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$Na_v1.5$ channels can reach the plasma membrane through distinct *N*-glycosylation states

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ABSTRACT

Background: Like many voltage-gated sodium channels, the cardiac isoform $Na_v1.5$ is well known as a glycoprotein which necessarily undergoes *N*-glycosylation processing during its transit to the plasma membrane. In some cardiac disorders, especially the Brugada syndrome (BrS), mutations in $Na_v1.5$ encoding gene lead to intracellular retention and consequently trafficking defect of these proteins. We used two BrS mutants as tools to clarify both $Na_v1.5$ glycosylation states and associated secretory behaviors.

Methods: Patch-clamp recordings and surface biotinylation assays of HEK293T cells expressing wild-type (WT) and/or mutant $Na_v1.5$ proteins were performed to assess the impact of mutant co-expression on the membrane activity and localization of WT channels. Enzymatic deglycosylation assays and brefeldin A (BFA) treatments were also employed to further characterize recombinant and native $Na_v1.5$ maturation.

Results: The present data demonstrate that $Na_v1.5$ channels mainly exist as two differentially glycosylated forms. We reveal that dominant negative effects induced by BrS mutants upon WT channel current result from the abnormal surface expression of the fully-glycosylated forms exclusively. Furthermore, we show that core-glycosylated channels can be found at the surface membrane of BFA-treated or untreated cells, but obviously without generating any sodium current.

Conclusions: Our findings provide evidence that native and recombinant $Na_v1.5$ subunits are expressed as two distinct matured forms. Fully-glycosylated state of $Na_v1.5$ seems to determine its functionality whereas core-glycosylated forms might be transported to the plasma membrane through an unconventional Golgi-independent secretory route.

General significance: This work highlights that *N*-linked glycosylation processing would be critical for $Na_v1.5$ membrane trafficking and function.

1. Introduction

$Na_v1.5$ channels represent one of the nine functionally pore-forming α-subunits ($Na_v1.1$–$Na_v1.9$) constituting the voltage-gated sodium channel (VGSC) family [1]. Each Na_v channel exists as multimeric complexes presumably composed of one α-subunit and one or several regulatory $Na_v\beta$-subunits ($Na_v\beta1$–$Na_v\beta4$) [2,3]. As the predominant cardiac VGSC isoform, $Na_v1.5$ channels play a key role in cardiac excitability and conduction as carrying large inward depolarizing sodium currents responsible for cardiac action potential initiation. During their biosynthetic trafficking, these transmembrane proteins are subject to extensive post-translational modifications like phosphorylations [4–8], methylations [9,10] as well as glycosylations [11–13].

VGSC α isoforms are assumed to be heavily glycosylated proteins containing approximately 20–30% by weight of carbohydrates [14–16]. Interestingly, $Na_v1.5$ channels might be considered as an exception since only 5% of their mature mass could be attributed to *N*-linked oligosaccharides [11]. The post-translational *N*-glycosylation of nascent Na_v channels would be initiated in the endoplasmic reticulum (ER). Sialic acid residues which account for 40% of Na_v complex carbohydrates [15,17] are thought to be subsequently transferred to acceptor oligosaccharides, presumably into the *trans*-Golgi subcompartment.

Previous reports indicated that *N*-glycans and particularly capping sialic acids could modulate the gating of different Na_v channels, including the cardiac isoform [12,13,16,18,19]. However, the presence of these residues does not seem to be not required for their proper membrane

Abbreviations: BFA, Brefeldin A; BrS, Brugada syndrome; Endo H, endoglycosidase H; ER, endoplasmic reticulum; HEK, human embryonic kidney cells; HMW, high-molecular weight; LMW, low-molecular weigh; PNGase, peptide *N*-glycosidase F; VGSC, voltage-gated sodium channel; WT, wild-type

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expression in native cardiac tissues [20]. Intriguingly, the modulation of $Na_v1.7$ membrane expression and resulting sodium current density was shown to be glycosylation-dependent [21]. Moreover, the completion of *N*-glycosylation process would be essential for proper membrane localization of other voltage-gated channels like potassium and more recently calcium channels [22–26]. Thus, studying the impact of *N*-glycosylation modifications in channel folding, trafficking and function allows to refine the understanding of the regulation of membrane excitability.

Several previous studies have already highlighted the existence of a negative dominance observed for $Na_v1.5$ mutants found in Brugada syndrome (BrS) patients over WT channels [27–31]. All findings suggest that this deleterious impact would be correlated with an alteration of wild-type (WT) channel trafficking. However, to date, the underlying causes and the fate of retained WT proteins remain unknown. The present study was initiated to provide a comprehensive understanding of membrane $Na_v1.5$ transport and maturation in the context of physiological or pathological situations such as BrS.

2. Experimental procedures

2.1. Genetic constructs

Co-transfection conditions involved the use of several plasmid vectors encoding WT or mutant $Na_v1.5$ channels and their auxiliary β_1-subunit. The cloning of the full length SCN5A/WT and SCN5A/R1432G into pcDNA vectors was described previously [32]. The cDNA encoding SCN5A/L325R subcloned in the plasmid pcDNA3 was a generous gift of Dr. Hugues Abriel (University of Bern, Bern, Switzerland). The human sodium channel $Na_v\beta1$-subunit and CD8 were constructed in pIRES bicistronic vector (pCD8-IRES-β_1).

2.2. Cell culture, transfection and treatments

Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker) containing 10% fetal bovine serum (Biowest), 100 μg/mL streptomycin and 100 U/mL penicillin (Gibco). In parallel, a stable cell line expressing $Na_v1.5$ (kindly provided by Prof. Mohamed Chahine, Laval University, QC, Canada) was maintained using identical culture conditions.

Plasmid constructs were transiently transfected into HEK293T cells using the calcium phosphate method as detailed in our previous study [31]. For biochemical and patch-clamp experiments, each transfection condition was adjusted to include 2.4 μg of DNA per 60 mm dish with HEK cells at 30% confluence. Transfection conditions were mimicking either monoallelic states (WT/(−), (−)/L325R, (−)/R1432G; with 0.6 μg SCN5A plasmids and 0.6 μg empty vectors) or patient heterozygosity (WT/L325R, WT/R1432G; with equivalent amounts (0.6 μg) of WT and mutant SCN5A constructs).

In indicated experiments and immediately after transfection, HEK293T cells were incubated with 50 to 500 ng/mL brefeldin A (LC Laboratories) or with ethanol vehicle at 37°C for 24 or 48 h before cell lysis or patch-clamp recordings.

For protein glycosylation inhibition, transfected cells were also treated in culture with tunicamycin (Sigma-Aldrich) for 18 h at varying concentrations (0.2, 0.5 or 1 μg/mL).

2.3. Protein extraction

Stably and transiently transfected cells were washed with cold PBS and lysed by scraping the cells into lysis buffer (10 mM Tris, 1% Nonidet P-40, 0.5% deoxycholic acid) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were then incubated for 30 min on ice and centrifuged at 20,000*g* for 5 min at 4 °C.

An alternative protocol was applied for the solubilization of cardiac tissue proteins. Human right atrial biopsies were removed during heart bypass surgery procedures as previously described [33–35]. All procedures were performed in accordance with the Declaration of Helsinki. Each biopsy was cut into small pieces in lysis buffer and then incubated under rotation at 4°C for 2 h. Samples were ground with a tissue grinder, and centrifuged at 250*g* during 5 min to remove tissue fragments. The homogenates were cleared by centrifugation at 20,000*g* for 10 min prior to determine protein contents in the supernatants.

Protein contents were measured using DC protein assay (Biorad) with BSA as a reference. Cleared cell lysates were denatured in 2× sample buffer (126 mM Tris HCl, 20% glycerol, 4% SDS and 0.02% bromophenol blue, pH 6.8) containing 5% 1-thioglycerol (Sigma-Aldrich) for 60 min at 37°C.

2.4. Deglycosylation assays

For deglycosylation experiments, peptide *N*-glycosidase F (PNGase F, cat. G5166, Sigma-Aldrich) and endoglycosidase H (Endo H, cat. 11 088 726 001, Roche Applied science) were used according to the manufacturer's instructions with some modifications.

Total cell lysates from transfected HEK293T cells (125 μg) or from cardiac biopsies (225 μg) were heated at 37°C for 30 min in denaturating phosphate buffer (0.1% SDS, 0.05% β-mercaptoethanol, 7 mM KH_2PO_4, 43 mM Na_2HPO_4, pH 7.5). After cooling on ice, denaturated proteins were supplemented first with 0.15% Triton X-100 and then with either 5 units of PNGase F or equivalent volume of phosphate buffer. Samples were finally incubated at 37°C for 5 h before denaturation step in 2× sample buffer and SDS-PAGE analysis.

For Endo H enzymatic assays, same protein lysate amounts were previously placed in denaturating sodium buffer (0.1% SDS, 0.05% β-mercaptoethanol, 10 mM $C_2H_3NaO_2$, 5 mM EDTA, 0.2% Nonidet P-40, pH 5.5) and then 100 milliunits of Endo H or sodium buffer were added to achieve similar final volumes. Enzymatic reactions were carried out as described for PNGase treatments.

2.5. Cell surface biotinylation

Biotinylation experiments were carried out on transfected HEK293T cells grown at 80–90% confluence in 60-mm dishes.

Forty-eight hours post-transfection, the cells were washed twice with ice-cold PBS (Phosphate Buffered Saline: 137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl and 8 mM Na_2HPO_4, pH 7.4) and once with PBS containing 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 8 (PBSCM). Plates were incubated with 0.5 mg/mL EZ-Link Sulfo-NHS-SS-Biotin (Pierce) freshly diluted in PBSCM for 30 min at 4°C. The biotinylation reaction was ended by washing the cells three times 5 min with PBSCM containing 50 mM glycine and 5 mg/mL BSA. After three PBS washes, cells were scraped in lysis buffer containing protease inhibitor cocktail (Sigma). Cell lysates were then incubated on ice for 30 min and clarified at 20,000*g* for 5 min at 4°C. Biotinylated proteins were isolated by incubating overnight cleared lysates (100 μg of proteins) with 15 μL of streptavidin-agarose resins (Pierce) at 4°C in lysis buffer. Beads were pelleted by centrifugation and washed three times with NET buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA and 0.05% Nonidet P-40, pH 7.4). Biotinylated cell surface proteins were eluted at 37°C for 60 min in 15 μL of 2 × sample buffer containing 5% 1-Thioglycerol (Sigma). Total cell lysates and biotinylated proteins were subjected to SDS-PAGE before immunoblotting analysis as described below.

2.6. Immunoblotting

Proteins from biotinylated samples and cell lysates were separated by SDS-PAGE using 5% polyacrylamide gels and transferred to 0.45 μm nitrocellulose sheets. Membranes were blocked 1 h in TBS-Tween blocking solution (100 mM Tris–HCl, 150 mM NaCl and 0.1% Tween-20, pH 7.6) with 5% nonfat dry milk and then probed overnight at 4 °C with primary antibodies rabbit polyclonal SP19 anti-pan-Na_v (1:1000, Alomone Labs),