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Na^+ Channel I–II Loop Mediates Parallel Genetic and Phosphorylation- Dependent Gating Changes

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In this issue of *Circulation*, Koval *et al.*[1] show that two arrhythmogenic human cardiac Na^+ channel ($hNa_V1.5$) variants mimic the altered channel gating effects induced by Ca^{2+}-calmodulin dependent protein kinase (CaMKII). They also show that phosphorylation of an adjacent CaMKII target site on $Na_V1.5$ is enhanced in human heart failure (HF) samples and in the border zone of post-infarcted canine hearts.

The cardiac Na^+ channel, $Na_V1.5$, is responsible for inward Na^+ current (I_{Na}) that drives the cardiac action potential (AP) upstroke and electrical impulse propagation.[2] Genetic variants of the SCN5A gene encoding $Na_V1.5$ are associated with long QT syndrome-3 (LQTs; gain of function), Brugada syndrome (BRs; loss of function), conduction system disease, SIDS, sick sinus syndrome, and dilated cardiomyopathy.[3,4] These inherited channelopathies have been tremendously important to our understanding of normal $Na_V1.5$ function and arrhythmia mechanisms. However, "acquired" forms of altered $Na_V1.5$ function due to post-translational modification (e.g. phosphorylation or oxidation) may have pathophysiological consequences during ischemia/ reperfusion or HF, and thus reach a larger patient population. Indeed, half of all HF deaths are sudden and presumed to be due to lethal ventricular arrhythmias.[5,6]

The pore forming α subunit (~220Kd predicted MW; $Na_V1.5$) has four homologous domains (I–IV) with six transmembrane segments each (S1–S6; Figure 1), is glycosylated and has auxiliary regulatory β subunits (β1–β4, ~30–35Kd).[7] The S5–S6 linker includes the P-loops or pore region, the four S4 segments serve as voltage sensors (involved in activation gating), while an IFM motif in the DIII–IV linker is important for fast inactivation gating. Importantly, $Na_V1.5$ forms a macromolecular complex with interacting proteins that can regulate channel gating and localization, and mutations in many of these proteins can be pro-arrhythmic (reviewed in [3,7,8]).

CaMKII Regulation of Cardiac Na^+ Channels

CaMKII was shown to associate with and phosphorylate $Na_V1.5$, causing characteristic I_{Na} gating changes in mouse and rabbit ventricular myocytes.[9] Specifically, CaMKII shifted I_{Na} availability to more negative potentials, enhanced entry into intermediate inactivation and slowed recovery from inactivation, all of which are loss-of function effects (analogous to

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BRs). CaMKII also increased late I_{Na} (I_{NaL}), an acquired LQTs gain-of-function effect. These potentially arrhythmogenic I_{Na} effects were acutely abolished by CaMKII inhibitors KN93 or AIP in rabbit myocytes. CaMKII expression and activity are both increased in HF.[10,11] and CaMKIIδ overexpressing mice exhibit enhanced arrhythmogenesis.[9] Notably, the full set of CaMKII- induced changes in I_{Na} gating almost exactly phenocopies a human point mutation (Ins1795D in the C-terminus) that is linked with combined LQTs and BRs in the same patients.[12] In these contexts, the seminal Wagner *et al.*[9] study fueled the search for critical CaMKII target sites on $Na_V1.5$ that could explain these gating effects and identify potential therapeutic targets for arrhythmias in cardiac disease.

Based on the above, one might look for a CaMKII target site in the C-terminal tail (near residue 1795), but Aiba *et al.*[13] provided evidence that the I–II loop might be a major CaMKII phosphorylation target. Utilizing a computer based scan for the traditional CaMKII consensus sequence, RXXS/T, Hund *et al.*[14] identified S571 as a potential CaMKII target (Figure 1). They demonstrated that CaMKII phosphorylates S571 in vitro and that, in a heterologous cell system expressing $Na_V1.5$, CaMKII shifts WT steady state inactivation to negative potentials. This effect on channel inactivation was abolished when S571 was mutated to a non-phosphorylatable alanine and mimicked when S571 was mutated to a phospho-mimetic glutamine reside. Our group[15] found that only the I–II loop of $hNa_V1.5$ was substantially phosphorylated by CaMKII (i.e. neither other loops nor N-or C-tail were targets), and systematic analysis of the entire I–II loop showed that S516 and T594 were the main in vitro CaMKII phosphorylation sites. In patch-clamp analysis, we found that alanine substitution of S516, S571 and T594 could all inhibit the CaMKII-dependent negative shift in I_{Na} availability and accumulation of intermediate inactivation observed in myocytes. However, only S516E and T594E phospho-mimetic mutants could recapitulate CaMKII effects on I_{Na} availability. Thus there may be three sites in this stretch of the I–II loop that participate in CaMKII-dependent regulation of cardiac I_{Na} gating (Figure 1).

There are two major points raised by Koval *et al.*[1] in extending their work on the S571 site. First, they provide evidence that phosphorylation of S571 on $Na_V1.5$ is increased in the canine post-infarct border zone, may be slightly increased in human HF and is increased in isolated mouse myocytes after acute stress (isoproterenol + phosphatase inhibition), but not in mice expressing CaMKII inhibitor AC3-I. These observations have potentially important implications for the genesis of acquired arrhythmias in cardiac disease. Second, they show that two rare, naturally occurring $Na_V1.5$ point mutations (A572D and Q573E) might functionally mimic the negative charge induced by S571 phosphorylation, resulting in similar channel biophysics. While these experiments only indirectly suggest this mimicry, electrostatic potential maps are also consistent with this idea. These channels also evoked AP prolongation in cultured neonatal mouse ventricular myocytes. The authors thus posit that the region comprising residues 571–573 functions as a sort of negatively charged “hot spot”, with a negative charge at any of these three residues (due to phosphorylation or a naturally occurring mutation) conferring similar results on channel gating and function. The 571–573 hot spot referred to here could extend to encompass the other CaMKII modulation sites (S516 and T594), which may also have a local charge basis such that multiple local sites might contribute to altered I_{Na} gating. Importantly, this may provide a new therapeutic target downstream of CaMKII for heart failure and arrhythmias.[16] Further studies will be required to clarify these molecular mechanisms.

CaMKII and Arrhythmias in HF

CaMKII transgenic mice exhibited tachyarrhythmias[9] and CaMKII inhibition may be protective against electrical[17] and structural[18] remodeling in HF. Interestingly, heterozygous SCN5A+/− knock-out[19] and Ins1795D knock-in[20] mice also have conduction disturbances

and increased propensity to arrhythmias. Since CaMKII can cause both LQTs-like I_{NaL} and loss of function BRs-like effects,[3,4] it is worth considering how CaMKII effects contribute to arrhythmogenesis and increase the risk of SCD in HF. Channelopathies associated with $Na_V1.5$ loss of function, such as BRs, and gain of function, LQT3, provide a nice framework for discussion.

Koval et al.[1] found that A572D and Q573E arrhythmia variants expressed in cultured neonatal mouse cardiomyocytes exhibited enhanced I_{NaL} and APD that was sensitive to TTX and the I_{NaL} specific inhibitor Ranolazine. Early afterdepolarizations were even seen in 2 of the 9 A572D neonatal mouse myocytes, and their computer simulations produced comparable results, and resemble earlier LQT3 models of I_{NaL} effects gain of function mutations in SCN5A.[21] In our computational model of CaMKII effects on I_{Na} in rabbit,[22] an LQT3-like phenotype was present at low heart rates (where APD is long) and thus integrated I_{NaL} is large. At higher heart rates (> 1Hz), the APD effect disappeared, and a BRs-like loss of I_{Na} availability and slower AP rate of rise was more apparent. Several studies have demonstrated increased I_{NaL} in HF [23,24] and some of this may be dependent on CaMKII activity.[25] More mechanistic work will be needed to test which (if any) of the CaMKII phosphorylation sites is critical for enhanced I_{NaL}. Regardless, selective I_{NaL} inhibition may be an important therapeutic strategy to treat some LQT-like arrhythmias in HF.[26]

What about the I_{Na} loss of function effects caused by CaMKII? CaMKII and all of the identified CaMKII sites (S571, T594, and S516) seem to mediate loss of I_{Na} function effects (reduced I_{Na} availability, increased intermediate inactivation and slowed recovery from inactivation). These effects may also contribute to arrhythmogenesis analogous to BRs, but have attracted less attention than the I_{NaL} and LQT3-like effects. The loss of I_{Na} function induced by CaMKII is likely to be exacerbated at high heart rates[22] and could slow conduction, create conduction block in vulnerable tissue, shorten APD and enhance the substrate for reentrant arrhythmias (Figure 1). In that sense, the CaMKII effects would match the Ins1795D and ΔK1500 human mutations, where patients exhibit LQT symptoms at low heart rate and Brugada-like symptoms at high heart rate.[12,27]

CaMKII effects on Other Channels Contribute to Arrhythmias

CaMKII also modulates I_{Ca} and I_{to} and I_{K1} properties[16] (Figure 1). CaMKII mediates I_{Ca} facilitation, which enhances peak I_{Ca} and slows inactivation (gain-of-function), and tends to prolong APD (along with I_{NaL}), favoring EADs. CaMKII also enhances I_{to} recovery from inactivation, thereby hastening repolarization. When the CaMKII effects on I_{Ca}, I_{to}, and I_{Na} are combined, APD shortening is predicted in epicardial rabbit myocytes.[22] However, in endocardial or HF myocytes, with reduced I_{to} expression and function, APD would be prolonged because of I_{Na} and I_{Ca} effects. This would enhance the normal transmural dispersion of repolarization, thereby enhancing the substrate for reentrant arrhythmias. Additionally, regional differences in CaMKII activity, such as around myocardial infarct zones, may translate into heterogeneity of phosphorylated channels. Re-entrant arrhythmias could thus ensue as in BRs. Clearly the combined effects of CaMKII in cardiac electrical remodeling are complex and further studies are needed.

CaMKII also phosphorylates phospholamban (PLN) to stimulate SR Ca-ATPase (enhancing SR Ca^{2+} loading) and the SR Ca^{2+} release channel (RyR2) enhancing arrhythmogenic spontaneous SR Ca^{2+} release.[16] Moreover, when the putative RyR2 CaMKII phosphorylation is mimicked (S2814D) or prevented (S2818A) in knock-in mice, there is increased or decreased (respectively) DADs and triggered arrhythmias.[28] Indeed, the combination of enhanced SR Ca^{2+} uptake and sensitized RyR2 may synergize in developing CaMKII-dependent DADs. In the setting of HF, where Na/Ca exchange is increased and I_{K1}

is reduced (both of which increase DAD amplitude), there is an increased propensity for triggered arrhythmias.[29]

All of the above pathways could degenerate into lethal ventricular tachyarrhythmias (Figure 1). These mechanisms are certainly not mutually exclusive, and a mixture of several factors plays a role. Inherited mutations at Ins1795D and ΔK1500 in $hNa_V1.5$ with combined LQTs and BRs and RyR2 sensitizing inherited mutations linked to catecholaminergic polymorphic ventricular tachycardia (CPVT), both of which phenocopy CaMKII effects, may identify these channel targets as especially important.

Future Studies

Koval *et al.*[1] furthers our understanding of the role and importance of CaMKII based $Na_V1.5$ phosphorylation at S571, but several key questions remain. What is the relative role of S571 and the other two CaMKII phosphorylation sites S516 and T594 (additive, synergistic, redundant, antagonistic)? Differences in the mode or location of CaMKII activation could differentially influence targets and their impact. For example, binding of CaMKII to β-spectrin may enhance site phosphorylation[14] and activation state of CaMKII may alter its binding affinities for certain substrates.[30] Further studies will are needed to evaluate the multiple phosphorylatable CaMKII sites on the Na^+ channel.

Going forward, it will be valuable to understand CaMKII effects on other Na^+ channel isoforms (Figure 2), some of which may be expressed in the heart ($Na_V1.1$, 1.3 and 1.6).[8] The S571 and T594 CaMKII phospho-sites are well conserved among mammalian $Na_V1.5$ (and in chicken). The S516 phospho-site, in contrast, appears to be exclusive to human $Na_V1.5$ (in others there is no basic residue at the important P-3 position, except in rabbit). Skeletal muscle $Na_V1.4$ (SCN4A) has none of these phosphorylation sites. Some neuronal Na^+ channels (SCN1A, 2A, 8A and 9A or $Na_V1.1$, 1.2, 1.6 and 1.7) contain the S571 site, but the T594 site is only the same in SCN8A ($Na_V1.6$). This opens up new avenues of study about potential roles of CaMKII-dependent regulation of Na^+ channels.

It will also be important to understand which CaMKII channel targets are the most important arrhythmogenic influences (among Na^+, K^+ and Ca^{2+} channels or RyR2). Furthermore, studies are needed to determine if different populations of e.g. Na^+ channels are present even within the same myocyte that differ in their signaling interactions and regulation by CaMKII. It may also be important to test whether these sites are targeted by other kinases relevant to cardiac pathophysiology, such as PKA and PKC. While much work remains to fully understand the complexities of phospho-regulation of voltage gated Na^+ channels, the work from Koval *et al.*[1] is an important step forward in our understanding of this process and its relevance to cardiac disease.

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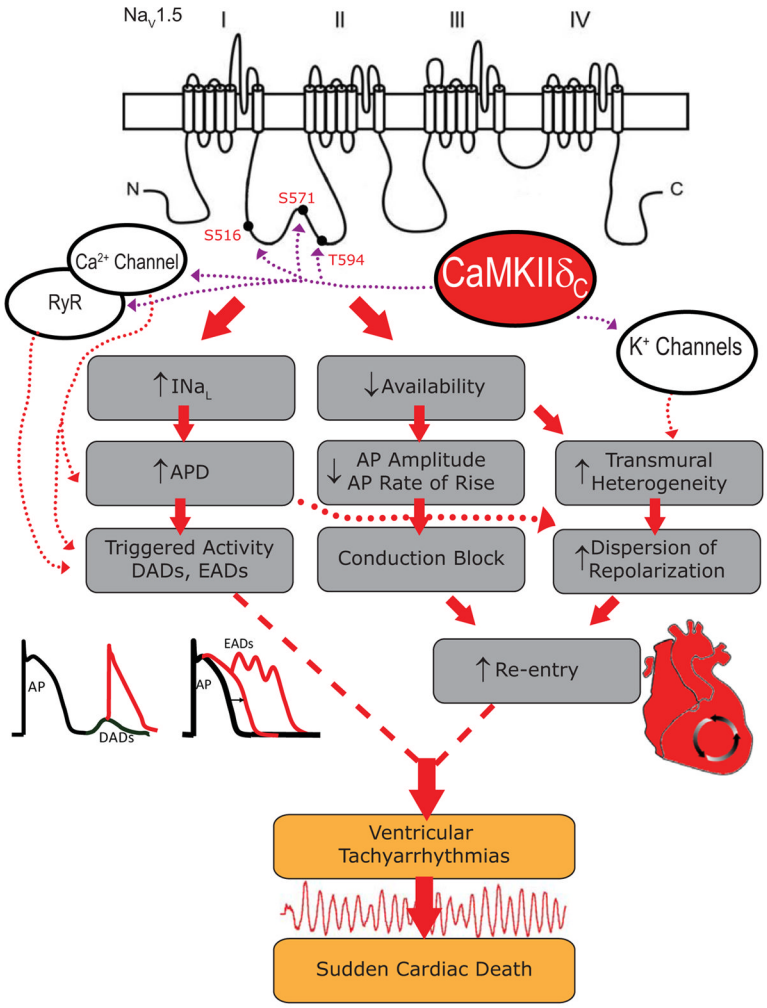

Figure 1.

CaMKII Phosphorylation of NaV1.5 is Pro-Arrhythmogenic. Arrhythmogenic mechanism of CaMKII based regulation of I_{Na}, showing different CaMKII based alterations in cardiac ion channel targets and contributions. The emphasis is on CaMKII sites on $Na_V1.5$ at S571, S516, and T594 on the DI–II loop of $Na_V1.5$. When loss- and gain-of-function effects combine with other CaMKII ion channel targets, this can further enhance ventricular arrhythmias and sudden cardiac death.

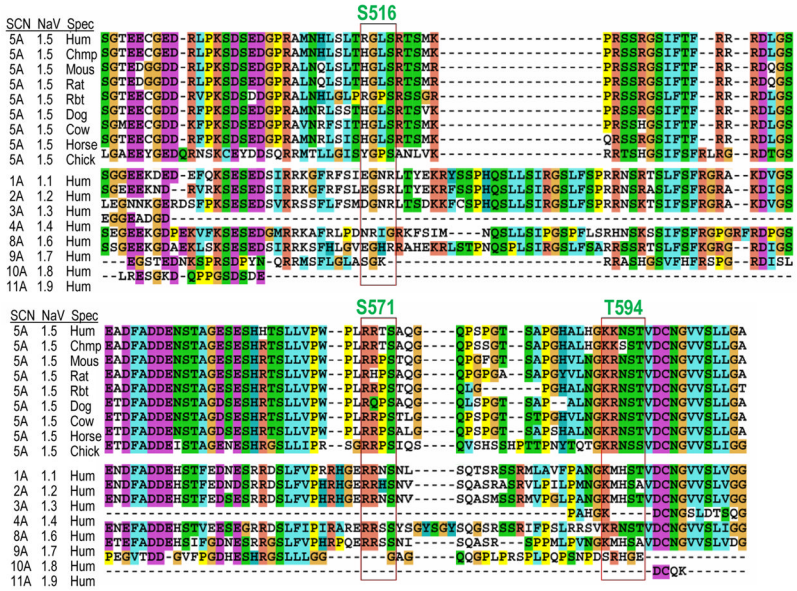

Figure 2.

Differential Conservation of CaMKII Phosphorylation Sites Across Species and Isoform. ClustalX2 protein sequence alignments of human $Na_V1.5$ across species and Na_V isoform. The I–II loop is shown with CaMKII consensus sequence (red bounding box) and Ser or Thr phospho-site highlighted. Species and isoform gene names as indicated.