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Backbone Resonance Assignments of Complexes of Human Voltage-Dependent Sodium Channel Na_v1.2 IQ Motif Peptide Bound to Apo Calmodulin and to the C-Domain Fragment of Apo Calmodulin

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Abstract

Human voltage-gated sodium channel Na_v1.2 has a single pore-forming α -subunit and two transmembrane β -subunits. Expressed primarily in the brain, Na_v1.2 is critical for initiation and propagation of action potentials. Milliseconds after the pore opens, sodium influx is terminated by inactivation processes mediated by regulatory proteins including calmodulin (CaM). Both calcium-free (apo) CaM and calcium-saturated CaM bind tightly to an IQ motif in the C-terminal tail of the α -subunit. Our thermodynamic studies and solution structure (2KXW) of a C-domain fragment of apo ¹³C,¹⁵N-CaM (CaM_C) bound to an unlabeled peptide with the sequence of rat Na_v1.2 IQ motif showed that apo CaM_C (a) was necessary and sufficient for binding, and (b) bound more favorably than calcium-saturated CaM_C. However, we could not monitor the Na_v1.2 residues directly, and no structure of full-length CaM (including the N-domain of CaM (CaM_N)) was determined. To distinguish contributions of CaM_N and CaM_C, we used solution NMR spectroscopy to assign the backbone resonances of a complex containing a ¹³C,¹⁵N-labeled peptide with the sequence of human Na_v1.2 IQ motif (Na_v1.2_{IQP}) bound to apo ¹³C,¹⁵N-CaM or apo ¹³C,¹⁵N-CaM_C. Comparing the assignments of apo CaM in complex with Na_v1.2_{IQP} to those of free apo CaM showed that residues within CaM_C were significantly perturbed, while residues within CaM_N were essentially unchanged. The chemical shifts of residues in Na_v1.2_{IQP} and in the C-domain of CaM were nearly identical regardless of whether CaM_N was covalently linked to CaM_C. This suggests that CaM_N does not influence apo CaM binding to Na_v1.2_{IQP}.

Keywords

Voltage-Gated Sodium Channel; Molecular Recognition; Domain Interactions; Allosteric; EF-hand protein

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Biological Context

The human type 2 voltage-gated sodium channel (Na_v1.2) is a large transmembrane protein that is primarily expressed in the central nervous system. In neurons, Na_v1.2 is found as a heterotrimer composed of a single pore-forming α -subunit interacting with two auxiliary β -subunits that are also transmembrane proteins (Messner and Catterall 1985). In the central nervous system, the function of Na_v1.2 is critical for the initiation and propagation of action potentials. At the molecular level, this depends on conformational responses of the Na_v1.2 α -subunit to changes in membrane potential. These cause the pore to open and allow sodium to flow rapidly from the extracellular space into the cytosol (Yang, George et al. 1996, Yu and Catterall 2003, Yarov-Yarovoy, DeCaen et al. 2012). Within milliseconds of opening, the flow of sodium through the channel is halted by a process known as fast inactivation (Gawali and Todt 2016). Mutations within the channel that alter the rapid inactivation of the α -subunit have been linked to multiple debilitating conditions, highlighting the importance of tight control of sodium entry (Ogiwara, Ito et al. 2009, Shi, Yasumoto et al. 2012, Nakamura, Kato et al. 2013).

Fast inactivation is modulated by numerous protein-protein interactions including intramolecular domain interactions between cytosolic elements of the α -subunit itself, and intermolecular association with the β -subunits, and several other auxiliary proteins (O'Malley and Isom 2015, Wildburger, Ali et al. 2015, Ahern, Payandeh et al. 2016). One regulator is calmodulin (CaM), a small, essential, and ubiquitously expressed calcium-sensing protein that binds 4 calcium ions. CaM is composed of two domains (CaM_N and CaM_C), each of which has a pair of calcium-binding EF-hands.

CaM recognizes two intracellular sites in most Na_v α -subunits in a calcium-dependent manner. (Ca²⁺)₄-CaM, but not apo CaM, binds to the highly conserved inactivation gate (also called the DIII-DIV linker) of Na_v1.5 with a dissociation constant (K_D) of $\sim 0.6 \mu\text{M}$ mediated by CaM_C (Sarhan, Tung et al. 2012). In contrast, the IQ motif [IQxxx[R,K]Gxxx[R,K] found in the C-terminal domain (CTD) of Na_v1.2 binds more tightly to apo CaM than (Ca²⁺)₄-CaM (Mori, Konno et al. 2000, Theoharis, Sorensen et al. 2008, Feldkamp, Yu et al. 2011, Wang, Chung et al. 2014). Our recent thermodynamic studies of CaM binding to the IQ motif of rat Na_v1.2 determined that the K_D for apo CaM binding was $\sim 3 \text{ nM}$, while the K_D for (Ca²⁺)₄-CaM was $\sim 85 \text{ nM}$ (Hovey, Fowler et al. 2017). The physiological importance of apo CaM binding to the IQ motif was highlighted by the finding that this interaction limits persistent current through Na_v1.2, 1.5, and 1.6 (Yan, Wang et al. 2017).

High-resolution structures of apo CaM bound to the IQ motif of Na_v1.5 (2L53, 4OVN, 4DCK) and 1.6 (3WFN) showed that CaM_C made the majority of contacts with the IQ motif. In contrast, CaM_N adopted different positions relative to CaM_C and the channel fragment in crystallographic structures (Wang, Chung et al. 2012, Reddy Chichili, Xiao et al. 2013, Gabelli, Boto et al. 2014), and sampled many positions in the only available solution structure (Chagot and Chazin 2011). Currently there is no high-resolution structure available for apo CaM bound to the Na_v1.2 IQ motif from any species, although there is one (2KXW) for apo CaM_C bound to the rat Na_v1.2 IQ motif (Feldkamp, Yu et al. 2011).

To determine whether and how apo CaM_N interacts with the Na_V1.2 IQ motif in solution, and whether it affects the interface between apo CaM_C and the Na_V1.2 IQ motif, we have used solution NMR spectroscopy to assign the backbone resonances of ¹³C, ¹⁵N- apo CaM and ¹³C, ¹⁵N-apo CaM_C bound to a ¹³C, ¹⁵N-labeled peptide that corresponds to the human Na_V1.2 IQ motif. These complexes are referred to as apo CaM+Na_V1.2IQ_p and apo CaM_C+Na_V1.2IQ_p throughout this report.

A comparison of the assignments of apo CaM+Na_V1.2IQ_p to assignments of free apo CaM indicated that residues in CaM_C underwent large chemical shift perturbations upon binding the peptide, while chemical shifts for residues in CaM_N were almost identical to those observed for the same residues in free CaM. Additionally, the chemical shifts of residues in the C-domain of CaM and Na_V1.2IQ_p were nearly identical in apo CaM+Na_V1.2IQ_p and apo CaM_C+Na_V1.2IQ_p. These data are consistent with the interpretation that only residues in the C-domain of CaM mediate binding to Na_V1.2IQ_p, while apo CaM_N remains free to interact with other regulatory components near the intracellular opening of the Na_V1.2 pore.

Materials and Experiments

Protein Expression and Purification

The complexes CaM+Na_V1.2IQ_p and CaM_C+Na_V1.2IQ_p, were obtained by bacterial co-expression of CaM or CaM_C with a GST-tagged sequence corresponding to the IQ motif of Na_V1.2. Full-length human CaM (residues 1-148) or CaM_C (residues 76-148) were expressed using a pT7-7 vector (Ampicillin^R) (Sorensen and Shea 1998). The peptide Na_V1.2IQ_p was expressed using a modified form of a pBG101 vector (Kanamycin^R) containing an N-terminal GST-tag and a 3C protease cleavage site (Damo, Feldkamp et al. 2013). It was a generous gift from Walter Chazin, Director of the Center for Structural Biology at Vanderbilt University. The plasmid was modified to code for residues 1901–1927 of human Na_V1.2 [KRKQEEVSIIIQRAYRRYLLKQKVKK]. Because the 3C protease site is between the sequence of GST and the IQ motif, Na_V1.2IQ_p contains a 4-residue N-terminal tag having the sequence G-P-G-S; these residues were labeled with negative integers (−4 to −1) in the BMRB deposits. The final sequence of Na_V1.2IQ_p was GPGSKRKQEEVSIIIQRAYRRYLLKQKVKK.

Escherichia coli BL21(DE3) cells co-transformed with plasmids for both CaM (or CaM_C) and GST-Na_V1.2IQ_p were grown in four baffled flasks containing 1 L cultures of LB at 37°C to an OD₆₀₀ of 0.7. Cells were pelleted by centrifugation at 4,000 rpm, 4°C for 20 min, and exchanged into 1 L of minimal media containing 1 g/L ¹⁵NH₄Cl (CIL) and 4 g/L ¹³C₆-glucose (CIL) (Marley, Lu et al. 2001). After a 1 hr recovery period, expression was induced with 1 mL of 0.8 M IPTG. Four hours after addition of IPTG, cells were pelleted by centrifugation at 4,000 rpm, 4°C, for 20 min, and stored at −20°C.

The cell pellet was thawed and resuspended in 30 mL of lysis buffer (50 mM HEPES, 100 mM KCl, 1 mM MgCl₂, 5 mM NTA, 50 μM EGTA, 0.01% NaN₃ (w/v), pH 7.4), and sonicated. Lysate was centrifuged at 20,000 rpm, 4°C for 50 min; supernatant was passed through a 0.22 μm sterile PVDF filter, added to 10 mL of Glutathione Sepharose resin (Amersham Biosciences), and gently rocked at 4°C. Resin was washed with 50 mL of lysis

buffer, 50 mL of a salt wash buffer (lysis buffer with 500 mM KCl), and 50 mL of lysis buffer at 4°C.

To release CaM+Nav1.2_{IQP} or CaM_C+Nav1.2_{IQP}, resin was incubated with rhinovirus 3C protease for 4 h at 22 to 25 °C with gentle rocking. The released complex was collected, concentrated and exchanged into a buffer of 10 mM imidazole, 100 mM KCl, 100 μM EDTA, and 0.01% (w/v) NaN₃, at pH 6.5. Protein purity (> 95%) was assessed by SDS-PAGE, UV/Vis spectroscopy, and rpHPLC. Protein concentrations were determined with the BCA assay (Pierce Biotechnology; Rockford IL).

NMR Experiments

All NMR spectra were collected at 25°C (298 K) using a 4-channel Unity Inova 600 MHz Oxford AS600 NMR spectrometer (Varian) equipped with a triple resonance gradient probe. The NMR samples consisted of 850 μM apo ¹³C,¹⁵N-labeled CaM_C+Nav1.2_{IQP} or 950 μM apo ¹³C,¹⁵N-labeled CaM+Nav1.2_{IQP} in 10 mM imidazole, 100 mM KCl, 100 μM EGTA, 0.01% (w/v) NaN₃, at pH 6.5. All spectra were processed using NMRPipe (Delaglio, Grzesiek et al. 1995) and analyzed using CCPN Analysis (Vranken, Boucher et al. 2005). Backbone resonance assignments for both complexes were made using 2D ¹H-¹⁵N HSQC and 3D HNCOC, HNCACB, and CBCA(CO)NH experiments in 90% H₂O/10% D₂O.

Order parameters were predicted using TALOS+ (Shen, Delaglio et al. 2009). Chemical shift perturbations and differences δ (ppm) were calculated with Eq. 1

$$\Delta\delta = \sqrt{(\Delta\delta_{HN})^2 + (\Delta\delta_N \cdot 0.10134)^2} \quad [1]$$

where δ_{HN} and δ_N are the observed changes in chemical shifts for ¹H_N and ¹⁵N respectively.

Extent of Assignment and Data Deposition

Assigned ¹H-¹⁵N HSQC spectra are shown for apo CaM_C+Nav1.2_{IQP} (Fig. 1A) and apo CaM+Nav1.2_{IQP} (Fig. 1B). Given the largely α-helical secondary structure of both CaM and Nav1.2_{IQP}, the spectra are reasonably well dispersed but the central region is crowded. Panels to the right of each spectrum expand those regions to provide greater clarity in labeling the peaks. For apo CaM_C+Nav1.2_{IQP} (Fig. 1A), 95% of the backbone ¹H_N and ¹⁵N resonances were assigned for CaM_C and 97% for Nav1.2_{IQP}. For apo CaM+Nav1.2_{IQP} (Fig. 1B), 97% of the backbone ¹H_N and ¹⁵N resonances were assigned for both CaM and Nav1.2_{IQP}.

Crosspeaks were not observed for the first two residues of CaM (M76 and K77 in CaM_C, and A1 and D2 in CaM), as well as residues D131 and G134 in calcium-binding site IV in both CaM and CaM_C. Additionally, no crosspeak was observed for I27 in calcium-binding site I in the apo CaM+Nav1.2_{IQP} complex. For Nav1.2_{IQP}, all backbone ¹H_N and ¹⁵N resonances of non-proline residues were assigned in both complexes, except for the first residue, G(-4).

For apo CaM_C+Nav1.2IQ_p, 96% of the ¹³C_α and ¹³C_β resonances, and 93% of the ¹³C' resonances were assigned for CaM_C, and 94% of ¹³C_α, 93% of ¹³C_β, and 90% of ¹³C' resonances were assigned for Nav1.2IQ_p. For the apo CaM+Nav1.2IQ_p complex, 95% of the ¹³C_α, 93% of the ¹³C_β, and 92% of the ¹³C' resonances were assigned for CaM, and 96% of the ¹³C_α and ¹³C_β, and 94% of the ¹³C' resonances were assigned for Nav1.2IQ_p.

TALOS+ was used to predict order parameters for both apo CaM and Nav1.2IQ_p (Fig. 2A and 2B) in apo CaM+Nav1.2IQ_p using ¹H_N, ¹⁵N, ¹³C_α, ¹³C_β, and ¹³C' chemical shifts (Shen, Delaglio et al. 2009). The values predicted for apo CaM agree well with previously determined high-resolution structures of apo CaM such as 1CFD (Kuboniwa, Tjandra et al. 1995). An inset schematic drawing of the typical secondary structure of CaM in Fig. 2A shows that positions predicted to have lower order parameters correspond to the calcium-binding loops (yellow), the loops between the EF-hands within each domain (gray), and the linker connecting CaM_N and CaM_C (gray). Nav1.2IQ_p is predicted to have reduced order parameters only at the N- and C-termini (Fig. 2B). This is consistent with Nav1.2IQ_p forming a single, basic, amphipathic α-helix when interacting with apo CaM, as was observed in high-resolution structures of apo CaM bound to several Nav IQ motifs (Chagot and Chazin 2011, Feldkamp, Yu et al. 2011, Wang, Chung et al. 2012, Reddy Chichili, Xiao et al. 2013, Gabelli, Boto et al. 2014).

To determine the effect of binding Nav1.2IQ_p on apo CaM, we calculated the backbone chemical shift perturbations (CSPs) of CaM residues in apo CaM+Nav1.2IQ_p relative to free apo CaM (1CFD) (Kuboniwa, Tjandra et al. 1995). The histogram shows that the largest CSPs are found in CaM_C, while those in CaM_N are smaller than the standard deviation. The dashed line in Fig. 2C corresponds to the standard deviation (0.229 ppm) of the chemical shift changes (average value of 0.163 ppm; median of 0.055 ppm).

Mapping the CSPs onto 1CFD (Fig 2D) indicates that the largest perturbations were in the turn between helices F and G in CaM_C, consistent with what was found previously in a solution structure of apo CaM_C bound to the Nav1.2 IQ motif (2KXW (Feldkamp, Yu et al. 2011)). The large and numerous CSPs in CaM_C, and the very small CSPs in CaM_N, suggest that the interaction between apo CaM and Nav1.2IQ_p is mediated primarily through CaM_C.

To determine whether the presence of CaM_N alters the interface between CaM and Nav1.2IQ_p, we calculated chemical shift differences between CaM residues in apo CaM +Nav1.2IQ_p and apo CaM_C+Nav1.2IQ_p (Fig. 2E) and between Nav1.2IQ_p residues bound to apo CaM or apo CaM_C (Fig. 2F), plotted on the same scale as the comparison of apo CaM +Nav1.2IQ_p to apo CaM alone (1CFD). The dashed line in Fig. 2E depicts the standard deviation of the chemical shift changes for apo CaM_C (0.044 ppm, average value of 0.025 ppm, median value of 0.015 ppm). The dashed line in Fig. 2F corresponds to the standard deviation for Nav1.2IQ_p (0.033 ppm, average value of 0.029 ppm, median value of 0.018 ppm).

Mapping the chemical shift differences between apo CaM_C+Nav1.2IQ_p (Fig. 1A) and apo CaM+Nav1.2IQ_p (Fig. 1B) onto 1CFD (apo CaM alone; Fig. 2G) showed that the largest changes were near the linker between CaM_N and CaM_C. These residues are at the N-

terminus of the CaM_C construct, but are tethered to CaM_N in full-length CaM (Fig. 2E and 2F). Very minor differences were found in comparing Na_V1.2_{IQp} in the two complexes (Fig G). The small differences indicate that the interface between apo CaM_C and Na_V1.2_{IQp} is unaltered by the presence of CaM_N. With the chemical shifts of CaM_N being independent of peptide binding (Fig. 2C), these data (Fig. 2E, 2F) indicate that the interaction between apo CaM and Na_V1.2_{IQp} is independent of CaM_N, consistent with the solution NMR structure of apo CaM bound to the IQ motif of Na_V1.5.(Chagot and Chazin 2011)

Backbone NMR assignments were deposited with BioMagResBank. Apo CaM+Na_V1.2_{IQp} has accession number 27095, and apo CaM_C+Na_V1.2_{IQp} 27094.

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Figure 1. ^1H , ^{15}N HSQC Spectra for apo CaM_C +Nav1.2IQP (A) and apo CaM +Nav1.2IQP (B) Backbone resonance assignments are labeled in black for residues of CaM or CaM_C , and green for Nav1.2IQP. Numbers correspond to the full-length sequence of each protein. Identifiable residues in the N-terminal tag (GPGS) of Nav1.2IQP are labeled with negative numbers. Peaks contoured in red were aliased. Crowded central regions (denoted by a dashed box) were expanded to the right for clarity of labeling.

Figure 2. Predicted Order Parameters and Analysis of Chemical Shift Perturbations

A, B. Order parameters predicted by TALOS+ (Shen, Delaglio et al. 2009) for apo CaM (**A**) and NaV1.2IQP (**B**) using the assigned chemical shifts of the apo CaM+NaV1.2IQP complex. The colored bars in (**A**) depict a schematic of CaM, with the helices of CaM_N shown in blue, the helices of CaM_C shown in red, the four calcium-binding sites shown in yellow, and the loops and inter-domain linker shown in gray. The helices and calcium-binding sites are labeled A–H and I–IV, respectively. In (**B**), NaV1.2IQP is shown in green.

C. Histogram of the chemical shift perturbations ($\Delta\delta$, ppm, Eq. 1) of apo CaM residues upon binding NaV1.2IQP. The dashed line indicates the standard deviation (0.229 ppm) of $\Delta\delta$ values.

D. Chemical shift perturbations attributable to NaV1.2IQP binding are mapped onto the solution structure of free apo CaM in (1CFD.pdb). Residues in calcium-binding sites are yellow; otherwise, residues 1–75 are blue, linker residues 76–80 are black, and residues

81-148 are red. Unassigned residues are gray. Cartoon thickness corresponds to δ values in panel **2C**. The image was generated using PyMOL (Schrödinger Software).

E. Histogram of the chemical shift differences (δ , ppm, Eq. 1) of CaM_C residues calculated by comparing apo CaM+Na_V1.2_{IQP} to apo CaM_C+Na_V1.2_{IQP}. The dashed line depicts the standard deviation of the chemical shift change (0.044 ppm).

F. Histogram of the chemical shift differences (δ , ppm, Eq. 1) between the peptide residues in apo CaM+Na_V1.2_{IQP} and apo CaM_C+Na_V1.2_{IQP}. The dashed line depicts the standard deviation of the chemical shift change (0.033 ppm). To facilitate comparisons, the same vertical scale was used in panels **2C**, **2E** and **2F**.

G. Chemical shift differences between apo CaM+Na_V1.2_{IQP} and apo CaM_C+Na_V1.2_{IQP} are illustrated on the solution structure of free apo CaM (1CFD.pdb). Residues 1-75 (not present in CaM_C) and unassigned residues in CaM_C are gray. CaM residues 76-80 are black, calcium-binding sites III and IV are yellow, and other residues in CaM_C are red. Cartoon thickness corresponds to δ values in panel **2E**. The image was generated using PyMOL (Schrödinger Software).