-S2, and IVS3-S4) inactivated at more negative potentials than WT rIIA, but at more positive potentials than rH1 (Fig. 2B). These were the only chimeras that had a shifted voltage dependence of inactivation (Fig. 2D). The three chimeras whose
voltage dependence of inactivation was negatively shifted (IISS2-S6, IIS1-S2, and IVS3-S4) also had a negatively shifted voltage dependence of activation (Fig. 2, C and D), suggesting that the change in inactivation results from coupling to the negatively shifted activation. Thus, these three extracellular loops are important determinants of the voltage dependence of inactivation as well as activation. Together, the negative shifts in steady-state inactivation observed for these three chimeras would be more than sufficient to account for the difference in the voltage dependence of inactivation between the rIIA and rH1α subunits.

**Kinetics of Inactivation and Recovery from Inactivation—Expression of the WT rIIA α subunit alone in Xenopus oocytes (7, 26, 27). It is not thought to be related to the distinct slow inactivation process of sodium channels, which requires longer depolarization and involves a different gating mechanism than fast inactivation (28–32).** Most chimeras displayed a similar time course of inactivation to that of WT at a test potential of 0 mV. However, five chimeras (IS5-SS1, ISS2-S6, IISS2-S6, IIS1-S2, and IVS3-S4) inactivated faster than WT (Fig. 3, A–D). As for WT, analysis of these sodium currents revealed that they could be fit by two exponential functions with fixed time constants, but with an increased fraction of channels inactivating with the faster time constant (Fig. 3E). These results indicate that the differences of these chimeric channels from WT are caused by a shift of the chimeric channels from the slow to the fast gating mode. Thus, these five extracellular segments are important determinants of the channel gating mode.

**The rate of recovery from inactivation at negative holding potentials was studied by repolarization to –200 mV following a conditioning pulse to –10 mV for 300 ms.** As for inactivation, the time course of recovery from inactivation for WT rIIA channels expressed without β1 subunits can be described by a

| Extracellular loop | Sequence |
|-------------------|---------|
| IS1-S2 rIIα       | SNPPDNKTN |
| Changes           | HD--P--Y |
| IS3-S4 rIIα       | FVDLGNVSL |
| Changes           | --------- |
| IISSS1 rIIα       | NKCLQPPDN SFNENITSF FNFNSDWGF ANRVTVMFN |
| Changes           | H--VR---- NFT-L-G-- --G-VFAD-L W------NS |
| IISS2-S6 rIIα     | WDEYIEIDSKH FYFDLEGQNDAG LCCGNSDAG QCPEGVICYC AGRNPNGYGT SF |
| Changes           | L-V-LN-FAN YLLKN-TT-V -- --R-L-- E--DH---- |
| IIS1-S2 rIIα      | NLYQLTLRAA GKTY |
| Changes           | R--Q--S-- I-- |
| IIS1-S2 rIIα      | ENYMTEQFS SV |
| Changes           | --N--AE--E EM |
| IIS3-S4 rIIα      | ANVEGLS |
| Changes           | SMGN-- |
| IISS2-S6 rIIα     | KSYECEVCKI SNDCELRWH MHH |
| Changes           | --N--LRHR-- DSGL--MD |
| IIIS1-S2 rIIα     | ETWMDCEMVA GQT |
| Changes           | --S--S |
| IIIS3-S4 rIIα     | EDIYIEQRTK IKT |
| Changes           | --L--E-- V |
| IIIS5-S4 rIIα     | ALGYSE |
| Changes           | --T--YA |
| IISS5-SS1 rIIα    | KEYHCINQTT GEM.FDVSVV NNYSECQALI ESNQTRAWKN VKNFD |
| Changes           | --GR--Q--E DLPLNYTT-- K--ESFN VTGEYWT-- |
| IISS2-S6 rIIα     | DJMYYADVSR NVELOQKYESD |
| Changes           | --GY--E--W-- |
| IVS1-S2 rIIα      | ETDDQSQEMT N |
| Changes           | --P--KV-- |
| IVS3-S4 rIIα      | KYFVSIGHTF |
| Changes           | --F--F-- |
| IVS5-SS1 rIIα     | REVGiddMFFN FE |
| Changes           | W--A--Q-- |
| IVSS2-S6 rIIα     | GLLAPIINNG PFDDPFDKH PGSSVKGDGC NPS |
| Changes           | --S--T-- Y--N--NLPN SNFG--R--N-- S--A |

**Amino acid sequences of sodium channel chimeras**

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sum of two exponentials, with approximately half of the channels recovering rapidly from inactivation (Fig. 4A). Using this protocol, the fraction of fast recovery from inactivation is 0.6 for rIIA and 0.9 for rH1 (Fig. 4E). For most chimeric channels, the fraction of fast recovery from inactivation was similar to that of WT rIIA a subunits (Fig. 4E). However, four chimeras that inactivated rapidly (IS5-SS1, ISS2-S6, and IIIS1-S2) also had faster kinetics of recovery from inactivation compared with WT rIIA channels (Fig. 4A–D, closed circles for chimeras versus open circles for rIIA). This accelerated recovery from inactivation was caused by a shift of channels from the slow gating mode to the fast gating mode, as illustrated in the measurements of $F_{fast}$ in Fig. 4E.

The effects of these chimeric substitutions on the kinetics of inactivation and recovery from inactivation indicate that four extracellular loops in domains I and III are important determinants of the sodium channel gating mode and that segment IVS3-S4 also has a minor effect. These are the first regions of the sodium channel structure found to have specific effects on the gating mode. As for the effects on voltage dependence, these effects of chimeric substitutions on the gating mode would be sufficient to fully account for the difference between rIIA and rH1 if they were additive.

**Modulation of Sodium Channel Gating by Coexpression of β1 Subunits**

**Differential Modulation of Cardiac and Brain Sodium Channels by β1 Subunits**—When either brain or skeletal muscle sodium channel a subunits are expressed alone in Xenopus oocytes, the resulting sodium currents are small, and they activate and inactivate abnormally slowly (6, 26, 27, 33). Coexpression of the β1 subunit increases the current amplitude, negatively shifts the voltage dependence of activation and inactivation, and decreases the inactivation rate constant.

**Fig. 2. Effects of substitution of extracellular segments on the voltage dependence of activation and inactivation.**

| Segment | Voltage Dependence of Activation | Voltage Dependence of Inactivation |
|---------|----------------------------------|-----------------------------------|
| ISS1-S2 | Open circles | Open squares |
| IIS1-S2 | Closed circles | Inverted closed triangles |
| ISS2-S6 | Closed squares | Closed diamonds |
| IVS2-S6 | Closed triangles | Closed hexagons |

**Fig. 3. Effects of substitution of extracellular segments on the kinetics of inactivation.**

- **A–D**: Average normalized sodium currents recorded at +10 mV in the absence (slow traces) or presence (fast traces) of β1 subunits. Solid lines, rIIA; dotted lines, chimeras IS5-SS1 (A), ISS2-S6 (B), IIS2-S6 (C), and IIIS1-S2 (D). $E$, mean fractions of the fast component of inactivation in the absence of β1 subunits. $F$, mean fractions of the fast component of inactivation in the presence of β1 subunits. Data are presented for a 1:4 molar ratio of a to β1 mRNA except as noted. Dashed lines indicate mean values derived from either rIIA alone in $E$ or rIIA + the β1 subunit (1:4 molar ratio) in $F$. Significant differences from rIIA are denoted by asterisks.
activation, and accelerates the rate of inactivation and recovery from inactivation (8, 34–39), as illustrated for type IIA sodium channels in Figs. 1A, 3A, and 4A. The acceleration of inactivation and recovery from inactivation are thought to result from a shift of the rIIA α subunit from a slow gating mode to a fast gating mode upon coexpression of β1 subunits (35, 37, 38, 40). β1 subunit mRNA is expressed in cardiac myocytes, as assessed by high resolution in situ hybridization (22). Coexpression of the rat β1 subunit with the rH1 α subunit significantly increases the current amplitude in Xenopus oocytes, but the voltage dependence and kinetics of gating are not dramatically altered (22). Similar experiments with human cardiac α and β1 subunits revealed significant effects of coexpression of β1 subunits on the voltage dependence of inactivation, but these effects were much smaller than those observed for brain or skeletal muscle sodium channels (41, 42). These differences in response to coexpression of β1 subunits suggested that analysis of brain/cardiac sodium channel chimeras may reveal extracellular loops required for β1 subunit binding or modulation of α subunit function.

Kinetics of Inactivation and Recovery from Inactivation—Effects of β1 subunits on the sodium channel gating mode are most easily assessed from measurement of the kinetics of inactivation and recovery from inactivation by analysis of exponential curve fitting. Coexpression of the β1 subunit substantially accelerated the kinetics of inactivation of rIIA (Fig. 3A, fast solid lines). The increased rate of inactivation was fit by an increase in $F_{\text{fast}}$ from 0.1 to 0.9 for an α/β1 subunit RNA ratio of 1:4 (Fig. 5, E and F). Coexpression of β1 subunits at that level substantially accelerated the rate of inactivation of all chimeras (Fig. 3, A–D, dotted lines). With an α/β1 subunit RNA molar ratio of 1:4, most chimeric channels displayed a fraction of fast inactivation near 0.9, comparable to WT rIIA in the presence of β1 subunits (Fig. 3F). In contrast, chimera IVSS2-S6 showed a substantially decreased fraction of fast inactivating channels compared with WT in the presence of β1 subunits (Fig. 3P). Since its voltage dependence of inactivation was not significantly different from WT rIIA α subunits in the absence of β1 subunits, this observation suggests that IVSS2-S6 is an important extracellular structure determining the functional effect of β1 subunits on gating mode.

Coexpression of the β1 subunit also substantially increased the fraction of rIIA channels recovering rapidly from inactivation (Fig. 4, A–D, open symbols). The fraction of fast recovery from inactivation increased from −0.6 to 0.9 at an α/β1 subunit RNA ratio of 1:4 (Fig. 4, E and F). For all the chimeras tested, the fraction of channels with fast recovery from inactivation in the presence of the β1 subunit was ~0.9, similar to WT, except for chimera IVSS2-S6 (Fig. 4, A–D, open symbols; and Fig. 4F). Chimera IVSS2-S6 had a similar recovery from inactivation compared with WT in the absence of the β1 subunit (Fig. 4E), whereas in the presence of the β1 subunit, it showed slower recovery from inactivation due to a reduced fraction of channels recovering in the fast gating mode (Fig. 4F). Considered together with the reduced β1 subunit effect on the inactivation kinetics of chimera IVSS2-S6, these results demonstrate a decreased efficiency of β1 subunit modulation of inactivation and recovery from inactivation for chimera IVSS2-S6 due to a decreased modulation of the sodium channel gating mode.

Reduced Effects of Coexpression of β1 Subunits on Chimera IVSS2-S6—To further investigate the ability of β1 subunits to modulate chimera IVSS2-S6, we carried out experiments with a 1:1 molar ratio of α to β1 subunit mRNA to emphasize differences in β1 subunit modulation. In the absence of the β1 subunit, chimera IVSS2-S6 and WT rIIA sodium channels inactivated at a similar rate (Fig. 5A, traces 1 and 4). In contrast, after co-injection with an α/β1 subunit molar ratio of 1:1, chimera IVSS2-S6 inactivated at a substantially slower rate than WT rIIA (Fig. 5A, traces 2 and 5), and a smaller, but still significant difference in the rate of inactivation was observed when a molar ratio of 1:4 was used (traces 3 and 6).

In the absence of the β1 subunit, chimera IVSS2-S6 had a time course of recovery similar to that of WT (Fig. 5B, circles). However, the β1 subunit was less effective at accelerating the recovery from inactivation for chimera IVSS2-S6 than for WT channels when coexpressed either at a 1:1 (Fig. 5B, squares) or at a 1:4 (inverted triangles) molar ratio of α to β1 subunit mRNA. Thus, as for measurement of inactivation, the difference between WT and chimera IVSS2-S6 is more pronounced at a lower level of coexpression of β1 subunit mRNA. These results imply a reduced affinity of the chimera for the expressed β1 subunit since the deficit in β1 subunit modulation can be at least partially compensated by increased expression of the β1 subunit.

Chimera IVSS2-S6 had a more positive voltage dependence of activation in either the presence or absence of β1 subunits (Fig. 2 and data not shown), which could slow inactivation due to the coupling of inactivation to activation. The voltage dependence of inactivation was also similar to that of WT rIIA in either the presence or absence of β1 subunits (Fig. 2 and data...
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FIG. 5. Effect of substitution of extracellular segment IVSS2-S6 on modulation of inactivation and recovery from inactivation by the β1 subunit. A. Average normalized sodium currents recorded at +10 mV in either the absence or presence of β1 subunits (n = 6 for each current trace). Solid lines, rIIA; dotted lines, chimera IVSS2-S6. Traces 1 and 4, α subunits alone; traces 2 and 5, α subunits plus β1 subunits at an α/β1 subunit molar ratio of 1:1; traces 3 and 6, α/β1 subunit molar ratio of 1:4. B, recovery from inactivation at −100 mV in either the absence or presence of β1 subunits. Solid lines are two-exponential fits from representative cells. Open symbols, rIIA; closed symbols, IVSS2-S6; circles, α subunits alone; squares, α/β1 subunit molar ratio of 1:1; inverted triangles, α/β1 subunit molar ratio of 1:4.

not shown). Evidently, coexpression of β1 subunits with chimera IVSS2-S6 was able to cause a similar shift in the voltage dependence of inactivation as for WT. These results show that substitution of the extracellular loop in chimera IVSS2-S6 causes decreased effectiveness of β1 subunit modulation of the kinetics of inactivation and recovery from inactivation specifically, without major effects on the voltage dependence or kinetics of gating in the absence of the β1 subunits. This might occur by weakening the association of α and β1 subunits and/or by decreasing the functional modulation of the gating mode of the α subunit by the bound β1 subunit.

DISCUSSION

Our experiments provide the first evidence for functional roles of the extracellular loops of the sodium channel in its normal gating. We consider below each of the observed effects on channel gating in light of other data on the gating of brain and cardiac sodium channels and its modulation by coexpression of β1 subunits. A molecular map of the functional effects of chimeric substitutions in extracellular loops of the sodium channel α subunit is presented in Fig. 6 for reference.

Conversion of rIIA Extracellular Loops into rH1 Sequences Causes Changes in the Voltage Dependence of Gating Similar to the Cardiac Isoform—Cardiac sodium channels activate and inactivate at more negative membrane potentials than brain sodium channels when expressed in Xenopus oocytes (11, 23, 24). Our results show that molecular differences in the extracellular loops of the α subunits make an important contribution to this difference in activation gating. The sum of the voltage shifts caused by substitution of the IISS5-S1, IISS2-S6, IIS1-S2, IISIISS2-S6, and IVS3-S4 segments is −22.7 mV, more than enough to account for the entire shift in the voltage dependence of activation between these two channels if the changes are truly additive when combined in a single channel construct. These results provide the first evidence that extracellular loops are important determinants of the gating properties of sodium channel isoforms, but previous work on L-type calcium channels has shown that the kinetics and voltage dependence of gating of the cardiac and skeletal muscle calcium channel isoforms are controlled by the IS3 segment and IS3-S4 loop (43). Evidently, isoform-specific differences in extracellular loop sequences can have important effects on activation gating, even though the S4 voltage sensors whose movements drive the activation process are located within the membrane. Requirements for movements of these extracellular loops during the gating process are likely to be responsible for the observed effects on the voltage dependence of activation.

Cardiac sodium channels also inactivate at more negative membrane potentials than brain sodium channels when expressed in Xenopus oocytes (11, 23, 24). The voltage dependence of inactivation is derived primarily from its coupling to the highly voltage-dependent activation process (44). Therefore, it would be expected that some of the chimeric substitutions that alter the voltage dependence of activation gating would also alter the voltage dependence of steady-state inactivation. We found that chimeric substitutions in segments IISS2-S6, IIS1-S2, and IVS3-S4 all shifted the voltage dependence of steady-state inactivation negatively, toward the Vh value for cardiac sodium channels. The sum of the negative shifts (−21.6 mV) observed would more than account for the difference in Vh observed between the WT rIIA and rH1 α subunits expressed in Xenopus oocytes. Thus, it is likely that these extracellular segments are involved in the conformational changes that couple activation to inactivation in sodium channels.

Chimeric Substitutions of Four rIIA Extracellular Loops with rH1 Sequences Cause Changes in Modal Gating Similar to the Cardiac Isoform—Sodium currents due to expression of the

FIG. 6. Summary of the functional effects of substitution of the extracellular loops of the sodium channel. Upper, the functional effects of chimeric mutations are illustrated for each extracellular loop. Under each domain (I–IV), the first column reflects the S1-S2 loop, the second column the S3-S4 loop, the third column the S5-S61 loop, and the fourth column the S62-S6 loop. Vh, voltage for half-maximal activation; V/2, voltage for half-maximal inactivation; Ff, fraction of channels in the fast gating mode determined for recovery from inactivation; α-ScTx, effects on scorpion α-toxin binding; β-ScTx, effects on scorpion β-toxin binding; β1, effects on modulation by the β1 subunit. + denotes an effect; − denotes no effect. Lower, the structure of the sodium channel α subunit correlated with the functional effects of substitution of each extracellular loop.
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rH1 α subunit alone in Xenopus oocytes have rates of fast inactivation and recovery from inactivation that are comparable to those of native cardiac sodium channels (23, 24), consistent with channel function in the fast gating mode. Chimeric substitutions of rH1 sequences in the IS5-S1, ISS2-S6, IVSS2-S6, or IIIS1-S2 loop of rIIA caused a greater fraction of sodium channels to inactivate and recover from inactivation in the fast gating mode compared with WT rIIA channel in the absence of β1 subunits. Thus, these chimeric sodium channels behaved more like the cardiac isof orm in terms of the gating mode. As the inactivation process itself takes place on the intracellular side of the sodium channel (44) and involves closure of an inactivation gate formed by the intracellular loop between domains III and IV (45–47), it is unlikely that these extracellular sequences contribute directly to the inactivation process. Instead, they are likely to be involved in the conformational changes that determine the inactivation gating mode and that control coupling of voltage-dependent activation to inactivation. In calcium channels, amino acid residues in the IS6 transmembrane segments and the ISS2-S6 loop also affect the kinetics of inactivation of channel chimeras (48), suggesting a role for these corresponding segments of calcium channel α1 subunits in controlling the rate of voltage-dependent inactivation.

The IVSS2-S6 Extracellular Loop Is Important for Modulation of the Gating Mode by β1 Subunits—β1 subunits are single membrane-spanning proteins with a small intracellular domain and a larger extracellular domain that is composed primarily of an immunoglobulin-like motif resembling those of cell adhesion molecules (8, 9, 49). Multiple lines of evidence indicate that the immunoglobulin-like motif in the extracellular domain of the β1 subunit is primarily responsible for the functional modulation of sodium channels (17). First, deletion of the extracellular domain of β1 subunits does not alter their modulation of skeletal muscle (15, 16) or brain (17) sodium channel function. Second, deletion of all or part of the extracellular domain prevents the functional modulation of skeletal muscle or brain sodium channels by β1 subunits (15–17). Third, clustered mutations in the core of the immunoglobulin fold and in the A–A' β strand on one edge of the immunoglobulin fold of the β1 subunit prevent or reduce the functional modulation of brain sodium channels (17). Fourth, the extracellular domain of the β1 subunit is sufficient for sodium channel modulation when membrane-anchored by an unrelated transmembrane segment or a lipid anchor (50). Thus, it is likely that β1 subunits interact with α subunits and modulate their function through a site formed by one or more of the extracellular loops of the α subunit.

Of the 13 extracellular chimeras studied here, only substitution of the IVSS2-S6 segment had a major effect on the modulation of sodium channel modal gating properties by the β1 subunit. This chimera had inactivation properties comparable to those of WT in the absence of β1 subunits, but it had significantly slower inactivation than WT when coexpressed with β1 subunits and significantly slower recovery from inactivation. Because these effects on rIIA α subunits have previously been shown to result from a shift from a slow to fast gating mode upon expression of β1 subunits in Xenopus oocytes (35), our results indicate that this chimera is less responsive to the effect of the β1 subunit to shift channels to the fast gating mode. Therefore, our results implicate the IVSS2-S6 segment in interaction with β1 subunits and in modulation of the gating mode by β1 subunits. Since the reduced β1 subunit effect on chimeras IVSS2-S6 can be partially restored by expression of a higher level of β1 subunits, it is likely that the affinity of the chimeric α subunit for the β1 subunit is reduced. Therefore, we propose that the IVSS2-S6 segment is one point of interaction between the immunoglobulin-like domain of the β1 subunit and the rIIA α subunit. A similar conclusion was reached by analysis of a different set of channel chimeras between cardiac and skeletal muscle sodium channels (Ref. 16 and see below).

Comparison with Studies of Chimeric Cardiac and Skeletal Muscle Sodium Channels—There are comparable functional differences between cardiac and skeletal muscle sodium channels to those described here for cardiac and brain sodium channels. Previous work on chimeras with substitutions of the large intracellular loops connecting the four homologous domains of the sodium channel α subunit found that none of the three cytoplasmic interdomain sequences was responsible for the functional differences between cardiac and skeletal muscle sodium channels (51). The functional and pharmacological properties of chimeras based on exchanges of entire homologous domains between skeletal muscle and cardiac sodium channels have also been studied. The exchange of domain I produced sodium channels with intermediate inactivation kinetics between skeletal muscle and cardiac channels (52), and exchanges of domains III and IV also had effects on inactivation (51). These results are consistent with our findings that substitutions of the IS5-S81, ISS2-S6, IIIS1-S2, and IVS3-S4 extracellular loops all affect inactivation properties.

The site of interaction of β1 subunits with α subunits of sodium channels has also been analyzed in studies with chimeras of human heart and skeletal muscle sodium channels (16). Substitution of the SS-S6 loops in domains I and IV of the skeletal muscle sodium channel with the corresponding segments of the cardiac channel abolished response to coexpression of the β1 subunit, and the reciprocal transfer yielded a partial response of the cardiac sodium channel to the β1 subunit. Further dissection of the SS-S6 loop in domain IV suggested that the IVSS2-S6 extracellular loop was important for functional interaction with β1 subunits. These results are consistent with the conclusion that extracellular interactions are most important for modulation of sodium channel gating by β1 subunits and are in agreement with our conclusion from analysis of extracellular chimeras of brain and cardiac sodium channels that the IVSS2-S6 loop is critical for this functional interaction.

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