Mapping of voltage sensor positions in resting and inactivated mammalian sodium channels by LRET

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Voltage-gated sodium channels (Navs) play crucial roles in excitable cells. Although vertebrate Nav function has been extensively studied, the detailed structural basis for voltage-dependent gating mechanisms remain obscure. We have assessed the structural changes of the Nav voltage sensor domain using lanthanide-based resonance energy transfer (LRET) between the rat skeletal muscle voltage-gated sodium channel (Nav1.4) and fluorescently labeled Nav1.4-targeting toxins. We generated donor constructs with genetically encoded lanthanide-binding tags (LBTs) inserted at the extracellular end of the S4 segment of each domain (with a single LBT per construct). Three different Bodipy-labeled, Nav1.4-targeting toxins were synthesized as acceptors: β-scorpion toxin (Ts1)-Bodipy, KIIIA-Bodipy, and GIIA-Bodipy analogs. Functional Nav-LBT channels expressed in Xenopus oocytes were voltage-damped, and distinct LRET signals were obtained in the resting and slow inactivated states. Intramolecular distances computed from the LRET signals define a geometrical map of Nav1.4 with the bound toxins, and reveal voltage-dependent structural changes related to channel gating.

Significance

Physical activities of our body and extremities are achieved by the propagation of electrical signals called action potentials from our brain, through nerves, to skeletal muscles. Voltage-gated sodium channel (Navs) play essential roles in the generation and propagation of action potentials in such excitable cells. Although mammalian Nav function has been studied comprehensively, the precise structural basis for the gating mechanisms has not been fully clarified. In this study, we have used lanthanide-based resonance energy transfer to obtain dynamic structural information in mammalian Nav gating. Our data define a geometrical map of Navs with the bound toxins and reveal voltage-induced structural changes related to channel gating, which lead us further toward an understanding of the gating mechanism in mammalian Navs.

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KIIIA-Bodipy and GIIIA-Bodipy (Fig. 1B). The function and binding sites in mammalian Navs for these three toxins have been well characterized by electrophysiological experiments and computational modeling; therefore, they function as trustworthy references for dynamics of the donor movement (21–26). We generated a total of 12 different Nav1.4 clones as LRET donors, which retained functional activity. Each clone has one inserted LBT, which binds a Tb³⁺ ion with high affinity in one of three different strategic locations on the extracellular side of the S4 segment on DI, DII, DIII, or DIV of Nav1.4 (Nav-LBTs). Multiple distances obtained from the Tb³⁺ in Nav-LBTs to each
toxin-dye constrain the intramolecular geometrical maps of Nav1.4 with bound toxins. In addition, we observed changes in the distance, from either DI or DIV LBTs to the bound KIIIA-Bodipy, between the resting and relaxed states (27), usually associated with a slow inactivated state of the pore under voltage clamp conditions. (Relaxed and slow inactivated states will be used interchangeably). Our results provide direct evidence for voltage-dependent structural changes in mammalian sodium channels (Fig. 1C).

Results

Generating Optimal Nav-LBT Clones as LRET Donors. To obtain stable Nav-LBT clones that produce a robust donor (D) signal, we optimized the insertion site of the LBT. First, we generated eight different clones with the LBT in domain I (DI-LBTs), from DI-LBT-R(0) to DI-LBT-R(−7), with the LBT located zero to seven residues from the first S4 gating charge “R1” of DI. The numbers in parentheses indicate the number of residues counting back from the first gating charge. R; thus, DI-LBT-R(0) has the LBT inserted immediately before the first charge and DI-LBT-R(−7) has seven residues between the LBT and the first charge (SI Appendix, Fig. S1A). D-only signals were measured from each clone, and the signal decay time constants were evaluated. As shown in SI Appendix, Fig. S1B, constructs DI-LBT-R(0) to DI-LBT-R(−3) did not show robust D signals. On the other hand, constructs DI-LBT-R(−4), DI-LBT-R(−5), DI-LBT-R(−6), and DI-LBT-R(−7) produced robust D signals with time constants that are longer than 2.2 ms. Among them, the oocytes expressing DI-LBT-R(−4) had a lower survival rate (30–50%) than the others (>65% for DI-LBT-R(−5), DI-LBT-R(−6), and DI-LBT-R(−7)). These results are consistent with the idea that the LBT motif must maintain its conformation to exclude water from the chelated terbium, which may be affected depending on where the motif is inserted. It is empirically assumed that introduction of sequential glycine residues increases polypeptide flexibility while conserving the appropriate conformation of the LBT to chelate the Tb³⁺ ion. Therefore, in an attempt to improve the magnitude of the D signal, we created Nav1.4-LBT constructs containing additional glycine residues attached to the N terminus of the LBT in DI-LBT-R(−5). We found that a triple-glycine insertion optimized the D signal of the DI-LBT clones (SI Appendix, Fig. S1C). This engineered motif, here referred to as 3G-LBT, was implemented in each of the three positions of S4 in all other Nav1.4 domains (DII, DIII, and DIV) for a total of 12 functional donor clones (Fig. 2). All constructs were functionally characterized under cut-open oocyte voltage clamp (COVC), and confirmed that most of them maintained the voltage-dependent properties of the Na⁺ conductance, although some [e.g., DIV R(−6), DII R(−7) in SI Appendix, Fig. S2] showed altered voltage-dependent activation and/or modified fast inactivation (Fig. 2B and SI Appendix, Fig. S2).

Pharmacological Properties of Acceptor Toxins. Ts1 is a β-scorpion toxin isolated from the Brazilian scorpion, *Tityus serrulatus* (28). Such scorpion toxins bind to Navs through the S1–S2 and the S3–S4 linkers of the VSD in DII and the S5–S6 pore linker in DIII (25, 26). Their mechanism of action has been described as a “voltage sensor trapping effect,” wherein the β-scorpion toxin traps the DII VSD in an activated state, facilitating Nav opening (23, 24). Consequently, β-scorpion toxins shift the threshold of Nav activation in the direction of hyperpolarization.

We prepared Bodipy site-specifically labeled Ts1 toxin as previously reported (29). Briefly, Ts1 bearing the mutation W50Pra (+) was chemically synthesized as Ts1-W50Pra, which enabled the bioconjugation with fluorescent dye using “click chemistry” (Fig. 1B). The basic pharmacological and optical properties of Ts1-Bodipy were reported previously (29). Interestingly, whereas most toxins reported to date that bind to Nav1.4 do so reversibly, Ts1-W50Pra’s binding was essentially
irreversible in our hands; therefore, we tested whether this property is preserved for Nav-LBTs. Here, we defined the activation threshold as the voltage where the conductance is 5% of the full conductance. The activation threshold of wild-type Nav1.4 channels is −30 mV (black symbols in Fig. 3A). After pretreatment with 1 μM Ts1-W50Pra, the activation threshold shifted to approximately −45 mV, even after washing with toxin-free recording solution (green symbols in Fig. 3A). This result indicates that the threshold of Nav1.4 activation was shifted in the hyperpolarizing direction by 15 mV, which is equivalent to the saturating effect caused by wild-type Ts1 (Fig. 3). Similar results were obtained for all three Nav-LBTs tested: DI-3GLBT-R(−5), DII-3GLBT-R(−5), and DIV-3GLBT-R(−5), which indicates that the pharmacological properties of the Nav1.4–Ts1 interaction are preserved in the Nav-LBT constructs (Fig. 3 B–D). We tested whether this property is maintained with the labeled Ts1. Although we tested only one cell because of the limited amount of Ts1-Bodipy available, the effect of Ts1-Bodipy remained stable even after washing with toxin-free solution. This property enabled us to conduct LRET measurements in toxin-free solution after pretreatment with Ts1-Bodipy, which has the advantage of decreasing nonspecific transfer in LRET signals.
and GIIIA-Bodipy (SI Appendix, Supplemental Experimental Procedures and Figs. S3 and S4). We verified the pharmacological properties of KIIIA-Bodipy and GIIIA-Bodipy by functional recording under COVC on wild-type Nav1.4. We found IC50 values of 108.9 ± 50.4 nM for KIIIA-Bodipy and of 336.4 ± 75.4 nM for GIIIA-Bodipy (Fig. 3E). Although these apparent affinities are somewhat lower than the unlabeled μ-conotoxins, these derivatives remain well-suited for LRET experiments.

**Geometrical Parameters of Toxin Bodipy-Bound Nav1.4-LBT.** Intact, unclamped oocytes were used to perform LRET measurements between the Nav-LBT constructs and each Bodipy-labeled toxin. In parallel experiments using identical solutions, resting potentials were measured and found to be in the range −20 to −15 mV. Therefore, a significant population of the Navs described in this type of experiments is assumed to be mostly in the slow inactivated state.

In LRET, the donor/acceptor distance, R, is obtained from the Förster relation:

$$R = R_0 \left[ \frac{\tau_{DA}}{(\tau_D - \tau_{DA})} \right]^{1/6} = R_0 \left[ \frac{\tau_{SE}}{(\tau_D - \tau_{SE})} \right]^{1/6},$$

where $R_0$ is the distance for 50% energy transfer efficiency, and $\tau_{DA}$ and $\tau_D$ are the lifetimes of Tb3⁺ luminescence decay in the presence and absence of an acceptor, respectively (20). $\tau_{SE}$ is the sensitized emission (SE) lifetime or fluorescence decay from the acceptor dye (here, Bodipy). The $R_0$ values for each toxin-dye/LBT Tb3⁺ pair were: 41.8 Å for Ts1-Bodipy; 41.4 Å for KIIIA-Bodipy, and 41.2 Å for GIIIA-Bodipy (Fig. 4).

LRET signals from Ts1-Bodipy were obtained as follows. First, we measured all oocytes expressing Nav-LBTs and selected cells showing robust D signals (TD > 2.25 ms). The selected oocytes were pretreated with 1 μM Ts1-Bodipy for 15 min. After rinsing the oocytes twice, using fresh standard oocyte saline (SOS) and then fresh recording solution, SE signals in toxin-free LRET recording solution were measured. Representative LRET decays, including the D (black trace) and SE (green traces) signals, are shown in Fig. 5A (Right). Based on the time constant of the SE signal decay, the distance between DI-3GLBT-R(−5) and Ts1-Bodipy was calculated as 44.2 ± 0.3 Å. Similarly, the distance between DIII-3GLBT-R(−5) and Ts1-Bodipy was 53.9 ± 1.2 Å. The SE signal of DIV-3GLBT-R(−5) was too small to determine reliable time constants (SI Appendix, Fig. S5). Instead, the distance between DIV-3GLBT-R(−5) and Ts1-Bodipy was calculated using the time constant of D in the presence of acceptor decay (DA) signal, resulting in 63.7 ± 1.8 Å. LRET assays using DII-LBT clones and Ts1, on the other hand, were not conducted because preliminary experiments using DI-3GLBT-R(−5) showed no Ts1 effect, suggesting that LBT insertion in DII prevents Ts1 binding. Even though the distance between DIV-3GLBT-R(−5) and Ts1-Bodipy falls within a region where LRET is relatively less sensitive, all three distances serve to provide the general position of the toxin, given that the distances measured were sufficiently different between them. However, because two of the three distances measured with Ts1-Bodipy do
not fall within a region of high sensitivity in LRET (i.e., within ~5 Å from the R0 value in both directions; R0 ~ 42 Å; Fig. 4C), and because the LRET signals are also smaller, Ts1-Bodipy was less suitable than the other toxin-dyes to serve as an acceptor for measurements of small movements during gating transitions among different conformations under voltage clamp (discussed in the following section).

To obtain SE signals from KIIIA-Bodipy, we first recorded D signals in the same way as described above for Ts1-Bodipy. Then, we measured SE signals in the presence of 0.85 μM KIIIA-Bodipy in the LRET recording solution to avoid toxin dissociation (concentration of approximately eightfold IC90, ~90% of channels bound). Similarly, we obtained SE signals from GIIIA-Bodipy in the presence of 0.85 μM GIIIA-Bodipy (concentration of approximately threefold IC90, ~75% of channels bound). Representative LRET signals are shown in Fig. 5A. The SE transfer signals from KIIIA-Bodipy to DII-3GLBT or DII-3GLBT clones showed faster decay than the SE transfer signals from GIIIA-Bodipy to the same donor constructs (Fig. 5A and SI Appendix, Fig. S5). This result indicates that, comparatively, KIIIA-Bodipy “sits” closer to DII-VSDs and DIII-VSDs than GIIIA-Bodipy. The time constant values measured and the computed distances from all donor/acceptor pairs measured are shown in Tables 1–3. Collectively, these results establish the geometrical locations of toxin-dyes bound to Nav1.4 as shown in Fig. 5B.

Gating-Dependent Conformational Changes in Nav Channel Detected by LRET Between Functional Nav-LBTs and KIIIA-Bodipy. DI-LBTs and DIV-LBTs as donors and KIIIA-Bodipy as acceptor were chosen for the detection of voltage-dependent changes in LRET signals in functional gating channels. The choice of donor/acceptor pairs for these experiments was guided by the following: (i) computed distances between KIIIA-Bodipy and DI-LBTs or DIV-LBTs for unclamped oocytes were close to the R0 (41–42 Å) that is the most sensitive range for detection of changes in distance with high precision using LRET, and (ii) SE signals from KIIIA-Bodipy were larger than SE signals from other toxin-dyes, which results in better signal resolution.

Two functional gating states of the mammalian Nav1.4 were assessed by LRET measurement in voltage-clamped oocytes. The slow inactivated (or relaxed) state and the resting state were induced by changes in the membrane potential under voltage clamp (27, 33–35) (Fig. 6). To ensure that the recorded changes in LRET signals were induced by the membrane potential manipulation and not due to changes in cell morphology by oocyte decay, only oocytes with matching slow inactivated signals at the beginning and end of the run were included in our analysis. A protocol to assess the Nav1.4 resting state was flanked by the two slow inactivation protocols (as shown in Fig. 6D, Right). Briefly, the oocyte was held at ±20 mV for over 5 min to induce Nav slow inactivation before LRET signal recordings (orange traces in Fig. 6A). The channels were then relaxed of inactivation by holding the membrane potential at −80 to −90 mV for 4 min, and thus driving channels to the resting state. The voltage was held at −90 mV for another minute before recording the LRET signal in the resting state (red traces in Fig. 6A). Finally, the LRET signal corresponding to the slow inactivated state was recorded once again (green traces in Fig. 6A). As shown in Fig. 6A, the SE signals were different depending on the functional state in which the channels were driven by the voltage protocol. However, the D signal from some of the clones also showed voltage-dependent changes, as shown in Fig. 6B, which might be related to direct quenching of the Tb3+ ions (LBT-bound) by voltage-dependent conformational changes. We collected D signal data in the presence of unlabeled KIIA (KIIA-N3X) independently and computed all distances using time constants for both D and SE signal decays at each voltage, with error propagation as described previously (18). The voltage-dependent donor/acceptor distance changes in DI-LBTs and DIV-LBTs are summarized in Fig. 6B and in Table 4.

Discussion
We have obtained two major pieces of structural information from functionally active mammalian Nav1.4 in live cells using LRET. First, LRET-based distance estimates, using variants of three different fluorescently labeled peptide toxins as acceptors, are consistent with various other experimental studies and structure-based predictions of the sites of action of these toxins.
physiological assays, and theoretically by computational molecular data as they emerge. Provide important constraints on the functional interpretation of such data have not previously been experimentally accessible. This structural information regarding sites of action of these toxins, however, remains somewhat imprecise. Our results provide a stable reference to measure VSD movement. A major goal of this project was to provide distance measurements from functional Navs in different gating states that are determined by voltage. Under voltage clamp, LRET showed significant voltage-dependent changes in the distance between KIIIA-Bodipy and the LBT/Tb3− signal obtained in the first and second slow inactivated state showed a voltage-dependent change. The D signal obtained in the first and second slow inactivated state recordings are shown as black and blue traces, respectively; the resting state D signal is the red trace. (D) Pulse-laser protocols used to induce and record from the slow inactivated and resting states are shown. (E) Distances between KIIIA-Bodipy and DI-LBTs or DIV-LBTs showed significant voltage-dependent changes. Data from Dx-3GLBT-R(−5) clones are shown in blue, data from Dx-3GLBT-R(−6) are shown in red, and data from Dx-3GLBT-R(−7) are shown in green. Error bars represent SEMs. Asterisks indicate significant distance changes (⁎P < 0.05, unpaired t test).

This structural information regarding sites of action of these toxins has been obtained directly from functioning Nav proteins. Such data have not previously been experimentally accessible. Second, LRET measurements, under voltage clamp, enabled us to directly assess the structural differences between two functional Nav states, resting and slow inactivated. These results will provide important constraints on the functional interpretation of new structural data as they emerge. Binding sites for Nav-specific toxins, as well as their binding characteristics, have been described functionally by electrophysiological assays, and theoretically by computational molecular modeling based on currently available crystal structures (21–26). Current knowledge for the physical locations of the toxins, however, remains somewhat imprecise. Our results provide details about the locations of toxin-binding sites. For instance, mutations in the voltage sensor of DII, which alter the function of toxin Ts1, imply that this region may be part of the toxin-binding site (25, 26). However, some of the effects of the mutations may be due to long-range allosteric modulations of a distant toxin-binding site. To date, homology modeling has been based on crystal structures of homotetrameric prokaryotic Navs or eukaryotic Kvαs because no high-resolution structures of a mammalian Nav have been available. Nonetheless, although the detailed architecture of mammalian Navs may differ somewhat from the current homology models, the currently proposed binding sites and orientations for well-characterized peptide toxins generally show good agreement among different studies.

From mutagenesis-based structure/function studies, the Nav-binding site for the Ts1 toxin has been proposed to be formed mainly by the DII VSD as mentioned above, with a minor contribution from the DIII pore linker (25, 26). The μ-conotoxins are thought to bind to the outer vestibule of the channel and to occlude the Na+ ion-conducting pathway physically (30). For the smallest of these μ-conotoxins, KIIIA, blockade of single-channel conductance is less than complete, and simultaneous occupancy by the classic all-or-none Nav blocker, TTX, is possible. Homology models for Navs indicate that the larger, archetypal GIHIA functionally occludes the ion-conducting pore, whereas KIIIA’s center of mass is slightly shifted toward the VSDs of DII and DIII (21, 22). The positions of the Bodipy acceptor in our toxin-containing dyes are effectively constrained by site-specific conjugation to Ts1-W50X, to the N terminus of KIIIA-N3X, or to GIHIA-TSA-1X (Fig. 1B). Thus, relative to the size of the functional Nav, their positions are defined well. Our experimental LRET data, from the use of multiple toxin-dye/LBT pairs, indicate locations of the bound toxins on Nav1.4 that are in good agreement with those locations in recently proposed toxin docking models (21, 22). Positions of the bound Bodipy-labeled toxins are shown schematically in Fig. 5. In the absence of a high-resolution structure of any mammalian Nav, this study lays a foundation for future studies to elucidate the mechanisms of toxin action in greater detail. When high-resolution structures are available, our data will allow direct comparison of geometric parameters measured in selected functional states with geometric parameters from purely structural studies.

Table 4. LRET results using KIIIA-N3X for D signal and GIHIA-Bodipy for SE signal from oocytes under voltage clamp

<table>
<thead>
<tr>
<th>Clones</th>
<th>Voltage</th>
<th>τSE, ms (n)</th>
<th>τD50, m</th>
<th>Distance, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1-3GLBT-R(−5)</td>
<td>−150mV</td>
<td>2.13 ± 0.03 (6)</td>
<td>1.19 ± 0.02 (5)</td>
<td>43.0 ± 0.5</td>
</tr>
<tr>
<td>D1-3GLBT-R(−6)</td>
<td>−150mV</td>
<td>2.24 ± 0.03 (6)</td>
<td>1.29 ± 0.03 (5)</td>
<td>42.3 ± 0.5</td>
</tr>
<tr>
<td>D1-3GLBT-R(−7)</td>
<td>−150mV</td>
<td>2.34 ± 0.01 (5)</td>
<td>1.11 ± 0.03 (7)</td>
<td>40.8 ± 0.4</td>
</tr>
<tr>
<td>D2-3GLBT-R(−5)</td>
<td>−150mV</td>
<td>2.37 ± 0.02 (5)</td>
<td>1.32 ± 0.03 (7)</td>
<td>43.0 ± 0.5</td>
</tr>
<tr>
<td>D2-3GLBT-R(−6)</td>
<td>−150mV</td>
<td>2.26 ± 0.06 (5)</td>
<td>1.04 ± 0.03 (6)</td>
<td>40.3 ± 0.7</td>
</tr>
<tr>
<td>D2-3GLBT-R(−7)</td>
<td>−150mV</td>
<td>2.35 ± 0.01 (5)</td>
<td>1.25 ± 0.02 (6)</td>
<td>42.3 ± 0.4</td>
</tr>
<tr>
<td>D4-3GLBT-R(−5)</td>
<td>−150mV</td>
<td>2.18 ± 0.04 (5)</td>
<td>0.99 ± 0.01 (5)</td>
<td>40.2 ± 0.4</td>
</tr>
<tr>
<td>D4-3GLBT-R(−6)</td>
<td>−150mV</td>
<td>2.30 ± 0.02 (5)</td>
<td>1.19 ± 0.03 (5)</td>
<td>41.9 ± 0.4</td>
</tr>
<tr>
<td>D4-3GLBT-R(−7)</td>
<td>−150mV</td>
<td>2.21 ± 0.08 (6)</td>
<td>1.01 ± 0.03 (6)</td>
<td>40.3 ± 0.8</td>
</tr>
<tr>
<td>D4-3GLBT-R(−6)</td>
<td>−150mV</td>
<td>2.38 ± 0.03 (6)</td>
<td>1.30 ± 0.04 (6)</td>
<td>42.7 ± 0.7</td>
</tr>
<tr>
<td>D4-3GLBT-R(−7)</td>
<td>−150mV</td>
<td>2.21 ± 0.04 (7)</td>
<td>1.03 ± 0.01 (5)</td>
<td>40.5 ± 0.3</td>
</tr>
<tr>
<td>D4-3GLBT-R(−7)</td>
<td>−150mV</td>
<td>2.39 ± 0.05 (7)</td>
<td>1.13 ± 0.01 (5)</td>
<td>40.7 ± 0.4</td>
</tr>
</tbody>
</table>

Values shown are averages ± SE of means. n, number of cells measured.
It is important to reiterate that our method measures distances along a line connecting the donor and acceptor. Thus, the computed distance changes will become smaller if the physical movement occurs perpendicular to the line connecting the donor and acceptor, and will approach zero as the motion becomes symmetrical around the vector connecting the LBT donor and Bodipy acceptor (SI Appendix, Fig. S6). For that reason, the measured distance changes can be considered a lower limit on the distance over which the terbium, chelated to the LBT, and Bodipy, attached to the toxin, approach one another in transitions between resting and relaxed states.

To interpret the LRET results quantitatively in three dimensions, high-resolution structural data from a eukaryotic Nav will indeed be required. At this stage, we can make a semiquantitative assessment of the conformational changes using the following assumptions: (i) The region of S4 preceding the first charge toward the N terminus is helical in structure, as in other voltage-gated ion channels (36), and (ii) the positions of LBTs follow the same helical structure. In SI Appendix, Fig. S1, the detection of D signals showed slight periodicity, which may reflect the helical structure of this region: for DI-LBT-R(−1), the periodicity was more strongly apparent than for DI-LBT-R(0), DI-LBT-R(−2), and DI-LBT-R(−3). Constructs DI-LBT-R(−4) again showed stronger periodicity. Considering all data from three different LBT clones for each domain [R(−5), R(−6), and R(−7)], a simple model containing a rotational and translational movement of the S4 segments is consistent with the LRET results, as represented schematically in Fig. 7. Importantly, our results, most clearly apparent in Fig. 6E, suggest a functional asymmetry of the VSDs in Nav1.4, with respect to KIIIA-Bodipy as follows: (i) For DI, the position of R(−5) in the resting state lies further from KIIIA-Bodipy than the distances of R(−6) and R(−7), whereas there are no significant differences between distances of DI R(−5), DI R(−6), and DI R(−7) with respect to KIIIA-Bodipy in the slow inactivated state, and (ii) for DIV, on the other hand, there is essentially no difference in the distances between KIIA-Bodipy and R(−5), R(−6), or R(−7) in the resting state, whereas the R(−7) position seems to be closest to KIIIA-Bodipy in the slow inactivated state. This reciprocal change observed between DI and DIV may underlie the different functional roles of DI and DIV VSDs previously proposed based on site-directed fluorimetry (2, 3, 37).

Another possible implication of our LRET results to be considered is that LBT insertion and/or toxin binding, per se, could modify Nav gating. Most of the 12 Nav-LBTs tested showed gating similar to wild-type Nav1.4, but some showed modified properties, including modest shifts of the voltage dependence of ionic conductance and changes in fast inactivation kinetics, as shown in SI Appendix, Fig. S2.

We conducted LRET analysis, under voltage clamp, in both resting and relaxed states (the latter reflects a slow inactivated state), but we did not obtain clear evidence that Nav-LBTs have exactly the same slow inactivated state as wild-type channels. Possible changes in the relaxed state in Nav-LBTs might generate some underestimation of the distances measured in our LRET analysis. On the other hand, we cannot exclude the possibility that toxin-Bodipy modifies the rate of entry to a slow inactivated state. There is evidence showing that toxins can modify Nav gating currents (34). Therefore, our LRET data might be partly determined by the presence of bound toxin-Bodipy. If so, this effect must be relatively small, and our results should mainly represent the intrinsic Nav gating process.

Other approaches to the estimation of conformational movements, which are subject to different constraints, might offer additional insights. For example, one possibility is to estimate the distance that VSD charges would have to move to account for voltage sensitivity of activation or measured gating currents. Energetic calculations, based on activation shifts resulting from discrete perturbations in the local electric field sensed by the VSDs when a GIIIA derivative binds to and dissociates from the pore, are consistent with a net movement of the center of the gating charge of a few angstroms (14) but require assumptions of the shape of the potential profile across the VSD and the resting position of the center of the gating charge. The present results are consistent with those previous estimates, but further experiments will be needed to construct a complete picture of mammalian Nav VSD charge movement and gating dynamics.

Technically diverse approaches to the measurement of spatial rearrangements in the coupled VSD-PD system will, in general, fall most conveniently on different coordinate systems. For LRET measurements, the estimated distances lie directly along a vector connecting the donor and acceptor, but, a priori, we know neither the spatial locations of the probes in a given functional state nor the direction of movement during the transition between two states. Our calculated changes are ∼2 Å, but amplitudes of movements between different well-defined reference points could be either larger or smaller, and would depend on the direction of movement of the molecular probes used in a particular assay.

Fig. 7. Schematic model of Nav1.4 DI and DIV movement that satisfies the LRET data. A simplified model, where the S4 segment does not change its tilt, can be built considering all of our LRET data under voltage clamp from three different LBT clones [R(−5), R(−6), and R(−7)]. The results are consistent with a rotation of the S4 helices in DI and DIV. Significant movements are indicated in the figure, alongside the corresponding distance measurement, for the slow inactivated state. (Upper) Blue helix represents the position of the DI-S4 helix at rest, and red indicates its slow inactivated state. (Lower) Purple helix is the DIV-S4 helix in the resting state, and red indicates its slow inactivated state. Circles indicate the Tb3+ ion position from R(−5) (blue), R(−6) (red), and R(−7) (green), respectively. The star indicates the chromophore of KIIIA-Bodipy. Long dashed lines depict the distance between donor (colored circles) and acceptor (star) in the resting state, and fine dotted lines depict the distance between donor and acceptor in the slow inactivated state.
(compare SI Appendix, Fig. S6). Nevertheless, our explicit distance measurements, and their voltage dependence, will provide clear and critical constraints on interpretation (38) of future structural and functional results, and thus will help us lead others to a comprehensive understanding of the intricate coupling involved in voltage-dependent gating in eukaryotic Navs.

Conclusions

The voltage sensor segments of Nav1.4 DI and DIV occupy distinguishably different locations in resting and slow inactivated eukaryotic Navs. Voltage-clamp-LRET constitutes an invaluable technique to assess structural dynamics of functional ion channels in live cells. The approach presented here provides an important step toward understanding the voltage-driven structural dynamics of mammalian Nav gating; the results will be especially pertinent as structures of a mammalian Nav are solved in additional specific conformations.

Experimental Procedures

Molecular Biology. The α and β1 subunits of Nav1.4 were cloned into pBSTA vector. For protein expression, ORNAs for Nav1.4 α and β1 subunits were transcribed in vitro using the T7 message CRNA kit (Ambion) and injected at a 1:1 molar ratio into Xenopus laevis oocytes. For electrophysiological experiments, freshly isolated oocytes were injected with 1 ng of cRNA and kept in SOS solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 10 mM Hepes, 200 μg/mL sodium pyruvate (pH 7.4)) for 1–2 d at 18 °C. For LRET experiments, the oocytes were injected with 75 ng of CRNA and incubated in SOS for 5–6 d at 12 °C, after which they were incubated for 1–2 d at 18 °C.

Chemical Synthesis of Dye-Conjugated Peptide Toxins. Ts1-Bodipy. Ts1-Bodipy was prepared as previously described (29). Bodipy was incorporated by click-conjugation of an azide-containing polypeptide precursor with Bodipy-alkyne (Lumiprobe). The X building block was incorporated in position 3 of KIIIA or added to the N terminus of GIIIA-TSA. In preliminary tests, the IC50 of GIIIA-TSA (9.8 mM) was found to be similar to the IC50 reported for the wild-type GIIA (6.3 mM) (SI Appendix, Supplemental Experimental Procedures). NMR spectroscopy was used to confirm the correct fold of the synthetic analogs. NMR experiments on all analogs were performed on a Bruker Avance 600 spectrometer equipped with a cryoprobe. The obtained Hu-chimera shifts provide a sensitive probe of peptide secondary and tertiary structure and were compared with literature values reported previously for wild-type KIIIA and GIIA (39, 40) (SI Appendix, Figs. S3 and S4). More details of the synthesis are provided in SI Appendix, Supplemental Experimental Procedures.

Preparation of Dye-Labeled Toxins. All Bodipy-conjugated toxins were diluted in external recording solution for electrophysiology (Experimental Procedures, Electrophysiology) or in the LRET recording medium (Experimental Procedures, LRET Recording). Unlabeled toxin concentrations were obtained by spectrophotometry (Nanodrop; Thermo Scientific). Concentration of toxins conjugated with Bodipy were calculated based on the extinction coefficient values and the peak values of their absorption spectra obtained with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies) (also Experimental Procedures, Extinction Coefficient Measurements in the Recording Solution).

Electrophysiology. Ionic currents were recorded using a COVC setup (41). The external solution contained 103.5 mM NMG-MS, 11.5 mM Na-MS, 10 mM Hepes, and 2 mM Ca-MS (pH 7.4). The internal solution contained 103.5 mM NMG-MS, 11.5 mM Na-MS, 10 mM Hepes, and 2 mM EGTA (pH 7.4). Ts1 derivatives were dissolved in external solution containing 1 μM Ts1-Bodipy. Both signals were obtained by averaging 20 measurements collected from oocytes without any treatment. SE signals were obtained by averaging 40 measurements collected from oocytes with Ts1 pretreatment, in the presence of 0.85 μM KIIIA-Bodipy or 0.85 μM GIIIA-Bodipy. For LRET recordings with voltage clamp, D signals were obtained from oocytes in the presence of 0.85 μM unlabeled KIIIA, KIIIA-N3X. The SE signals were obtained from oocytes in the presence of 0.85 μM KIIIA-Bodipy. Both signals were obtained by averaging 20 measurements for the D signal and 40 measurements for the SE signal; measurements under poor voltage control were excluded from the average.

Data were analyzed with an in-house program, DECAY ANALYSIS (17). For D signals and DA signals, a three-exponential fit was applied. The SE signals were fit with two exponentials. The slowest time constants of this fit represent LRET signals. The two faster components in D and DA signals and the faster component in SE signals are supposed to be the intrinsic signals from the LRET setup and/or oocytes (17).

Extinction Coefficient Measurements in the Recording Solution. A serial dilution of free dye, BODIPY FL-γ-azido-Abu, was prepared at linearly spaced concentra-


Supplemental Information

Mapping of voltage sensor positions in resting and inactivated mammalian sodium channels by LRET

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Supplemental Information

(A) Sequence alignment of all DI-LBT clones tested. Insertion of residues LI occurred during the subcloning process when the LBT was inserted. When DI-2GLBT-R(-5) and DI-3GLBT-R(-5) were generated, the LI insertion was replaced by glycines. (B) Time constant of $D$ signal from eight different DI-LBT clones with the LBT in different positions. These data were collected from at least three different batches of oocytes. Figures inside the columns are the number of cells tested. Error-bars indicate SEM. (C) Time constant of $D$ signals from DI-LBT-R(-5), DI-2GLBT-R(-5) and DI-3GLBT-R(-5). Red bars (overall) indicate the average values from all cells examined. The blue bars (selected) indicate the average values of cells selected from the whole set that showed time constants slower than 2.2 ms. The number of cells evaluated in the overall and selected groups are shown within the corresponding bars. The percentage of cells with time constants slower than 2.2 ms is shown at the bottom (R(-5) = 19/35, 2GR(-5) = 25/32 and 3GR(-5) = 88/101, respectively). Error bars indicate SEM.
Supplemental Fig. S2 related to Fig. 2. Representative Na\textsuperscript{+} ionic current from Nav-LBTs.

Representative Na\textsuperscript{+} ionic currents from wild type (WT) and all Nav-LBTs are shown. Right upper inset shows the pulse protocols used for the measurements. The protocol to the left was used for wild type (WT) and for DIII-LBTs recordings. The protocol to the right was used for DI-LBTs, DII-LBTs and DIV-LBTs. Horizontal and vertical scale bars throughout indicate 10 ms and 0.5\(\mu\)A, respectively. A small reversal potential shift is apparent for some families of traces shown. We attribute this variability to incomplete equilibration of Na concentration in the internal solution in the cut open oocyte within the time window chosen during the experiment. Slowing of fast inactivation was observed in DIV-3GLBT-R(-6) and (-7).

Supplemental Fig. S3 related to Fig.3. Hα-chemical shift analysis of synthetic KIIIA and analogues.

Despite minor differences near the site of modification (residue 3), the overall fold of all analogues is highly similar and in agreement with literature data reported previously.
Supplemental Fig. S4 related to Fig. 3. Hα-chemical shift analysis of synthetic GIIIA and analogues.
Supplemental Fig. S5 related to Fig. 5. Representative LRET signals in all LBT-Navs using three different acceptors.

In the case of KIIIA-Bodipy as the acceptor, black traces indicate the donor (D) signal before applying the acceptor, and orange trace indicates the sensitized emission (SE) signal from an identical cell in the presence of 0.85 μM KIIIA-Bodipy. Similarly, in case of GIIIA-Bodipy as the acceptor, the gray trace represents the D signal and blue signal is SE signal from a separate cell in the presence of 0.85 μM GIIIA-Bodipy. In LRET signal from DIV-3GLBT-R(-7) using Ts1-Bodipy, the black trace indicates the D signal, the red one indicates the donor in the presence of acceptor (DA) signal, the and green one is the SE signal after pretreatment with Ts1-Bodipy.
Supplemental Fig.S6 related to Fig.6 and Fig 7. Diagram illustrating why LRET measurements in this work may be insensitive to movements perpendicular to the membrane

The figure shows a case in which the donor moves perpendicular to the plane of the membrane (gray sheet), such that the distances between donor and acceptor are equal in the resting and slow inactivated states. Blue balls represent the positions of the donor, and the star represents the acceptor. Blue solid lines indicate the distance measured by LRET in the resting state ($L_{\text{rest}}$) and in the slow-inactivated state ($L_{\text{slow}}$). The black dashed line indicates the actual donor movement.

In this extreme case, the LRET measurement will not detect a movement. If the donor moves between positions placed asymmetrically with respect to the acceptor, a distance change will be detected.
Supplemental Experimental Procedures

Chemical synthesis of dye-conjugated peptide toxins

KIIIA, KIIIA-Bodipy and GIIIA-Bodipy:

Site-specific incorporation of Bodipy was achieved by click-conjugation of an azide-containing polypeptide precursor with Bodipy-alkyne (Lumiprobe, Germany). The β-azido alanine (X) building block was incorporated in position 3 of KIIIA or added to the N-terminus of GIIIA-T5A. A preliminary test of blocking activity by μCTX GIIIA-T5A was performed using two-microelectrode voltage clamp to record from rat Nav1.4 channels expressed in oocytes. The half-blocking concentration (IC$_{50}$) was estimated as follows using [GIIIA-T5A] = 10 nM:

\[
\text{IC}_{50} = \frac{(1/fb)-1}{\text{[GIIIA-T5A]}},
\]

where fraction blocked = fb = I$_{tx}$/I$_{o}$, and I$_{tx}$ = peak I$_{Na}$ in the presence of the GIIIA, and I$_{o}$ = peak I$_{Na}$ in the absence of the peptide. For GIIIA-T5A, IC$_{50}$ (nM) = 9.8 ± 3.8 (mean ± s.d., n=5). For comparison, Shon et al (1998) reported a value of 6.3 nM for wildtype GIIIA in similar experiments.

The following peptides were chemically synthesized by Fmoc/tBu solid phase peptide synthesis on an Applied Biosystems automated synthesizer using Rink-amide resin: KIIIA (CCNCSSKWRDHSRCC-NH2), KIIIA_N3X (CCXCSSKWRDHSRCC-NH2) and GIIIA_N5A_NX (XRDCCAOOKKKCKDRQCKQQRCCA-NH2; O = 4-hydroxyproline). Following chain assembly, peptides were cleaved from the solid support and side-chain deprotected using TFA:Triisopropylsilane:water (95:2.5:2.5 (v/v/v)) for 1.5 h at room temperature. The crude and fully reduced peptide was precipitated with diethylether, redissolved in acetonitrile (ACN):water (50:50) containing 0.1% TFA and lyophilized. The desired products were purified by RP-HPLC and lyophilized. ESI-MS for reduced peptides (average isotope composition) KIIIA Mcalc: 1890.2 Da, Mobsd: 1889.5 Da; KIIIA_N3X Mcalc: 1888.1 Da, Mobsd: 1887.4 Da; GIIIA_N5A_NX Mcalc: 2697.1 Da, Mobsd: 2696.7 Da).
Folding and formation of disulfides was carried out by first dissolving the peptide in 10% ACN/water at a concentration of 4 mg/mL and subsequent 1:40 dilution into folding buffer (100 mM Tris-HCl, 1 mM reduced glutathione, 1 mM oxidized glutathione, pH 7.5). The folding mixture reached equilibrium after 2h (HPLC), after which the solution was acidified with TFA to give a pH of 2-3, and the products were purified by preparative HPLC. In the case of KIIIIA analogues, two closely eluting disulfide isomers were isolated, of which the earlier eluting isomer was identified by NMR spectroscopy as the desired analogue (disulfide connectivity I-V, II-IV, III-VI). MALDI-MS (most abundant isotope composition) KIIIIA Mcalc: 1882.7 Da, Mobsd: 1882.6 Da; KIIIIA_N3X Mcalc: 1880.6 Da, Mobsd: 1880.5 Da; GIIIA_N5A_NX Mcalc: 2689.1 Da, Mobsd: 2688.9 Da).

Click conjugation of the purified, azide-labeled conotoxins was performed in 50% ACN/water containing 3.5 mM peptide, 7 mM Bodipy-alkyne, 10 mM aminoguanidine, 20 mM ascorbate, 5 mM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methylamine (TBTA) and 5 mM CuSO4. The reaction reached completion within 2h after which the product was purified by RP-HPLC and isolated as an orange powder following lyophilization. MALDI-MS (most abundant isotope composition) KIIIIA-Bodipy Mcalc: 2209.6 Da, Mobsd: 2209.5 Da; GIIIA-Bodipy Mcalc: 3018.2 Da, Mobsd: 3018.1 Da).

NMR spectroscopy was used to confirm the correct fold of the synthetic analogues. NMR experiments of all analogues were performed on a Bruker Avance 600 spectrometer equipped with a cryoprobe. Peptides were dissolved in H2O/D2O (9:1) at a concentration of 2-3 mg/mL. 1D 1H, 2D TOCSY, NOESY as well as 1H-13C HSQC spectra were recorded at 280 K (KIIIIA) or 300 K (GIIIA) and used for sequential assignment of the spectra. The obtained Hα-chemical shifts provide a sensitive probe of peptide secondary and tertiary structure and were compared to literature values reported previously for wild-type KIIIIA and GIIIA (Supplemental Fig. S3 and S4).

Supplemental Reference: