



Review

# Calmodulin Interactions with Voltage-Gated Sodium Channels

Xin Wu and Liang Hong \*

Department of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA; xinwu@uic.edu

\* Correspondence: hong2004@uic.edu

**Abstract:** Calmodulin (CaM) is a small protein that acts as a ubiquitous signal transducer and regulates neuronal plasticity, muscle contraction, and immune response. It interacts with ion channels and plays regulatory roles in cellular electrophysiology. CaM modulates the voltage-gated sodium channel gating process, alters sodium current density, and regulates sodium channel protein trafficking and expression. Many mutations in the CaM-binding IQ domain give rise to diseases including epilepsy, autism, and arrhythmias by interfering with CaM interaction with the channel. In the present review, we discuss CaM interactions with the voltage-gated sodium channel and modulators involved in CaM regulation, as well as summarize CaM-binding IQ domain mutations associated with human diseases in the voltage-gated sodium channel family.

**Keywords:** calmodulin; voltage-gated sodium channel; IQ domain; sodium channel isoform; genetic mutation



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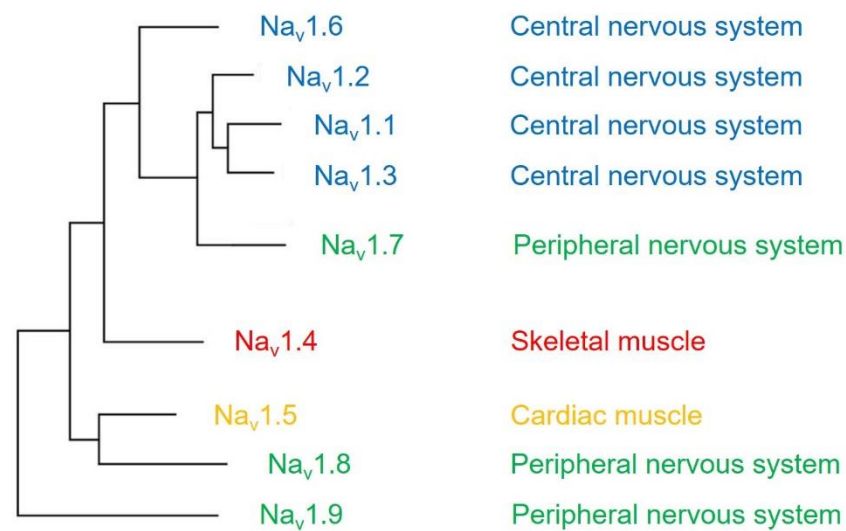
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## 1. Introduction

The voltage-gated sodium channel (Na<sub>v</sub>) plays a vital role in the generation and propagation of action potential in excitable cells such as neurons and cardiac myocytes [1]. The family of voltage-gated sodium channels has nine members named Na<sub>v</sub>1.1 through Na<sub>v</sub>1.9 [2]. Among them, Na<sub>v</sub>1.1, Na<sub>v</sub>1.2, Na<sub>v</sub>1.3, and Na<sub>v</sub>1.6 are predominantly expressed in neurons of the central nervous system [3,4]. Three isoforms (Na<sub>v</sub>1.7, Na<sub>v</sub>1.8, and Na<sub>v</sub>1.9) are widely expressed in neurons of the peripheral nervous system, such as dorsal root ganglia (DRG) neurons [5–7]. The isoform Na<sub>v</sub>1.4 is responsible for upstroke of the action potential in skeletal muscle [8], and Na<sub>v</sub>1.5 is a cardiac-specific isoform known as cardiac sodium channel (Figure 1) [9].

An essential property of voltage-gated sodium channels is “inactivation”, which prevents the reopening of the channel until complete recovery [10]. The inactivation regulates the frequency of action potential firing initiated by sodium channels in excitable cells. It reduces the breakdown of ionic gradients and cell death.

All voltage-gated sodium channels contain a calmodulin (CaM)-binding IQ domain necessary for the channel inactivation [11]. CaM is a small protein expressed in all eukaryotic cells [12]. It acts as a ubiquitous signal transducer and regulates essential processes such as neuronal plasticity, muscle contraction, and immune response [13]. CaM has two globular domains (i.e., N-terminal lobe and C-terminal lobe) connected by a linker. Each lobe contains two EF-hand motifs binding to Ca<sup>2+</sup> [14]. In the Ca<sup>2+</sup>-free state, the EF-hands are collapsed in a compact configuration. When Ca<sup>2+</sup> is bound to CaM, a conformational change occurs in the protein and rearranges the structural information of CaM.



**Figure 1.** A phylogenetic tree and tissue distribution of voltage-gated sodium channels. Isoforms predominantly expressed in the central nervous system (blue), peripheral nervous system (green), skeletal muscle (red), and cardiac muscle (orange) are highlighted.

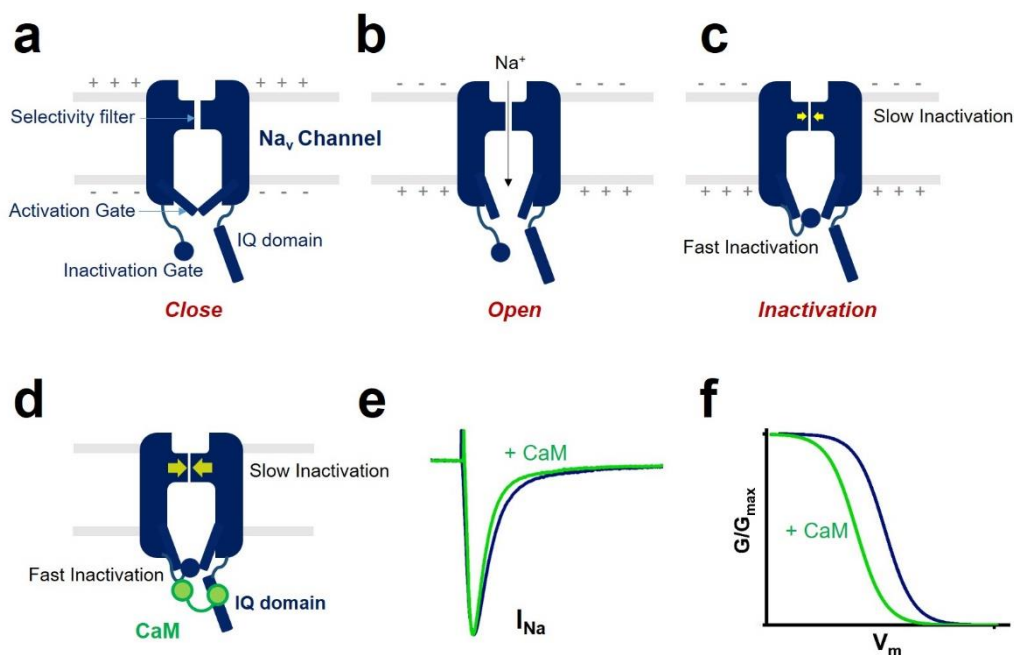
The CaM-binding IQ domain is located within the C-terminal domain of the voltage-gated sodium channel. It contains around 25 residues with two highly conserved amino acids, isoleucine (I) and glutamine (Q), in the middle of the motif. The residues in the IQ domain form a seven-turn  $\alpha$ -helix binding to CaM in a  $\text{Ca}^{2+}$ -independent manner. In addition to the IQ domain, a globular domain in the C-terminus of voltage-gated sodium channel consists of EF hand-like (EFL) motifs that interact with CaM in the absence of  $\text{Ca}^{2+}$  [15,16], whereas an intracellular loop connecting domains III and IV (III-IV linker) in some isoforms of the sodium channel is shown to interact with CaM in the presence of  $\text{Ca}^{2+}$  [17].

## 2. CaM Regulation of Voltage-Gated Sodium Channel

CaM binds to the voltage-gated sodium channel and modulates the sodium channel gating process [15], alters sodium current density [18], and regulates sodium channel protein trafficking and expression [19].

One of the CaM modulations of voltage-gated sodium channel gating is inactivation (Figure 2). Voltage-gated sodium channel has two different types of inactivation, known as fast inactivation and slow inactivation. Fast inactivation occurs by occlusion of the intracellular pore by an “inactivation gate” formed by the cytoplasmic III-IV linker [10,20]. Slow inactivation is related to conformational rearrangements of the selectivity filter of the channel [21,22]. CaM mediates the fast inactivation by an interaction between III-IV linker and C-terminal domain of the voltage-gated sodium channel [23]. It enhances the slow inactivation by inducing a hyperpolarized shift of voltage dependence of inactivation and reducing the channel’s availability. CaM interaction with the sodium channel affects channel inactivation kinetics. It strengthens the development of inactivation and slows the recovery from inactivated state of the sodium channel.

In some isoforms of the voltage-gated sodium channels, CaM influences the activation process. It was reported that co-expression of CaM and Na<sub>v</sub>1.1 produced a hyperpolarized shift of the voltage dependence of activation of the Na<sub>v</sub>1.1 sodium channel [24].



**Figure 2.** CaM regulation of sodium channel function by interaction with the IQ domain. (a–c) Voltage-gated sodium channel ( $\text{Na}_v$ ) has three states known as “close” (a), “open” (b), and “inactivation” (c). Several functional parts (inactivation gate, activation gate, and selectivity filter) are involved in channel gating. The “+” and “-” represent charge separation across the membrane. Differences in charges on opposite sides of the cellular membrane generate the membrane potential. At rest, the activation gate is closed (a). When the membrane is depolarized, the activation gate opens and allows  $\text{Na}^+$  to enter (b). At the same time, depolarization of the membrane causes the inactivation gate to close and induces the fast inactivation. The channel also shows slow inactivation related to conformational rearrangements of the selectivity filter (highlighted in yellow arrows in c). (d–f) CaM binds to the voltage-gated sodium channel IQ domain and modulates the sodium channel inactivation. CaM affects channel’s fast inactivation kinetics (e) by an interaction between the inactivation gate formed by cytoplasmic III–IV linker and the C-terminal domain of the channel (d). CaM enhances the slow inactivation (highlighted in bigger yellow arrows in d). It induces a hyperpolarized shift of voltage dependence of inactivation and reduces the channel’s availability (f).

Moreover, CaM can modulate sodium channel current density [18]. Studies reported that CaM regulation of sodium current density is  $\text{Ca}^{2+}$ -dependent [24,25]. CaM does not affect sodium current amplitude in the absence of  $\text{Ca}^{2+}$ . In contrast, it increases the current density in the presence of a high concentration of  $\text{Ca}^{2+}$ .

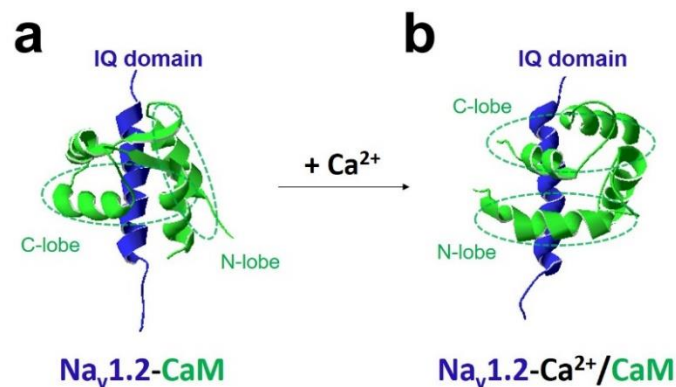
In addition to modulation of the gating process of the voltage-gated sodium channel, CaM influences sodium channel protein trafficking and expression. It was shown to mediate the cell surface expression of  $\text{Na}_v1.4$  protein through the interaction with the IQ domain of the sodium channel [19].

### 3. Modulators Involved in CaM Regulation of Sodium Channel

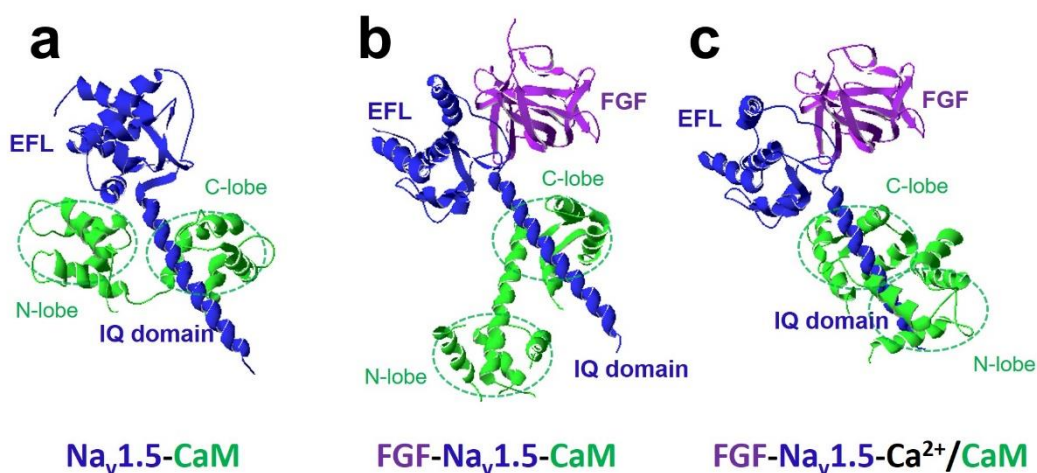
There are several cellular partners involved in CaM regulation of the voltage-gated sodium channel function such as FHF (fibroblast growth factor homologous factor) [26], CaMKII (calcium/calmodulin-dependent protein kinase II) [27], and cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) [11,28,29].

The most crucial regulator is  $\text{Ca}^{2+}$  [11,28]. In the presence of  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}/\text{CaM}$  ( $\text{Ca}^{2+}$ -binding form of CaM) has a different regulation of sodium channel function compared with apo-CaM ( $\text{Ca}^{2+}$  free CaM), and  $\text{Ca}^{2+}$  binding to CaM induces rearrangements between the voltage-gated sodium channel and CaM lobes. In the absence of  $\text{Ca}^{2+}$ , the N-lobe of CaM does not target the IQ domain (Figures 3a and 4a,b). In the presence of  $\text{Ca}^{2+}$ , the structure of the N-lobe is rearranged by  $\text{Ca}^{2+}$ . It generates an allosterically conformational change binding to the distal IQ domain of the channel (Figures 3b and 4c). However, the C-lobe

of CaM can bind to the voltage-gated sodium channel IQ domain in a  $\text{Ca}^{2+}$ -independent manner.



**Figure 3.**  $\text{Ca}^{2+}$  modulates the interaction between CaM and  $\text{Na}_v1.2$  IQ domain. The NMR structures showed  $\text{Na}_v1.2$  IQ domain (blue) in complex with CaM (green) in the absence (a) or presence (b) of  $\text{Ca}^{2+}$ . Two lobes (N-lobe and C-lobe) of CaM are indicated. C-lobe of CaM bound to the  $\text{Na}_v1.2$  channel IQ domain independent of  $\text{Ca}^{2+}$  concentration. In the absence of  $\text{Ca}^{2+}$ , the N-lobe of CaM did not interact with the IQ domain (PDB: 2KXW) (a). When binding to  $\text{Ca}^{2+}$ , the N-lobe of CaM was induced to interact with the distal IQ domain of  $\text{Na}_v1.2$  channel (PDB: 2M5E) (b).



**Figure 4.** FGF and  $\text{Ca}^{2+}$  modulate the interaction between CaM and  $\text{Na}_v1.5$  IQ domain. (a) The structure of the complex of the C-terminal domain of  $\text{Na}_v1.5$  with CaM showed C-lobe of CaM bound to the IQ domain, and N-lobe of CaM interacted with EFL (PDB: 4OVN). (b) Crystal structure showed the interactions between the C-terminal domain of  $\text{Na}_v1.5$ , FGF, and CaM (PDB: 4DCK). When FGF bound to the channel, the N-lobe of CaM was rearranged. It did not make contact with EFL and was free to interact with other parts of the channel complex. The N-lobe of CaM likely interacted with the inactivation gate of the sodium channel and modulated the channel's inactivation. (c) In a high concentration of  $\text{Ca}^{2+}$ , the N-lobe of CaM was induced to interact with the distal IQ domain of  $\text{Na}_v1.5$  channel (PDB: 4JQ0).

The FHF2s were shown to form a complex with CaM and modulate CaM regulation of  $\text{Na}_v1.5$  function [26]. A recent study discovered fibroblast growth factor homologous factor FGF13 (fibroblast growth factor 13, also known as FHF2) tuned arrhythmic late sodium currents in CaM binding-deficient channels in cardiac myocytes [26]. The crystal structures in Figure 4 showed that FGF and  $\text{Ca}^{2+}$  mediated the interaction between CaM and  $\text{Na}_v1.5$  IQ domain. It indicated that the N-lobe of CaM interacted with the EFL of the channel in the absence of FGF. However, when FGF was bound to the channel, the N-lobe of CaM was rearranged. It did not make contact with the EFL and was free to interact with other parts of the channel complex. The N-lobe of CaM likely interacted with the III-IV linker of the sodium channel and modulated the channel's inactivation.

In addition, CaMKII has been reported to influence CaM interaction with the sodium channel. One study found CaMKII-mediated phosphorylation of the Na<sub>v</sub>1.1 sodium channel IQ domain increased CaM binding affinity to the channel [27]. Three phosphorylation sites (T1909, S1918, and T1934) were identified in the Na<sub>v</sub>1.1 IQ domain. Other studies demonstrated that CaMKII phosphorylated Na<sub>v</sub>1.5 at residue S571 to decrease channel availability. The CaMKII phosphorylation site S571 is located at the intracellular DI-DII loop of the Na<sub>v</sub>1.5 sodium channel, and mutation at position S571 abolished the hyperpolarized shift of the voltage dependence of inactivation produced by CaMKII phosphorylation. The effects of CaMKII-mediated phosphorylation of IQ domain on sodium currents have not been characterized. CaMKII-dependent IQ domain phosphorylation might confer normal sodium currents important for the function of cells where the Na<sub>v</sub> channels are expressed. The CaMKII-mediated effect seems to be isoform-specific. CaMKII phosphorylation enhances CaM affinity for sodium channel Na<sub>v</sub>1.1 but not for Na<sub>v</sub>1.2 [27,30].

Moreover, Mg<sup>2+</sup> is involved in the CaM-mediated modulation. In some neurons, low Mg<sup>2+</sup> treatment increases the effects of CaM on the voltage-gated sodium channel activity [29].

With these partners, CaM regulates voltage-gated sodium channel function by altering channel gating, sodium current density, and expression of the sodium channel protein. Studies have shown that CaM has isoform-specific modulation of voltage-gated sodium channel function [31–34].

#### 4. Interaction between CaM and Sodium Channel Isoforms

##### 4.1. Na<sub>v</sub>1.1

Na<sub>v</sub>1.1 is one of the voltage-gated sodium channels predominantly expressed in the central nervous system [35]. It is known to be highly expressed in the soma and apical dendrite of the Purkinje cells.

The effect of CaM on Na<sub>v</sub>1.1 channel function is Ca<sup>2+</sup>-dependent. CaM does not affect sodium current density of wild-type Na<sub>v</sub>1.1 channel in the absence of Ca<sup>2+</sup> [25], but significantly increases Na<sub>v</sub>1.1 current density in the presence of 10 μM intracellular Ca<sup>2+</sup> [24].

CaM mediates Na<sub>v</sub>1.1 channel gating process when binding to the IQ domain, and overexpression of CaM induces a hyperpolarized shift of the voltage dependence of activation of Na<sub>v</sub>1.1 [24]. However, it does not generate significant effects on the voltage dependence of inactivation at high intracellular Ca<sup>2+</sup> concentration but accelerates inactivation process of Na<sub>v</sub>1.1 with low Ca<sup>2+</sup> [24].

A GST pull-down assay reported CaM interaction with Na<sub>v</sub>1.1 sodium channel IQ domain [27]. The Na<sub>v</sub>1.1 IQ domain preferentially binds to apoCaM than Ca<sup>2+</sup>/CaM. In the absence of Ca<sup>2+</sup>, C-lobe of CaM is the predominant domain binding to Na<sub>v</sub>1.1, and N-lobe of CaM is proposed to interact with other parts of the channel [36]. In the presence of Ca<sup>2+</sup>, N-lobe of CaM is shown to be the predominant domain binding to Na<sub>v</sub>1.1 [27]. Moreover, one study reported that CaMKII-mediated phosphorylation of Na<sub>v</sub>1.1 enhanced CaM interaction with IQ domain of the Na<sub>v</sub>1.1 channel [27].

##### 4.2. Na<sub>v</sub>1.2

The neuronal sodium channel Na<sub>v</sub>1.2 is expressed in granule cells and interneurons in the central nervous system [37], and plays roles in excitatory neurons in the neocortex and hippocampus [38].

CaM has been shown to mediate Ca<sup>2+</sup>-dependent regulation of the Na<sub>v</sub>1.2 channel [39]. CaM binding to Na<sub>v</sub>1.2 reduces Ca<sup>2+</sup> binding affinity in the CaM-binding sites [40]. Although there is a Ca<sup>2+</sup>-binding EFL motif upstream of the IQ domain in the Na<sub>v</sub>1.2 channel, the Ca<sup>2+</sup>-mediated changes in modification of Na<sub>v</sub>1.2 function are proposed to be likely from the interaction between CaM and Na<sub>v</sub>1.2 IQ domain when Ca<sup>2+</sup> binding to CaM. The Na<sub>v</sub>1.2 channel EFL motif was indicated to enhance Ca<sup>2+</sup> binding to CaM interacting with the channel [41].



Structural study analysis showed the C-lobe of CaM anchored to the Na<sub>v</sub>1.2 channel IQ domain independent of Ca<sup>2+</sup> concentration. In the absence of Ca<sup>2+</sup>, the N-lobe of CaM did not interact with the IQ domain. When binding to Ca<sup>2+</sup>, the N-lobe of CaM was induced to interact with the distal IQ domain of the Na<sub>v</sub>1.2 channel (Figure 3) [42].

CaM has no notable effects on the Na<sub>v</sub>1.2 current density. Overexpression of CaM does not significantly decrease the peak sodium current amplitude of the Na<sub>v</sub>1.2 channel [43].

#### 4.3. Na<sub>v</sub>1.3

Although Na<sub>v</sub>1.3 is a neuronal sodium channel [44], the first paper reporting CaM regulation of Na<sub>v</sub>1.3 is from a study on microvessels of the kidney [45].

The study examined the expression of Na<sub>v</sub>1.3 in rat descending vasa recta, a series of blood vessels that perfuse the renal medulla. Further, it verified that CaM binding to the C-terminus of Na<sub>v</sub>1.3 by pull-down and immunoprecipitation assays [45]. The voltage-gated sodium currents in the vasa recta pericytes were remarkably suppressed by CaM inhibitors CIP (calmodulin inhibitory peptide) and W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride). However, CIP or W7 did not generate alteration of gating process of endogenous voltage-gated sodium currents in vasa recta cells [45].

Another study in hippocampal neurons showed that CaM regulates Na<sub>v</sub> channel function in an Mg<sup>2+</sup>-dependent manner. The Na<sub>v</sub>1.3 activities, together with Na<sub>v</sub>1.1 and Na<sub>v</sub>1.2, were more sensitive to Ca<sup>2+</sup>/CaM regulation in a low-Mg<sup>2+</sup> environment than normal neurons [29].

#### 4.4. Na<sub>v</sub>1.4

Na<sub>v</sub>1.4 is the predominant subtype of sodium channel initiating skeletal muscle action potential [8]. Na<sub>v</sub>1.4 mutations associated with periodic paralysis disorders affect skeletal muscle excitability [46].

CaM influences the gating process of the Na<sub>v</sub>1.4 sodium channel, and the effects of CaM on Na<sub>v</sub>1.4 inactivation are related to Ca<sup>2+</sup> [31]. In the presence of Ca<sup>2+</sup>, CaM shifts the voltage dependence of inactivation of the channel to hyperpolarizing direction. When the free Ca<sup>2+</sup> is removed, the CaM-induced shift of the steady-state inactivation curve is attenuated [31].

CaM regulates Na<sub>v</sub>1.4 sodium current density. The decreased sodium currents caused by Na<sub>v</sub>1.4 IQ mutations were shown to be rescued by overexpression of CaM. However, CaM does not significantly affect the sodium current density of the wild-type Na<sub>v</sub>1.4 channel [32].

In addition to functional regulation, CaM influences Na<sub>v</sub>1.4 channel trafficking and expression. One study demonstrated an intimate relationship between intact Na<sub>v</sub>1.4 channels and CaM in live cells and revealed that CaM participated in the regulation of cell surface expression of Na<sub>v</sub>1.4 protein through the interaction with the Na<sub>v</sub>1.4 channel IQ domain [19].

#### 4.5. Na<sub>v</sub>1.5

The most well-studied voltage-gated sodium channel interacting with CaM is the Na<sub>v</sub>1.5. The cardiac sodium channel Na<sub>v</sub>1.5, encoded by the *SCN5A* gene, plays a critical role in the fast depolarization of the cardiac action potential [47]. Cardiac sodium channel dysfunction caused by Na<sub>v</sub>1.5 mutations was reported to remodel abnormal action potential underlying arrhythmias [48].

CaM binds to Na<sub>v</sub>1.5 C-terminal domain and enhances the inactivation of Na<sub>v</sub>1.5 channel. It shifts the steady-state inactivation to hyperpolarization and influences inactivation kinetic [49]. CaM mediates Ca<sup>2+</sup> regulation of Na<sub>v</sub>1.5 sodium channel function [50]. In the presence of Ca<sup>2+</sup>, CaM is promoted to bind to the Na<sub>v</sub>1.5 inactivation gate. Perturbation of the interaction between the gate and CaM generates a decreased recovery from inactivation of the Na<sub>v</sub>1.5 channel [51,52].

The sodium channel  $Na_v1.5$  IQ domain mutations associated with arrhythmia reduce CaM binding affinity. In HEK293 cells, overexpression of CaM attenuates late sodium currents caused by  $Na_v1.5$  IQ domain mutations [43]. CaM binding to  $Na_v1.5$  channel IQ domain also modifies late sodium currents in cardiac myocytes [26]. Study in transgenic mouse models showed FHF-tuned cardiac late sodium currents in ventricular myocytes. FHF-tuned channels diminished late sodium current of the  $Na_v1.5$  channel IQ/AA mutation (substitution of two conserved residues isoleucine (I) and glutamine (Q) with double alanines (AA) in the IQ domain), IQ/AA mutation has been reported to disrupt CaM binding to the IQ domain of voltage-gated sodium channels.

Studies in guinea-pig ventricular myocytes revealed CaM enhances the cardiac sodium current density [53]; this is different from the result that CaM does not influence on sodium current amplitude when co-expressing with  $Na_v1.5$  channel in HEK293 cells [43]. It indicates that some environmental factors in cardiac tissues are involved in CaM regulation of  $Na_v$  function.

Additionally, a recent study proposed CaM can bind to the N-terminal domain of the cardiac  $Na_v1.5$  channel, wherein one Brugada syndrome-associated mutation in the N-terminal domain of  $Na_v1.5$  was shown to weaken the interaction between CaM and  $Na_v1.5$  N-terminal domain [54].

#### 4.6. $Na_v1.6$

$Na_v1.6$  is the primary voltage-gated sodium channel at the myelin-sheath gaps. It is important in the generation and propagation of the action potential along myelinated axons [55].

CaM binding to  $Na_v1.6$  is crucial for functional sodium current expression [32]. Disruption of CaM binding to the  $Na_v1.6$  channel significantly reduces sodium current amplitude. Overexpression of CaM rescues the decreased sodium currents caused by  $Na_v1.6$  channel CaM-binding domain mutations. CaM enhances the rate of  $Na_v1.6$  inactivation and switches  $Na_v1.6$  channel from fast mode to slow mode. It has no effects on the voltage dependence of activation of  $Na_v1.6$  [32].

The structural study reported that CaM interacted with different residues of the  $Na_v1.6$  channel, depending on the absence or presence of  $Ca^{2+}$ . However, three key residues (Arg1902, Tyr1904, and Arg1905) in the  $Na_v1.6$  channel IQ domain were identified to interact with CaM in a  $Ca^{2+}$ -independent manner [56].

#### 4.7. $Na_v1.7$

The voltage-gated sodium channel  $Na_v1.7$  is highly expressed in nociceptive dorsal root ganglion (DRG) neurons and superior cervical ganglion (SCG) neurons [57].

The electrophysiological study on CaM interacting with  $Na_v1.7$  has not yet been determined. One nuclear magnetic resonance (NMR) study analyzed complexes of calcium-free CaM bound to peptides of IQ motifs of  $Na_v1.7$ . It showed C-lobe of CaM contributed to the interface with the IQ motif of  $Na_v1.7$  and the N-lobe of CaM interacted with other parts of the sodium channel [36]. These results are consistent with findings in other voltage-gated sodium channels and suggest that an interaction interface between CaM and IQ domain of voltage-gated sodium channels is highly conserved in the C-lobe of CaM.

#### 4.8. $Na_v1.8$

$Na_v1.8$  channel is expressed in dorsal root ganglia (DRG) neurons [6], and genetic research has shown this channel is also implicated in cardiac function [58], in which  $Na_v1.8$  was identified in cardiomyocytes and intracardiac neurons, contributing to cardiac repolarization [59].

CaM is a functional partner of  $Na_v1.8$  sodium and interacts with  $Na_v1.8$  in vivo [18]. It was found that CaM can coimmunoprecipitate with endogenous  $Na_v1.8$  channels from native DRG. CaM mediated a frequency-dependent inhibition of sodium channels in the DRG neurons. The sodium currents in neurons were significantly decreased in the presence

of high cellular  $\text{Ca}^{2+}$  (10  $\mu\text{M}$  free  $\text{Ca}^{2+}$ ) and CaM antagonist calmodulin-binding peptide (CBP), suggesting CaM modulated  $\text{Na}_v1.8$  current density in neurons [18].

A recent study demonstrated the CaM enhanced slow inactivation of the  $\text{Na}_v1.8$  channel and reduced channel availability. It modulated  $\text{Na}_v1.8$  function through its interaction with the IQ domain. CaM mediated regulation was abrogated in mutations disrupting CaM binding to the channel [60].

#### 4.9. $\text{Na}_v1.9$

$\text{Na}_v1.9$  is expressed in sensory neurons of the DRG and trigeminal ganglion [61].

To date, there is no study investigating the interaction between CaM and  $\text{Na}_v1.9$  channel. The  $\text{Na}_v1.9$  IQ domain shares high sequence homology with other sodium channels (Figure 5). It remains to be determined if CaM regulation of  $\text{Na}_v1.9$  channel plays a role in channel function in sensory neurons.

| Channel          | Gene          |      | **          |                        |
|------------------|---------------|------|-------------|------------------------|
| $\text{Na}_v1.1$ | <i>SCN1A</i>  | 1912 | RKQEEVSAVI  | IQRAYRRHLLKRTVKQA 1938 |
| $\text{Na}_v1.2$ | <i>SCN2A</i>  | 1902 | RKQEEVSAII  | IQRAYRRYLLKQKVKV 1928  |
| $\text{Na}_v1.3$ | <i>SCN3A</i>  | 1897 | RKQEEVSAII  | IQRNFRCYLLKQRLKNI 1923 |
| $\text{Na}_v1.4$ | <i>SCN4A</i>  | 1724 | RKHEEVCAIKI | IQRAYRRHLLQRSMKQA 1750 |
| $\text{Na}_v1.5$ | <i>SCN5A</i>  | 1898 | RKHEEVSAMVI | QRAFRHLLQRSLKHA 1924   |
| $\text{Na}_v1.6$ | <i>SCN8A</i>  | 1892 | RKQEEVSAVVL | QRAYRGHLARRGFICK 1918  |
| $\text{Na}_v1.7$ | <i>SCN9A</i>  | 1886 | RKQEDVSATVI | IQRAYRRYRLRQNVKNI 1912 |
| $\text{Na}_v1.8$ | <i>SCN10A</i> | 1848 | WKQEDISATVI | QKAYRSYVLHRSMLS 1874   |
| $\text{Na}_v1.9$ | <i>SCN11A</i> | 1730 | RKEEERGAII  | IQKAFRKYMMKVTKGDQ 1756 |

**Figure 5.** Alignment of voltage-gated sodium channel family IQ domain and CaM-binding IQ domain mutations. Sodium channel mutations in the IQ domain associated with human diseases are highlighted in yellow. The conserved residues Ile (I) and Glu (Q) are indicated by stars.

### 5. CaM-Binding IQ Domain Mutations in Voltage-Gated Sodium Channel

A large number of voltage-gated sodium channel mutations have been linked with disorders of the nervous and cardiovascular systems [62]. Some mutations are located in the highly conserved CaM-binding IQ domain and cause severe neurological diseases and cardiac arrhythmia (Figure 5, Table 1).

**Table 1.** CaM-binding IQ domain mutations in  $\text{Na}_v$  channels.

| Pathological Mutation in CaM-Binding IQ Domain | Change in Nucleotide | Phenotype   | Effect On $\text{Na}_v$ Function  |
|--|----------------------|---|---|
| $\text{Na}_v1.1$ -I1922T                       | c.5765T>C            | Dravet syndrome   | unknown   |
| $\text{Na}_v1.1$ -R1928G                       | c.5782C>G            | Familial epilepsy syndrome<br>Familial hemiplegic migraine<br>Cryptogenic epileptic syndrome<br>Dravet syndrome | unknown   |
| $\text{Na}_v1.2$ -R1902C                       | c.5704C>T            | Familial autism   | (1) Abolished $\text{Ca}^{2+}$ -dependence of CaM binding; (2) Induced hyperpolarization shift of the voltage dependence of activation and inactivation of the channel. |
| $\text{Na}_v1.2$ -R1918H                       | c.5753G>A            | Idiopathic generalized epilepsy   | (1) Induced an increased late sodium current; (2) Overexpression of CaM reduced the late sodium current.  |
| $\text{Na}_v1.5$ -R1898C                       | c.5692C>T            | Brugada syndrome  | unknown   |



Table 1. Cont.

| Pathological Mutation in CaM-Binding IQ Domain | Change in Nucleotide | Phenotype   | Effect On Na <sub>v</sub> Function   |
|--|----------------------|---|--|
| Na <sub>v</sub> 1.5-R1898H                     | c.5693G>A            | Arrhythmogenic right ventricular dysplasia/cardiomyopathy | (1) Caused a reduction in peak sodium current; (2) Caused a structural deficit and decreased abundance of Na <sub>v</sub> 1.5 and N-Cadherin clusters. |
| Na <sub>v</sub> 1.5-E1901K                     | c.5701G>A            | Brugada syndrome  | unknown  |
| Na <sub>v</sub> 1.5-E1901Q                     | c.5701G>C            | Long QT syndrome  | (1) Induced an increased late sodium current; (2) Overexpression of CaM reduced the late sodium current.   |
| Na <sub>v</sub> 1.5-S1904L                     | c.5711C>T            | Long QT syndrome<br>Brugada syndrome                      | (1) Reduced binding affinity between channel and CaM; (2) Induced an increased late sodium current.  |
| Na <sub>v</sub> 1.5-Q1909R                     | c.5726A>G            | Long QT syndrome<br>Sudden infant death syndrome          | (1) Induced an increased late sodium current; (2) Overexpression of CaM reduced the late sodium current.   |
| Na <sub>v</sub> 1.5-R1913H                     | c.5738G>A            | Long QT syndrome  | (1) Induced an increased late sodium current; (2) Overexpression of CaM reduced the late sodium current.   |
| Na <sub>v</sub> 1.5-R1919C                     | c.5755C>T            | Long QT syndrome<br>Brugada syndrome                      | unknown  |
| Na <sub>v</sub> 1.5-A1924T                     | c.5770G>A            | Brugada syndrome  | (1) Induced a hyperpolarization shift in voltage-state activation; (2) Generated a reduced slow inactivation that was rescued by CaM.                  |
| Na <sub>v</sub> 1.8-R1863Q                     | c.5588 G>A           | Brugada syndrome  | (1) Reduced CaM-induced hyperpolarization shift of the voltage dependence of inactivation of the channel.  |
| Na <sub>v</sub> 1.8-R1869C                     | c.5605 C>T           | Brugada syndrome  | (1) Disrupted CaM-induced hyperpolarization shift; (2) Attenuated effects of CaM on development and recovery from slow inactivation.                   |
| Na <sub>v</sub> 1.8-R1869G                     | c.5605 C>G           | Atrial fibrillation                                       | (1) Disrupted CaM-induced hyperpolarization shift; (2) Attenuated effects of CaM on development and recovery from slow inactivation.                   |

### 5.1. Na<sub>v</sub>1.1 Mutations

Na<sub>v</sub>1.1 mutations are associated with familial epilepsies. The Na<sub>v</sub>1.1-I1922T was identified in patients with Dravet syndrome [63,64]. In this mutation, highly conserved residue isoleucine (I) that plays a critical role in CaM binding to the IQ domain was substituted by threonine (T). Another mutation, Na<sub>v</sub>1.1-R1928G, is linked with cryptogenic epileptic syndrome [65], and characterized in patients diagnosed with generalized epilepsy with febrile seizures plus (GEFS+) [66], familial hemiplegic migraine (FHM) [67], and severe myoclonic epilepsy of infancy (SMEI) [68].

Additionally, CaM was reported to generate partially functional rescue of Na<sub>v</sub>1.1-M1841T, an epileptogenic mutation located upstream of the CaM-binding IQ domain of the Na<sub>v</sub>1.1 channel [25]. M1841T was characterized as a loss-of-function mutant and generated very small sodium currents. Co-expression of CaM with M1841T partially rescued the loss of function of the channel and induced a 3.5-fold increase in sodium current amplitude [25].

### 5.2. Na<sub>v</sub>1.2 Mutations

The Na<sub>v</sub>1.2 mutation R1902C has been identified in patients with familial autism [69]. Na<sub>v</sub>1.2-R1902C perturbed Ca<sup>2+</sup>-dependent changes in the regulation of Na<sub>v</sub>1.2 function [39]. Protein pull-down assays showed R1902C abolished Ca<sup>2+</sup>-dependence of CaM binding [30]. In the presence of Ca<sup>2+</sup>, Na<sub>v</sub>1.2-R1902C induced a hyperpolarization shift of the voltage dependence of activation and inactivation of the channel [42].

The mutation Na<sub>v</sub>1.2-R1918H was associated with idiopathic generalized epilepsy [70]. This mutation produced a pathogenically increased late sodium current, indicating a gain-of-function mechanism in Na<sub>v</sub>1.2 mutations associated with epilepsy. The enhanced late sodium current mediated by Na<sub>v</sub>1.2-R1918H was able to be rescued by CaM overexpression [43].

### 5.3. Na<sub>v</sub>1.5 Mutations

The Na<sub>v</sub>1.5-R1898H is a mutation located at the N-terminal end of the CaM-binding IQ domain of the Na<sub>v</sub>1.5 channel and was characterized in a patient with arrhythmogenic right ventricular dysplasia/cardiomyopathy (AVRD/C) [71]. A study assessed this mutation's cellular and molecular phenotype using an induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) approach. It showed that R1898H was a loss-of-function mutation that caused around 40% reduction in peak sodium current [71]. Three-dimensional super-resolution fluorescence microscopy (3D-SRFM) experiments showed a structural deficit of Na<sub>v</sub>1.5 and N-cadherin clusters in the R1898H mutation cells, indicating a role that Na<sub>v</sub>1.5 dysfunction associated with R1898H mediated cardiomyopathy [71]. Another mutation at the same position, R1898 (Na<sub>v</sub>1.5-R1898C), was identified in a patient with Brugada syndrome [72]. It should be noted that the Na<sub>v</sub>1.5-R1898C corresponds to the autism mutation Na<sub>v</sub>1.2-R1902C (Figure 5). In Na<sub>v</sub>1.2, the mutation (Na<sub>v</sub>1.2-R1902C) affected the gating process of the channel and perturbed Ca<sup>2+</sup>-dependent regulation of Na<sub>v</sub>1.2 function [42]. It is likely that the same mutation in the Na<sub>v</sub>1.5 (Na<sub>v</sub>1.5-R1898C) generated similar consequences on the Na<sub>v</sub>1.5 function and played similar roles in cardiac diseases.

There are two mutations at the position Na<sub>v</sub>1.5-E1901 [73,74]. The Na<sub>v</sub>1.5-E1901K was associated with Brugada syndrome [74], and Na<sub>v</sub>1.5-E1901Q was identified in a patient with type 3 of the long-QT syndrome (LQT3). Na<sub>v</sub>1.5-E1901Q was shown to cause an increased late sodium current [73]. In the absence of Ca<sup>2+</sup>, the negative residue Glu at the position 1901 (E1901) might couple with a positive residue of CaM to form charge–charge interaction, stabilizing CaM binding to the IQ domain of the channel [75]. Replacement of Glu with Gln (E1901Q) or Lys (E1901K) likely disrupted the salt bridge formed by the charge–charge interaction between CaM and Na<sub>v</sub>1.5 IQ domain and produced a perturbation of CaM regulation of the channel, providing an explanation for the pathological roles of those mutations in Brugada syndrome and LQT3 syndrome.

The Na<sub>v</sub>1.5 mutation S1904L was associated with Brugada and long-QT syndromes [74,76]. The position of Ser1904 was proposed to use hydrogen bonds to interact with the main chain of the C-lobe of the CaM. Mutation at the position of 1904 (S1904L) perturbed the Na<sub>v</sub>1.5 channel interaction with CaM. In the absence of Ca<sup>2+</sup>, a flow cytometric FRET two-hybrid analysis between the S1904L and CaM demonstrated a weaker affinity compared to the wild-type Na<sub>v</sub>1.5 channel [15]. Additionally, S1904L induced an enhanced late sodium current, indicating a delayed inactivation of the channel caused by CaM dysregulation of the channel in this mutation [76].

Na<sub>v</sub>1.5-Q1909R was linked with sudden infant death syndrome (SIDS) and LQT3 syndrome [77,78]. Functional studies showed that Q1909R is a gain-of-function mutation [79]. Ventricular action potential (AP) simulations showed a frequency-dependent reduction of AP duration in Q1909R carriers [79]. Moreover, Q1909R resulted in an increase in late sodium current. Co-expression of CaM or higher intracellular Ca<sup>2+</sup> reduced the enhanced late sodium currents [43,79].

The mutation Na<sub>v</sub>1.5-R1913H was identified in a patient with LQT3 syndrome [80]. In the absence of Ca<sup>2+</sup>, the position Arg1913 interacts with negative residues of C-lobe of CaM. Mutation at this position (R1913H) resulted in an enhanced late sodium current, and overexpression of CaM can reduce the enhanced late sodium current [43]. Another mutation (R1919C) was reported to be associated with Brugada syndrome and Long-QT syndrome [81].

A Brugada syndrome mutation A1924T is located at the C-terminal end of the IQ domain. Na<sub>v</sub>1.5-A1924T induced a negative shift in voltage-state activation, which would cause some persistent depolarization of the channel [82]. Na<sub>v</sub>1.5-A1924T showed a reduced slow inactivation, and application of CaM rescued the decreased slow inactivation caused by Na<sub>v</sub>1.5-A1924T [49].

In addition to IQ domain mutations in the cardiac Na<sub>v</sub>1.5 channel, there are several mutations in other domains of Na<sub>v</sub>1.5 involved in the regulation of CaM interaction with the sodium channel. In the N-terminal domain of the Na<sub>v</sub>1.5 channel, one Brugada syndrome mutation R121W was reported to weaken the interaction between CaM and the channel [54]. In the III-IV linker of the Na<sub>v</sub>1.5 channel, Brugada syndrome mutation K1493del (a Lys was deleted at the position 1493) decreased Ca<sup>2+</sup>-dependent CaM-interaction with Na<sub>v</sub>1.5 channel [83]. Brugada syndrome mutations Y1494N and I1521K were shown to cause domain-specific perturbations of the interaction with CaM [51]. In the EF hand-like (EFL) motifs upstream of the IQ domain of Na<sub>v</sub>1.5 channel, mutations (L1825P and Y1795insD) associated with LQT and Brugada syndromes perturbed CaM regulation of the channel function [84]. The persistent sodium currents produced by another Brugada syndrome mutation E1784K in the EFL motif were modulated by CaM overexpression [23]. Moreover, the mutation F1759A near the EFL motifs tuned the late sodium currents in CaM binding-deficient channels in cardiac myocytes [26].

#### 5.4. Na<sub>v</sub>1.8 Mutations

Na<sub>v</sub>1.8 channel IQ domain mutations were reported to be linked with cardiac arrhythmias. One clinical research reported Na<sub>v</sub>1.8-R1863Q mutation in a patient with Brugada syndrome (BrS) [85]. The Na<sub>v</sub>1.8 mutation R1869C was identified in an index case with atrial fibrillation (AF) and BrS [86]. In addition, mutation Na<sub>v</sub>1.8-R1869G was identified to co-segregate with familial AF [87].

All IQ domain mutations influence CaM interaction with the Na<sub>v</sub>1.8 channel. Na<sub>v</sub>1.8-R1863Q reduced the CaM-induced hyperpolarization shift of the voltage dependence of inactivation of the channel. Na<sub>v</sub>1.8-R1869C and Na<sub>v</sub>1.8-R1869G disrupted CaM-induced hyperpolarization shift and attenuated effects of CaM on development and recovery from slow inactivation [60]. These results suggested that Na<sub>v</sub>1.8 IQ domain mutations weakened the interaction between CaM and Na<sub>v</sub>1.8 channel and perturbed CaM regulation of Na<sub>v</sub>1.8 function.

## 6. Conclusions and Perspectives

We have described the interaction between CaM and each isoform of voltage-gated sodium channel family and summarized mutations associated with human diseases in CaM regulation of voltage-gated sodium channels.

The effects of CaM on the sodium channels are complex. They are not only involved in various regulation ways but also related to multiple factors in the cells. CaM regulates Na<sub>v</sub> channel gating by binding to the IQ motif of the channel. Intracellular Ca<sup>2+</sup> is also reported to modulate cardiac Na<sub>v</sub>1.5 channel inactivation by binding to an EF hand-like

(EFL) motif of the sodium channel. The multiple binding sites of  $\text{Ca}^{2+}$  (either in the EFL of  $\text{Na}_v$  channel or in the EF-hand of CaM) and ability of CaM binding to the IQ domain of the  $\text{Na}_v$  channel result in various regulations of  $\text{Ca}^{2+}$  and/or CaM on the  $\text{Na}_v$  channel function. Therefore, delineating  $\text{Na}_v$  modulation by CaM will require developing novel approaches that exclude or minimize other factors' modulation on CaM regulation of sodium channel function. In addition, studies have shown that CaM has isoform-specific modulation of voltage-gated sodium channels [31–34]. Because sodium channel isoforms have specific tissue distribution (Figure 1), whether environmental factors in different tissues are involved in CaM regulation of  $\text{Na}_v$  function remains unclear. Studies in the field will help explore the regulatory mechanism underlying distinct properties of CaM modulation of voltage-gated sodium channel function. Moreover, although the canonical binding site of CaM is the C-terminal domain of the sodium channel, a recent study showed another interaction site between CaM and the channel. A putative CaM-binding sequence comprising 26 amino acids is located at the N-terminal domain of the sodium channel [54]. Future studies on the complexes of a  $\text{Na}_v$  N-terminal domain and CaM will extend and provide a complete understanding of the contribution of CaM on the  $\text{Na}_v$  function.

In summary, CaM plays a critical regulatory role in cellular electrophysiology by its ability to bind to voltage-gated sodium channels. Understanding how it regulates sodium channel function is a crucial step towards developing treatments for diseases associated with sodium channel mutations.

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## Abbreviations

|                       |  |
|-----------------------|--|
| $\text{Na}_v$         | Voltage-gated sodium channel   |
| CaM                   | Calmodulin   |
| Apo-CaM               | $\text{Ca}^{2+}$ -free CaM   |
| $\text{Ca}^{2+}$ /CaM | $\text{Ca}^{2+}$ -binding CaM  |
| IQ domain             | A domain with highly conserved residues isoleucine (I) and glutamine (Q) |
| CBP                   | Calmodulin-binding peptide   |
| CIP                   | Calmodulin inhibitory peptide  |
| EFL                   | EF hand-like motifs  |
| FHF                   | Fibroblast growth factor homologous factor                               |
| FGF                   | Fibroblast growth factor   |
| CaMKII                | Calcium/calmodulin-dependent protein kinase II                           |
| AP                    | Action potential   |
| DRG                   | Dorsal root ganglia  |
| SCG                   | Superior cervical ganglion   |
| NMR                   | Nuclear magnetic resonance   |
| GEFS+                 | Generalized epilepsy with febrile seizures plus                          |
| FHM                   | Familial hemiplegic migraine   |
| SMEI                  | Severe myoclonic epilepsy of infancy                                     |
| SIDS                  | Sudden infant death syndrome   |
| LQT3                  | Type 3 of long-QT syndrome   |

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