Interplay between R513 methylation and S516 phosphorylation of the cardiac voltage-gated sodium channel

Pedro Beltran-Alvarez,[1,*] Ferran Feixas,[2,†] Sílvia Osuna,[2,†] Rubí Díaz-Hernández,[1] Ramon Brugada,[1,*] Sara Pagans[1,*]

[1] Cardiovascular Genetics Center, Institut d'Investigació Biomèdica de Girona Dr. Josep Trueta, and Department of Medical Sciences, School of Medicine, University of Girona, ES-17003 Girona, Spain

[2] Institut de Química Computacional i Catàlisi (IQCC) and Departament de Química, Universitat de Girona, Campus Montilivi, ES-17071 Girona, Spain

* Contributed equally as corresponding authors. Sara Pagans: sara.pagans@udg.edu, Ramon Brugada: rbrugada@idibgi.org, Pedro Beltran-Alvarez: pbeltran@idibgi.org.

† Contributed equally.

Abstract

Arginine methylation is a novel post-translational modification within the voltage-gated ion channel superfamily, including the cardiac sodium channel, $Na_V1.5$. We show that $Na_V1.5$ R513 methylation decreases S516 phosphorylation rate by 4 orders of magnitude, the first evidence of PKA inhibition by arginine methylation. Reciprocally, S516 phosphorylation blocks R513 methylation. $Na_V1.5$ p.G514C, associated to cardiac conduction disease, abrogates R513 methylation, while leaving S516 phosphorylation rate unchanged. This is the first report of methylation - phosphorylation cross-talk of a cardiac ion channel.

Keywords: sodium channel, post-translational modification, arginine methylation, phosphorylation, cross-talk

Introduction

Arginine methylation is emerging as a common post-translational modification of voltage-gated ion channels (Beltran-Alvarez et al. 2014, Guo et al. 2014). Arginine methylation is catalyzed by protein arginine methyltransferases (PRMT)[§], which transfer methyl groups from *S*-adenosyl- L-methionine (SAM) to produce mono- or dimethylated arginines (Pang et al. 2010). We have recently described that the α subunit of the cardiac voltage-gated sodium channel, $Na_V1.5$, is methylated by PRMT3 (Beltran-Alvarez et al. 2013).

$Na_V1.5$ is responsible for the fast sodium current that initiates cardiomyocyte action potentials. It is a large protein (*ca*. 2,000 residues) containing 24 transmembrane segments allocated in four homologous domains, DI to DIV, which are joined by cytosolic, interdomain linkers. The linker between domains DI and DII has been identified as a *hot-spot* of post-translational modifications that modulate $Na_V1.5$ function, including arginine methylation, and phosphorylation. $Na_V1.5$ residues R513 and R526 are mono- or dimethylated (Beltran-Alvarez et al. 2011). Additionally, there are 11 phosphorylated serines, including S516, S524 and S525, which are targets for kinases such as cAMP-dependent protein kinase (PKA), (Marionneau et al. 2012, Herren et al. 2013). The fine-tuned regulation of $Na_V1.5$ current is vital for proper cardiac function, which is exemplified by the fact that single mutations in $Na_V1.5$ can lead to serious cardiac disorders, including cardiac conduction disease (Tan et al. 2001).

In our original report of $Na_V1.5$ methylation, we had speculated that arginine methylation could regulate phosphorylation of adjacent phosphosites (Beltran-Alvarez et al. 2011). Here, we describe the interplay between $Na_V1.5$ R513 methylation and S516 phosphorylation.

Materials and Methods

The manuscript does not contain clinical studies or patient data.

In vitro methylation and phosphorylation assays

Peptides were synthesized by the Laboratory of Proteomics and Protein Chemistry, University Pompeu Fabra, and isolated to 98 % purity. We used 15,000 recombinant murine PKA α isoform (EC 2.7.11.11) units, and 500 μM ATP (both from New England Biolabs, Ipswich, MA, product numbers P6000L and P0756L) in 20 μL phosphorylation reactions. PKA reaction buffer (pH 7.5) was provided by the manufacturer. Human PRMT3 (EC 2.1.1.125) was expressed in *E. coli* as GST fusion and purified following published protocols (Zurita-Lopez et

al. 2012). PRMT3 preparations contained (mM): 50 Tris, 150 NaCl, 10 reduced glutathione, pH = 7.4. SAM (Sigma) concentration was 200 μM.

All phosphorylation and methylation reactions were started by addition of the peptide, and then incubated at 30 ºC with shaking (400 rpm). At desired time points, aliquots were taken and reactions stopped by addition of 1 % formic acid. Samples were kept at -20 ºC until analysis. All reactions were done in triplicate.

To identify methylation sites, *RS_long* methylated by PRMT3 as above was incubated with trypsin (Promega) for 2 hours at 37 ºC, and we analyzed the resulting peptide fragments by matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) as described below.

MALDI-TOF analysis of methylation and phosphorylation

Peptides were directly spotted onto a Bruker Anchor Chip 384-spot MALDI target plate (Bruker Daltonics) using α-cyano-4-hydroxy-cinnamic acid as matrix. Mass spectra (*m*/*z* 900 - 4000) were acquired using an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics) in the positive ion mode. Between 500 and 1,000 shots / sample were performed. Spectra were analyzed using FlexAnalysis v.2.0. The extent of reaction was calculated as the ratio (area phosphorylated peptide) / (area unmodified peptide), and it was less than 15 % in all cases. For every substrate concentration, 5 - 7 time points were used to calculate initial rates. We used the Michaelis-Menten formalism to fit reaction rate data (shown as mean ± SD).

LC-MS/MS analysis of methylation and phosphorylation

Tandem mass spectrometry was done in the Proteomics Platform, University of Barcelona. *RS_short* phosphorylated by PKA (see above) was analyzed in a nanoAcquity liquid chromatographer (Waters) – LTQ Orbitrap Velos Pro (Thermo) mass spectrometer. Peptides (*m*/*z* 350-1700) were analyzed in data dependent mode in the Orbitrap with a resolution of 60,000 *FWHM* at 400 *m*/*z*. The 10 most abundant peptides were fragmented using collision induced dissociation (CID) in a linear ion trap. Data were collected with Thermo Xcalibur (v.2.1.0.1140). Searches were performed using the Sequest search engine (Proteome Discoverer v.1.3.0.339) against a database containing common laboratory contaminants and the sequence of *RS_short*. The PhosphoRS node was used to provide a confidence measurement of the phosphorylation site.

To search for mutual exclusivity of $Na_V1.5$ arginine methylation and phosphorylation in a cellular model, we used our previously obtained LC-MS/MS characterization of $Na_V1.5$ overexpressed in HEK293 cells (Beltran-Alvarez et al. 2011). Searches were done using

Sequest, as before, against a database containing common laboratory contaminants and $Na_V1.5$ sequence.

Molecular dynamics

Molecular dynamics simulations of *RS_short* and *RmeS_short* assessed the different conformational states explored by peptides in solution. See Supplemental Methods for details.

Results and Discussion

First, we used the synthetic peptide *RS_short* (Table 1) as a substrate for PKA *in vitro*. PKA readily monophosphorylated *RS_short* (Figure 1, Panel a, and Online Resources S1 and S2). The main phosphorylation site was S516 (pRS node site probability: 99.7 %, Online Resource S3) although, due to the resolution of our mass spectrometric analyses, we cannot rule out the possibility of a very small population of *RS_short* having an unexpected modification site. Introduction of a methyl group at position R513 decreased *RmeS_short* phosphorylation rate (Figure 1, Panel a, and Online Resource S2), and the specificity constant (V_{max} / K_M) was reduced by *ca*. 26-fold (Figure 1, Panel b). We propose that this effect was due to arginine methylation within the PKA consensus motif (RRxS/T), which may impose additional hydrophobic / steric constraints and jeopardize PKA binding. An alternative would be that arginine methylation alters peptide folding, but molecular dynamics simulations of *RS_short* and *RmeS_short* predicted superimposable secondary structures (Online Resource S4).

PRMT3 asymmetrically dimethylates its substrates (Tang et al. 1998). R513 asymmetric dimethylation in the peptide *Rme2S_short* reduced V_{max} / K_M by *ca*. 10,000-fold compared to *RS_short* (Figure 1, Panels a, and b). Given our evidence, it appears that S516 phosphorylation rate may be determined by R513 methylation state, although there could be other mechanisms occurring *in vivo* that cannot be explored with our current approach.

Our mass spectrometric analyses assumed that MALDI-TOF performance of *RS_short*, *RmeS_short*, and *Rme2S_short* was similar. This was supported by the observation that MALDI-TOF analysis of a mixture containing 0.1 µg / µL each peptide rendered peaks of comparable intensities (Online Resource S5). Although *RS_short*, *RmeS_short*, and *Rme2S_short* phosphorylation was not expected to affect mass spectrometric properties (Steen et al. 2006), kinetic parameters in this work should be regarded as apparent constants. In any event, our approach was suitable for comparing different substrates (Pahlich et al. 2005, Kang et al. 2008, Köbel et al. 2009).

Having described how R513 methylation defines S516 phosphorylation rate, we asked whether S516 phosphorylation could, in turn, modulate R513 methylation. We initially

performed methylation experiments using *RS_long*, which included 12 residues at the *C* terminus of *RS_short*, and which was a better substrate for PRMT3 (Online Resource S6). Methylation assays using *RS_long* indicated that R513 was the preferential methylation site (Online Resource S7). To eliminate any residual activity of PRMT3 towards non-R513 arginine residues, we synthesized the peptide *RS_long_K*, where R517, R523 and R526 were substituted for lysines. *RS_long_K* was efficiently mono- and dimethylated by PRMT3 (Figure 1, Panel c).

To test the effect of S516 phosphorylation on R513 methylation, we performed experiments using the peptide *RpS_long_K*. We could not detect methylation of *RpS_long_K* (1 µg / µL) after 8 h reactions (Figure 1, Panel d). This indicated that S516 phosphorylation abrogated R513 methylation.

To validate our results in a cellular system, and to assess whether the antagonistic relation of arginine methylation and phosphorylation could be generalized to other $Na_V1.5$ Arg-Ser pairs, we re-analyzed our original mass spectrometry data of $Na_V1.5$ overexpressed in HEK293 cells (Beltran-Alvarez et al. 2011). We set both arginine methylation, and serine phosphorylation, as dynamic post-translational modifications. We found methylated R526 only in peptides where S524 and S525 were not phosphorylated, and S524 and S525 phosphorylation only in peptides where R526 was not modified (Online Resource S8). These observations suggest that S524 / S525 phosphorylation, and R526 methylation, are mutually exclusive (Baek et al. 2014). Overall, our results suggest a model where arginine methylation may regulate $Na_V1.5$ phosphorylation, and *viceversa*.

Cross-talk between arginine methylation and phosphorylation has previously been described. For instance, methylation of arginines within the Akt kinase consensus motif (RxRxxS/T) blocks phosphorylation of BAD proteins (Sakamaki et al. 2011). Conversely, phosphorylation of RNA polymerase II inhibits arginine methylation *in vitro* (Sims et al. 2011). In one of the few examples of arginine methylation – phosphorylation interplay in membrane proteins, methylation of R1175 of the epidermal growth factor receptor enhanced auto-phosphorylation of Y1173 (Hsu et al. 2011).

Our results raise the possibility that pathogenic $Na_V1.5$ mutations alter $Na_V1.5$ methylation-phosphorylation equilibria. To test for this hypothesis, we evaluated the effect of $Na_V1.5$ p.G514C, associated to cardiac conduction disease (Tan et al. 2001), on R513 methylation and S516 phosphorylation rates. We could not observe methylation of *RS_long_K_G514C* (1 µg / µL) after 8 h reactions (Figure 1, Panel e). This was not entirely unexpected, because G514C disrupts the RG consensus methylation motif, thereby introducing a non-favored Cys at the +1 position (Guo et al. 2014). In contrast, PKA phosphorylated *RS_short_G514C* with the same efficiency as *RS_short* (Online Resources S2 and S9). We speculate that one of the mechanisms by which $Na_V1.5$ p.G514C leads to cardiac conduction disease could be S516 hyperphosphorylation due to R513 methylation blockade. Nevertheless,

the translation of our current results to an *in vivo* context must be done with care. Future studies, including mutagenesis of R513 and S516 in the full-length protein, should shed light on how post-translational modification of these residues affect *e.g.* the structure and protein-protein interactions of this $Na_V1.5$ region.

As this manuscript was in preparation, proteomic studies by the group of Trimmer identified reciprocal changes in phosphorylation and arginine methylation of the rat brain sodium channel ($Na_V1.2$) after seizure (Baek et al. 2014). One of the three methylated arginines identified in $Na_V1.2$ was R563, equivalent to R513 in $Na_V1.5$ (Online Resource S10). The authors speculate that $Na_V1.2$ R563 methylation regulates phosphorylation of $Na_V1.2$ S568, equivalent to S516 or S519 in $Na_V1.5$. Using a completely different approach, we have herein characterized the interplay R513 methylation – S516 phosphorylation in $Na_V1.5$ as a model to understand how arginine methylation may regulate phosphorylation of voltage-gated sodium channels, and *viceversa*. At a broader level, our results open the door to pharmaceutical intervention to balance $Na_V1.5$ methylation - phosphorylation equilibria in cardiopathological states, and provide the first evidence that arginine methylation inhibits PKA-catalyzed phosphorylation reactions. In this sense, we are currently assessing the hypothesis that arginine methylation imposes steric constraints on peptide : PKA catalytic complexes, and our results will be published in due time.

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The authors declare that they have no conflict of interest.

§ Abbreviations. MALDI-TOF: matrix-assisted laser desorption ionization - time of flight; $Na_V1.5$: voltage-gated sodium channel, cardiac isoform, α subunit; PKA: cAMP-dependent protein kinase; PRMT: protein arginine methyltransferase; SAM: *S*-adenosyl-L-methionine

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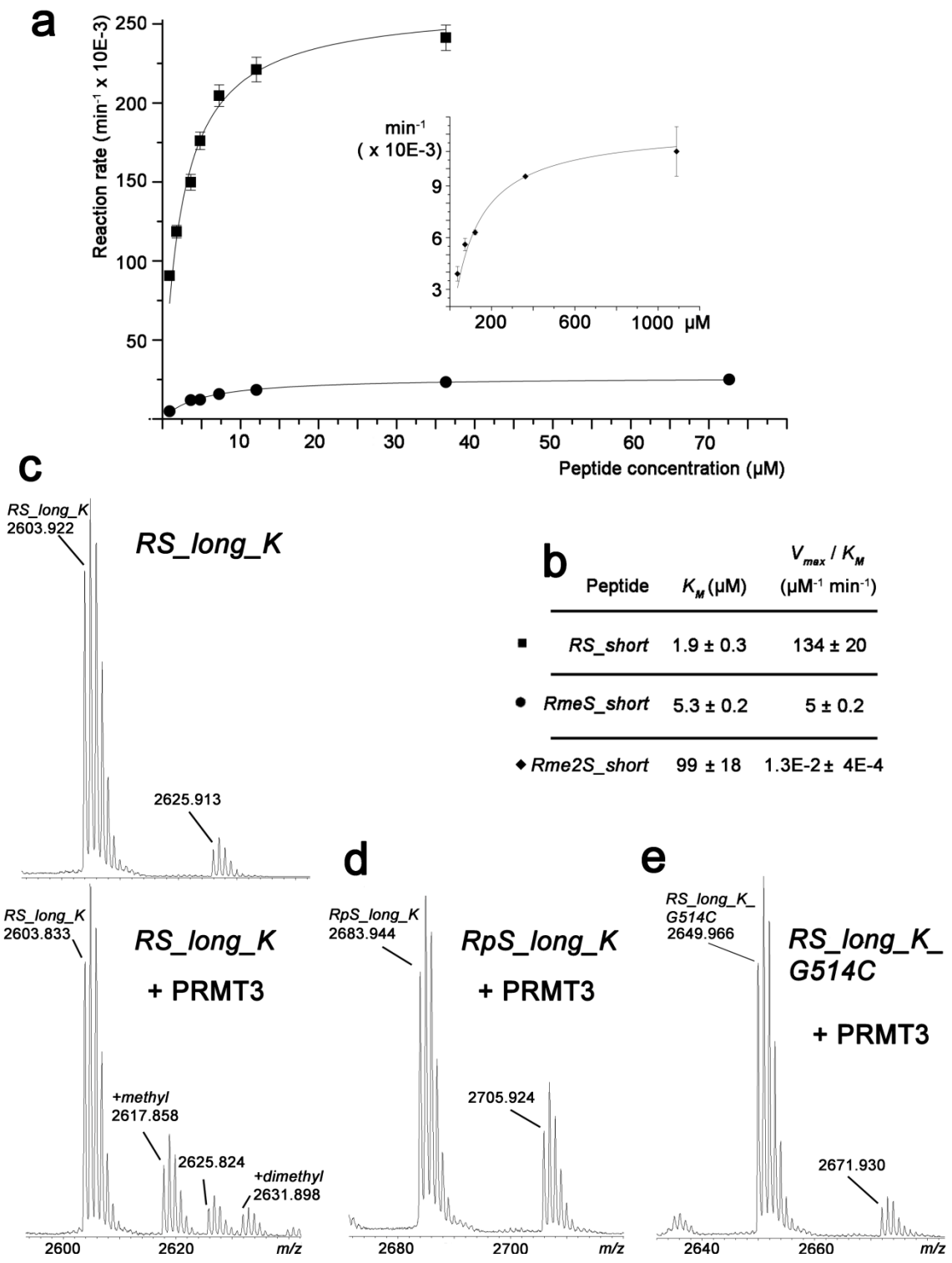
Table 1 Synthetic peptides used in this work. The *RS_short* peptide series was used in PKA reactions, but could not be used in methylation assays due to lack of activity of PRMT3 towards *RS_short*. The *RS_long* peptide series was used in PRMT3 reactions, but could not be used in phosphorylation assays because the introduction of four extra Ser residues in *RS_long* led to a complex mixture of di-, tri- and quatri-phosphorylated peptides upon incubation with PKA. R513 and S516 are bold throughout. Rme: methylated arginine. Rme2: asymmetrically dimethylated arginine. pS: phosphorylated serine

Fig. 1 a. Michaelis-Menten plots of *RS_short* (squares), *RmeS_short* (circles), and *Rme2_short* (inset, diamonds) phosphorylation by PKA. Note the scale change in the inset. **b**. Apparent kinetic parameters of *RS_short*, *RmeS_short*, and *Rme2_short* phosphorylation by PKA. **c**. Methylation of *RS_long_K* by PRMT3. Incubation of *RS_long_K* (expected mass: 2603.420 Da) with PRMT3 led to mono- and dimethylated *RS_long_K* (expected masses: 2617.447 Da and 2631.474 Da, respectively). The species at 2625 Da is most likely sodium adduct to *RS_long_K*. **d**. S516 phosphorylation blocks R513 methylation. MALDI-TOF spectrum of *RpS_long_K* (expected mass: 2683.750 Da) incubated with PRMT3. Methylated species were expected, but not observed, at 2697.8 Da (mono-) and 2711.8 Da (dimethylation). The species at 2705.924 and 2727.930 Da are most likely mono- and disodium adducts to *RpS_long_K*. **e**. G514C blocks R513 methylation. MALDI-TOF spectrum of *RS_long_K_G514C* (expected mass: 2649.410 Da) incubated with PRMT3. Methylated species were expected, but not observed, at 2663.4 Da (mono-) and 2677.4 Da (dimethylation). The species at 2671.930 Da is most likely sodium adduct to *RS_long_K_G514C*

Table 1

Peptide Name	Peptide Sequence
RS_short	NHLSLTR GL SRT
RmeS_short	NHLSLTR$_{me}$ GL SRT
Rme2S_short	NHLSLTR$_{me2}$ GL SRT
RS_short_G514C	NHLSLTR CL SRT
RS_long	NHLSLTR GL SRTSMKPRSSRGSIF
RS_long_K	NHLSLTR GL SKTSMKPKSSKGSIF
RpS_long_K	NHLSLTR GL$_{p}$SKTSMKPKSSKGSIF
RS_long_K_G514C	NHLSLTR CL SKTSMKPKSSKGSIF

Fig. 1


a
Reaction rate (min-1 x 10E-3)
250
200
150
100
50
min-1
(x 10E-3)
9
6
3
200
600
1000 µM
10
20
30
40
50
60
70
Peptide concentration (µM)
b
Peptide
KM (µM)
Vmax / KM
(µM-1 min-1)
RS_short
1.9 ± 0.3
134 ± 20
RmeS_short
5.3 ± 0.2
5 ± 0.2
Rme2S_short
99 ± 18
1.3E-2 ± 4E-4
c
RS_long_K
2603.922
RS_long_K
2625.913
RS_long_K
2603.833
RS_long_K
+ PRMT3
+methyl
2617.858
2625.824
+dimethyl
2631.898
2600
2620
m/z
d
RpS_long_K
2683.944
RpS_long_K
+ PRMT3
2705.924
2680
2700
m/z
e
RS_long_K_
G514C
2649.966
RS_long_K_
G514C
+ PRMT3
2671.930
2640
2660
m/z