Protein arginine methyl transferases-3 and -5 increase cell surface expression of cardiac sodium channel

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Abstract

The α-subunit of the cardiac voltage-gated sodium channel (NaVα1.5) plays a central role in cardiomyocyte excitability. We have recently reported that NaVα1.5 is post-translationally modified by arginine methylation. Here, we aimed to identify the enzymes that methylate NaVα1.5, and to describe the role of arginine methylation on NaVα1.5 function. Our results show that protein arginine methyltransferase (PRMT)-3 and -5 methylate NaVα1.5 in vitro, interact with NaVα1.5 in human embryonic kidney (HEK) cells, and increase NaVα1.5 current density by enhancing NaVα1.5 cell surface expression. Our observations are the first evidence of regulation of a voltage-gated ion channel, including calcium, potassium, sodium and TRP channels, by arginine methylation.

Abbreviations: AP, action potential; ArgMe, arginine methylation; bpm, beats per minute; CFP or YFP, cyan or yellow fluorescent protein; co-IP, co-immunoprecipitation; FRET, Förster resonance energy transfer; HEK, human embryonic kidney; IP, immunoprecipitation; LC-MS/MS, liquid chromatography-mass spectrometry; PRMT, protein arginine methyltransferase; SAM, S-(adenosyl)-L-methionine

1. Introduction

Cardiac ion channels are essential for the generation of cardiomyocyte action potentials (AP). The cardiac voltage-gated sodium channel is responsible for the sodium inward current that initiates the AP, and consists of an α-subunit (NaVα1.5), which is necessary and sufficient for the generation of sodium currents, and accessory proteins [1]. The fine regulation of NaVα1.5 current is vital for proper cardiac function, and single mutations in NaVα1.5 can lead to cardiac arrhythmia conditions [2]. NaVα1.5 is predicted to be composed of four homologous domains, DI to DIV, joined by interdomain linkers. Interdomain linkers, N and C termini of the protein are cytosolic, and are accessible to post-translational modifications such as phosphorylation [3], and ubiquitination [4] which modulate NaVα1.5 function. Noteworthily, we have recently reported initial (40% sequence coverage) mass spectrometry evidence that identified arginine methylation (ArgMe) as a novel post-translational modification of NaVα1.5 [5]. ArgMe was observed in Arg residues within the interdomain linker between DI and DIV of NaVα1.5 (L4–L5).

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ArgMe is catalysed by protein arginine methyltransferases (PRMT), which transfer methyl groups from the cofactor S-adenosyl-L-methionine (SAM) to the protein, leading to mono- or dimethylarginines [6]. In the latter case, methylation can be asymmetric or symmetric [7]. To date, 9 PRMTs have been identified in the human genome [8], namely PRMT1 to -9. PRMT1, -2, -3, -4, -6 and -8 are Type I PRMT that produce monomethyl and asymmetric dimethyl arginines [9]. PRMT5 is a Type II PRMT that produces monomethyl and symmetric dimethyl arginines [10], while PRMT7 leads to monomethyl arginines only [11]. PRMT9 has not been biochemically characterised to date. The addition of methyl groups modifies the shape of the arginine residue, turning it more bulky and hydrophobic, and may block a potential hydrogen bond donor.

Evidence indicating that NaV1.5 is modified by ArgMe has opened new questions in the sodium channel field. In this context, we set ourselves to identify the PRMTs that mediate NaV1.5 ArgMe, and to describe their effect on NaV1.5 function.

2. Materials and methods

2.1. Plasmids

A pcDNA3 plasmid that encodes NaV1.5 as a FLAG fusion, which has previously been described [12–14], was used in electrophysiological, co-IP, and biotin pull-down experiments. For Förster resonance energy transfer (FRET) measurements, a NaV1.5-CFP construct, which localises to the endoplasmic reticulum in HEK cells, was used [15].

PRMTs for in vitro methylation assays were cloned into pGEX or Myc vectors. GST-PRMT1, -2, -3, -4, -6, and -7, as well as Myc-PRMT5, have been described [11,16]. To perform methylation studies in cells, FRET, co-IP, electrophysiological, and biotin pull-down experiments, PRMT-YPF fusion proteins were produced. PRMT1, -2, -3, -5, -6 and -7 were cloned from human cardiac cDNA (the study was approved by the institutional ethical committee of the Dr. Josep Trueta Hospital, Girona, Spain) into pcDNA3.1 (Invitrogen) as C-terminal YFP fusion proteins. PRMT4 could not be amplified from cardiac cDNA and a plasmid template was used.

L1.5 (NaV1.5 residues 416–711) was cloned into pcDNA3.1 as L1.5-FLAG-YFP fusion. Plasmids for expression in Escherichia coli were pETM15b (EMBL) in which L1.5 or the C-terminal domain (NaV1.5 residues 1773–2016) were cloned as N-terminal Histagged proteins.

For electrophysiological and FRET assays, HEK cells were transfected using calcium phosphate precipitation. For all other experiments, cells were transfected using GeneCellin (BioCellChallenge).

2.2. Detection of methylated L1.5 in cells

HEK cells were transiently transfected with the L1.5-FLAG-YFP plasmid (2 µg), and harvested 30 h later. Soluble lysates were incubated with α-GFP antibody (Abcam) coupled to protein A beads (GE Healthcare). Immunoprecipitated proteins were detected with α-FLAG (M2, Sigma), α-ASYM24, or α-SYM11 (Merck).

2.3. In vitro methylation assays

L1.5 and the C-terminal domain of NaV1.5 were expressed in E. coli as 6×His fusions, and purified using Ni-NTA chromatography. In vitro methylation assays were conducted as described [17]. Recombinant GST-PRMT1, -2, -3, -4, -6 and -7 were expressed and purified from E. coli. Myc-tagged human PRMT5 was expressed and purified from HeLa cells. Reactions were done in the presence of 0.5 µCi S-adenosyl-L-[methyl-3H]methionine (3H-SAM, GE Healthcare) for 1 h at 30 °C in a final volume of 30 µl.

2.4. FRET between NaV1.5 and PRMTs

HEK cells expressing NaV1.5-CFP (0.5 µg plasmid) together with the corresponding PRMT–YPF (1.5 µg plasmid), or YFP as a negative control (0.5 µg plasmid), were grown for 24–48 h after transfection. For every NaV1.5/PRMT pair, 4 independent transfections were done. In every measuring session, 5–10 negative controls were measured in parallel. Cells were examined by confocal microscopy on a LSM 710 (Zeiss, Jena, Germany) using a C-Apochromat 40×/1.2 NA water-immersion objective lens at room temperature. FRET was calculated using the sensitised emission method. Reported apparent FRET efficiencies (FRETapp) are the mean ± S.E.M. of individual cell FRETapp values for a given NaV1.5-CFP/PRMT–YPF pair.

2.5. Co-IP of PRMT1 and -3, with NaV1.5

HEK cells were transiently transfected with FLAG NaV1.5 (1 µg plasmid), together with GFP (0.5 µg plasmid), or PRMT1-, or -3-YPF (1.5 µg plasmid). Cells were lysed 48 h after transfection, and NaV1.5 was immunoprecipitated (n = 3) using FLAG chromotography (Sigma). PRMTs were detected using an α-GFP antibody (Abcam), and NaV1.5 was detected using an α-NaV1.5 antibody (Alomone).

2.6. Electrophysiological recordings

Sodium currents in HEK cells transiently expressing FLAG-NaV1.5 (1.5 µg plasmid) together with the corresponding PRMT–YPF (0.5 µg plasmid), were measured between 25 and 35 h after transfection. The total number of independent transfections was 5 (PRMT3 and -5), 3 (PRMT1), or 2 (PRMT2, -4, and -7). The day after transfection, SAM (0.15 mM, final concentration) was added to the medium, and sodium currents were measured at room temperature. In every session, 3–10 negative controls (i.e. cells co-expressing NaV1.5 and YFP) were measured in parallel.

Currents were obtained by patch clamp whole-cell recording using an Axopatch 200B amplifier (Molecular Devices). Activation data at test potentials of −80 to +50 mV (in 5 or 10 mV steps) were fitted to a Boltzmann equation, of the form

\[ \frac{g}{g_{\text{max}}} = \frac{1}{1 + \exp \left( \frac{V_{1/2} - V_{m}}{\tau} \right)} \]

where \( g \) is the conductance, \( g_{\text{max}} \) the maximum conductance, \( V_{m} \) the membrane potential, \( V_{1/2} \) the voltage at which half of the channels are activated, and \( \tau \) the slope factor. Inactivation data at test potentials of −140 to −30 mV (in 5 or 10 mV steps, for 250 ms) were fitted to a Boltzmann equation of the form

\[ I = I_{\text{max}} \frac{1}{1 + \exp \left( \frac{V_{m} - V_{1/2}}{\tau} \right)} \]

where \( I \) is the peak current amplitude, \( I_{\text{max}} \) the maximum peak current amplitude, \( V_{m} \) the membrane potential, \( V_{1/2} \) the voltage at which half of the channels are inactivated, and \( \tau \) the slope factor. Kinetics of NaV1.5 inactivation were fitted to a monoexponential function, from which time constant \( \tau \) (inactivation) between −40 and 30 mV was estimated. NaV1.5 recovery from inactivation data (at recovery times of 250 µs, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 100, 250, 500, 1000 and 1900 ms at −120 mV) were fitted to monoexponential functions to determine recovery time constant \( \tau \). All reported errors are S.E.M.

2.7. Biotin pull-down of cell surface proteins

Biotin pull-down of FLAG-NaV1.5 was performed from transiently transfected HEK cells (n = 2) and from a previously developed [5] cell line stably expressing FLAG-NaV1.5 (n = 2). Cells were transfected with NaV1.5 (1 µg plasmid) together with YFP...
(0.5 µg plasmid), PRMT1, -3 or -5 (1.5 µg plasmid). After 24 h, cells were treated with reactive biotin (Pierce) for 60 min at 4 °C, and lysed. Soluble fractions were incubated with neutravidin (Pierce) overnight at 4 °C. Pull-downs were probed with α-NaV1.5 antibody. Protein bands were quantified using ImageJ (NIH).

2.8. Cardiac AP simulations

We used the ten Tusscher–Panfilov 2006 model [18]. The model was modified to reproduce the observed changes in sodium current density in cells overexpressing PRMT3 or -5. We multiplied the fast sodium current by a factor of \((1 + p)\), where \(p\) was 0.46 or 0.35 (PRMT3-, or -5-cardiomyocytes, respectively). Used protocols were an adaptation of recently reported protocols to simulate cardiac arrhythmias [19].

3. Results

3.1. LI–II is a substrate for Type I and Type II PRMTs

The linker between DI and DII (LI–II) of NaV1.5 is a hot-spot for ArgMe within NaV1.5 [5]. To test an isolated LI-II protein as substrate for PRMTs, we overexpressed LI–II in HEK cells. Antibodies specific to asymmetric, and symmetric, dimethyl arginines recognised LI–II (Fig. 1A), indicating that LI–II was a target for Type I and Type II PRMTs.

To identify the PRMTs that methylate LI–II, we performed in vitro methylation assays using recombinant PRMTs and \(^{3}\text{H-SAM}\). We purified untagged LI–II, from E. coli (Fig. 1B), as substrate. The C-terminal domain of NaV1.5 was included as negative control. We screened all known PRMTs excluding PRMT8, which is specifically expressed in the central nervous system [20], and PRMT9, which has not been characterised [9]. Consistent with our findings in cells, we observed methylation of LI–II by Type I (PRMT1, -3, -4 and -6), and Type II (PRMT5) PRMTs (Fig. 1C). PRMT5 showed weak activity towards LI–II in vitro, but the fact that LI–II was symmetrically dimethylated in cells strongly indicates that PRMT5, the only Type II PRMT [9,10,21], methylates NaV1.5.

The observation that PRMT1 methylates the C-terminal domain was unexpected. Methylated protein bands at lower molecular weight most likely corresponded to degradation products, which might serve as preferred recognition motifs.

3.2. NaV1.5 interacts with PRMT1, -3 and -5

Next, we screened for interactions between full length NaV1.5 and PRMTs. We observed significant FRET efficiency (FRETapp) between NaV1.5–CFP and PRMT1, -3, or -5–YFP, compared to control (YFP) cells, and cells co-expressing PRMT2, -4, and -7 (Fig. 2A and B, and Supplementary Fig. S1). PRMT6 was discarded for later experiments, because it localised exclusively to the cell nucleus (not shown), in agreement with previous reports [16,22–24].

To validate our FRET results, we performed co-IP experiments in HEK cells. After IP of NaV1.5 by FLAG-affinity, we detected co-IP of PRMT1 and -3–YFP (Fig. 2C). PRMT5 was excluded from FLAG co-IP experiments, as it cross-reacts with α-FLAG antibodies [9].

3.3. NaV1.5 cell surface expression is increased by PRMT3 and -5

To address the effect of PRMTs on NaV1.5 electrophysiology, we co-transfected HEK cells with NaV1.5 and PRMTs. We observed increased NaV1.5 current density (in pA/pF) in cells co-expressing PRMT3 (102 ± 10), or PRMT5 (95 ± 9), compared to control cells (70 ± 6), (Fig. 3A–C). NaV1.5 current density in cells co-expressing PRMT1 (77 ± 7) was not significantly higher than in control cells. NaV1.5 voltage-dependent activation and inactivation, recovery from inactivation (Supplementary Table S1), and kinetics of inactivation (Supplementary Fig. S2) were unchanged in all cases.

We hypothesised that the observed increase in NaV1.5 current density was due to higher NaV1.5 cell surface expression. Biotin pull-down experiments revealed ca. 30% increased NaV1.5 cell surface expression in cells co-expressing PRMT3 or -5, compared to control, and PRMT1 cells (Fig. 3, D–F), in reasonable agreement with electrophysiological findings (46% and 35% higher current density in the presence of PRMT3 and -5, respectively).

3.4. Increased NaV1.5 ArgMe changes cardiac AP dynamics in silico

To extend our findings to a cardiac context, we generated computer cardiomyocyte AP simulations. We considered control...
cardiomyocytes, as well as PRMT3- or -5-cardiomyocytes with 46% or 35% (respectively), higher sodium current density. We performed 6000 cycles at 60 beats per minute (bpm), and then increased pacing abruptly to ca. 150 bpm. Under these conditions, control cardiomyocytes led to alternans (Fig. 4), while PRMT3- and -5-cardiomyocytes generated APs at pacing rate, the duration of which was stabilised after 10 stimuli.

4. Discussion

Recently, we have revealed ArgMe as a novel Na\textsubscript{v}1.5 post-translational modification [5]. Now, we present the first systematic analysis of the effect of individual PRMTs on the function of a voltage-gated ion channel, and demonstrate that PRMT3 and -5 increase Na\textsubscript{v}1.5 cell surface expression. Future work will elucidate whether this is due to facilitated trafficking to the plasma membrane (a proposed effect of Na\textsubscript{v}1.5 phosphorylation [3]), or extended residence time at the cell surface (ArgMe by PRMT3 may inhibit ubiquitination and thereby proteolysis of target proteins [25]).

PRMT1 methylated L\textsubscript{L-H}, and interacted with Na\textsubscript{v}1.5 in cells, but did not regulate Na\textsubscript{v}1.5 cell surface expression under our experimental conditions. This may be related to the observation that PRMT1 was the only PRMT that methylated the C-terminal domain

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Fig. 2. Na\textsubscript{v}1.5 interacts with PRMT1, -3 and -5. (A) Representative cells co-expressing Na\textsubscript{v}1.5-CFP and YFP (control), PRMT1–, -3–, or -5–YFP. Shown: transmission image including CFP and YFP fluorescence; CFP and YFP channels at respective excitation and emission; and corrected FRET\textsubscript{app} image. Scale bars (of 40 μm length) represent low (red) to high (blue) FRET\textsubscript{app}; and low (white) to high (black) CFP or YFP intensity. (B) FRET\textsubscript{app} between Na\textsubscript{v}1.5–CFP and PRMT–YFP. Over each bar the number of evaluated cells is indicated. *Significant (P < 0.002 ANOVA test). (C) Representative gel picture (n = 3) before (input), and after IP of Na\textsubscript{v}1.5. PRMT1–YFP (67 kDa) and -3–YFP (86 kDa), but not YFP neither actin, co-IP with Na\textsubscript{v}1.5.
in vitro, however, the individual effect of ArgMe of specific Arg residues on NaV1.5 cell surface expression, needs be described by further work. PRMT2, -4, -6 and -7 are most likely not relevant to full length NaV1.5, and we relate the observed in vitro methylation of LI–II subunit as a substrate for ArgMe.

The observation of an effect on NaV1.5 cell surface expression upon co-expression of PRMT3 and -5 suggests that PRMT concentration is an important factor to regulate levels of NaV1.5 ArgMe. In the human heart, where NaV1.5 is modified by ArgMe [5], expression of NaV1.5, PRMT3 and -5 may be dysregulated e.g. in cardiac disease [26,27], which in turn may affect NaV1.5 ArgMe.
Our simulations suggested that cardiomyocytes with increased NaV1.5 ArgMe sustained action potentials at high pacing when control cardiomyocytes did not. Validation of our data, which have been obtained in accepted (but heterologous) systems, in the cardiac pathophysiology, will further the relevance of NaV1.5 ArgMe.

In conclusion, the observation of NaV1.5 regulation by PRMT3 and -5 contributes to growing evidence that NaV1.5 is modulated by multiple processes [28], continues to expand ArgMe to non-histone proteins [21], and should trigger studies of ArgMe in other members of the voltage-gated ion channel superfamily, which consists of 143 proteins [29].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.043.

References