N-glycosylation of the voltage-gated sodium channel β2 subunit is required for efficient trafficking of NaV1.5/β2 to the plasma membrane.

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Running title: β2 glycosylation in NaV1.5/β2 trafficking

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Abstract

The voltage-gated sodium channel is critical for cardiomyocyte function and consists of a protein complex comprising a pore-forming α subunit and two associated β subunits. It previously has been shown that the associated β2 subunits promote cell surface expression of the α subunit. The major α isoform in the adult human heart is NaV1.5, and germline mutations in the NaV1.5-encoding gene, sodium voltage-gated channel alpha subunit 5 (SCN5A), often cause inherited arrhythmias. Here, we investigated the mechanisms that regulate β2 trafficking, and how they may determine proper NaV1.5 cell surface localization. Using heterologous expression in polarized Madin-Darby canine kidney (MDCK) cells, we show that β2 is N-glycosylated in vivo and in vitro at residues 42, 66, and 74, becoming sialylated only at Asn-42. We found that fully non-glycosylated β2 was mostly retained in the endoplasmic reticulum, indicating that N-linked glycosylation is required for efficient β2 trafficking to the apical plasma membrane. The non-glycosylated variant reached the cell surface by bypassing the Golgi compartment at a rate of only approximately one-third of that of wild-type β2. YFP-tagged, non-glycosylated β2 displayed mobility kinetics in the plane of the membrane similar to that of wild-type β2. However, it was defective in promoting surface localization of NaV1.5. Interestingly, β2 with a single intact glycosylation site was as effective as the wild-type in promoting NaV1.5 surface localization. In conclusion, our results indicate that N-linked glycosylation of β2 is required for surface localization of NaV1.5, a property that is often defective in inherited cardiac arrhythmias.

Introduction

Genetic alterations leading to channelopathies are frequently found in the voltage-gated sodium (NaV) channel (1). A well-known ion channel disorder causing ventricular fibrillation is Brugada syndrome (BrS). In this regard, ~20% of BrS cases are caused by mutations in SCN5A, the gene encoding NaV1.5, i.e., the pore-forming, α subunit, of the major cardiac NaV channel (2). The NaV channel allows
fast influx of sodium ions, thus generating the rapid upward deflection of the action potential (AP). Therefore, it plays a central role in myocardial cell excitability. The abnormal electrocardiogram observed in BrS is due to Na\textsubscript{V} channel loss-of-function, often caused by defective Na\textsubscript{V}1.5 trafficking and localization to the cell surface (3).

Na\textsubscript{V}1.5 is localized at the sarcolemma, i.e. the cardiomyocytes’ plasma membrane. The differential localization of Na\textsubscript{V} channel pools at sarcolemma subregions is important for conduction velocity and cardiac impulse propagation (4). Large evidence shows that localization and function of the \( \alpha \) subunit are regulated by Na\textsubscript{V} channel auxiliary \( \beta \) subunits and other associated proteins (5). Analysis of Na\textsubscript{V}1.5 trafficking can be envisaged from at least three standpoints; first, to address how Na\textsubscript{V}1.5 is targeted to the plasma membrane; secondly, how Nav1.5 is retained at certain surface domains or subregions; and third, how Na\textsubscript{V}1.5 endocytosis and turnover are regulated. In this work, we mainly focused on the first two aspects, addressing the contribution of one of the associated \( \beta \) subunits. Five \( \beta \) subunits are known in mammals: \( \beta_1, \beta_2, \beta_3, \beta_4, \) and \( \beta_1B \) (the latter is an alternative splice variant of \( \beta_1 \)) (6). Interacting with Na\textsubscript{V}1.5 through their extracellular region (7), or even with their transmembrane domain (TMD) (8), \( \beta \) subunits are thought to assist \( \alpha \) for effective transport to the plasma membrane (3). In fact, various mutations in \( \beta \) subunits have been found associated with BrS, thereby causing loss-of-function of the Na\textsubscript{V} channel (9-12).

We focused here on \( \beta_2 \), whose case is of particular interest, since it is believed to influence Na\textsubscript{V}1.5 localization in post-Golgi compartments, just before, or during its targeting to the cell surface (13,14). In fact, we previously described the first BrS-associated mutation in SCN2B, the gene encoding \( \beta_2 \). Such mutation, D211G (substitution of Asp for Gly), causes a 40 % decrease in sodium current density due to reduced cell surface levels of Nav1.5 (10). Moreover, we have shown that exogenously expressed \( \beta_2 \) is transported in a polarized fashion, namely, to the apical domain in polarized Madin-Darby canine kidney (MDCK) cells. Both in MDCK cells and in cardiomyocyte-derived HL-1 cells, surface localization of Na\textsubscript{V}1.5 was promoted by wild-type (WT) but not D211G \( \beta_2 \) (15).

It is not known how \( \beta_2 \) is targeted to the cell surface and, more specifically, how it preferentially reaches the apical surface in MDCK cells. Indeed, it is subject of intense study to understand how apical targeting signals are recognized. Recognition can take place by association of the protein’s TMD to lipid rafts. It can also occur via N- or O-linked glycosylation of the luminal domain and consequent interaction with sugar-binding galectins. In addition, Ras-related Rab GTPases, microtubule motors and the actin cytoskeleton have been implicated (16,17).

\( \beta_2 \) is a type I transmembrane protein with an extracellular, immunoglobulin-like loop, likely performing a cell adhesion function (5), a single TMD, and a short cytoplasmic tail (18). The extracellular loop, maintained by an intramolecular disulfide bond between Cys-50 and Cys-127 (7), has three potential N-glycosylation sites, i.e. Asn-42, Asn-66 and Asn-74 (19). Within this region, a third cysteine, Cys-55, establishes a disulfide bond with the \( \alpha \) subunit (7). In addition, the short C-terminal intracellular domain has two potential phosphorylation sites, i.e. Ser-192 and Thr-204 (20); see UniProtKB - O60939.

Glycosylation, and more specifically sialylation, appears important for regulating channel biophysical properties. Thus, changes in sodium current density at the plasma membrane have been related with the sialic acid content of \( \beta_2 \) (19). For the \( \beta_1 \) subunit, which interacts non-covalently with \( \alpha \), it has been proposed that its glycosylation level, including its sialylation, may be differentially regulated in a tissue- and developmental-specific manner. Hence, different \( \alpha/\beta_1 \) subunit combinations would be differentially sialylated in various tissues throughout development, thereby contributing, to a different degree, to Na\textsubscript{V} channel gating. Such differences could even be linked to pathological alterations (21). Despite this evidence, to our knowledge, the contribution of \( \beta_2 \) glycosylation on its own trafficking, and importantly, how such posttranslational modification may influence trafficking of the \( \alpha \) subunit, have not been addressed in detail. Here, we found that N-linked glycosylation of \( \beta_2 \) is required for its efficient trafficking to the plasma membrane. Importantly, unglycosylated \( \beta_2 \) was
defective in promoting surface localization of Nav1.5.

Results

β2 is N-glycosylated and sialylated in vitro and in vivo

We previously showed that exogenously expressed β2 localizes almost exclusively at the apical domain in polarized MDCK cells (15). Here, we addressed how β2 is preferentially targeted to this surface domain. Both N- and O-linked glycosylation are common apical sorting signals (16,17). The extracellular domain of β2 has three predicted N-glycosylation sites, i.e. Asn-42, Asn-66 and Asn-74 (18), that follow the Asn-X-Ser/Thr (NxS/T) motif, being x any amino acid except Pro (22). We thus systematically mutated these to Gln, which is never glycosylated due to its different conformation, and transiently expressed YFP-tagged β2 in MDCK cells. Consequently, all mutants showed increased electrophoretic mobility, with N42Q displaying the highest increase, followed by N74Q and N66Q, the latter, with a minor, albeit measurable shift. This variable mobility may be due to different degrees of glycosylation on each site and/or changes on glycoprotein size or charge due to the sugar chain; the triple (fully) unglycosylated mutant showed complete reduction in apparent mass, no longer appearing as a smear, with double mutants migrating in between (Figure 1A). To verify that β2 variants were indeed N-glycosylated, cells were lysed and treated with peptide:N-glycosidase F (PNGase F), which cleaves off the bond between Asn and the first N-acetylglucosamine (GlcNAc) moiety, liberating the entire N-glycan (23). Upon treatment, WT and mutants displayed identical mobility to that of fully unglycosylated β2 (Figure 1B). To confirm that β2 glycosylation takes place in vivo, cells were treated with tunicamycin (TUN), or with benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (Gal-NAc-α-O-benzyl), to block N- or O-glycosylation, respectively. As a result, β2 WT became fully deglycosylated only with TUN, remaining unaffected with Gal-NAc-α-O-benzyl (Figure 1C). These data show that β2 is N-glycosylated in vitro and in vivo, but does not undergo O-glycosylation.

We next investigated the complexity of β2 N-glycosylation with endoglycosidase H (Endo H), which cleaves on high-mannose and hybrid, but not complex glycans, typically generated at late stages of Golgi glycosylation (23). When cells were analyzed early (1 day) after transfection, a faster-migrating band, also visible in single and double mutants, suggested the presence of immature β2-YFP still unprocessed in the endoplasmic reticulum (ER). Endo H treatment effectively increased the mobility of this band, which then coincided with unglycosylated β2, without affecting mature β2 (Figure 2A and Supplementary Figure S1A). Thus, at that moment a considerable fraction of β2 had not yet undergone processing by Golgi α-mannosidase II (23).

To further assess N-glycans complexity, cells were treated with broad-specificity sialidase, i.e. neuraminidase (NA), which cleaves terminal sialic acids from both N- and O-glycans (23). In consequence, the slower-migrating band displayed a noticeable increase in mobility in β2 WT, N66Q and N74Q mutants, but interestingly not in β2 N42Q. Similarly, no effect was seen in double mutants including the N42Q mutation, but it was clear in β2 N66Q / N74Q (Figure 2B and Supplementary Figure S1B). Because all variants with the N42Q mutation were insensitive to NA, we conclude that β2 is sialylated uniquely at Asn-42.

N-glycosylation is required for efficient cell surface localization of β2

Since glycosylation is a well-known mechanism for many proteins to efficiently reach the plasma membrane (16), we tested by protein biotinylation whether partially or fully unglycosylated β2 properly localizes to the cell surface. Uniquely the triple mutant displayed a substantial defect, and band quantitation showed that it reaches the surface at a rate of approximately one-third in comparison with the WT (Figure 3A and 3B). Moreover, the portion of unglycosylated mutant at the surface was around 8 % of total cellular β2 protein, contrasting with 25-30 % by the WT and single or double mutants. A comparable defect was found in fully polarized cells. In these, the rate by which unglycosylated β2 reached the apical surface was also approximately one-third when comparing with β2 WT or the partial mutants (Figure 3C and 3D). To note, all variants of β2
remained nearly undetected at the basolateral surface, or at least clearly de-enriched comparing with lysates (Supplementary Figure S2).

To determine the magnitude of glycosylation loss in trafficking deficiency of β2 overtime, we analyzed its surface levels along various days from transfection. Indeed, the defect was maintained throughout time. Therefore, these data show that total lack of glycosylation significantly prevents β2 localization to the surface (Figure 4). While a single glycosylation site appeared sufficient for proper surface localization of β2, TUN treatment further confirmed that unglycosylated β2 virtually does not reach the plasma membrane in vivo (Figure 3E and 3F).

We next determined in what subcellular compartment trafficking of unglycosylated β2 becomes interrupted. To this end, cells were immunostained for detection of various subcellular markers of the endocytic and exocytic pathways. These included the early endosome (EE) marker EEA1, the late endosomal lysobisphosphatidic acid (LBPA), the lysosome-associated membrane protein LAMP2, the cis-Golgi marker Golgi matrix protein of 130 kDa (GM130), and the trans-Golgi network (TGN) marker TGN46. None of them overlapped markedly with unglycosylated β2 (Supplementary Figure S3). However, an apparent overlap found with the ER chaperone calnexin indicates that a large portion of the triple mutant is retained in the ER membranes. Moreover, its pattern was highly comparable to that of β2 WT in cells treated with TUN (Figure 5A). Indeed, the Manders’ coefficient was around 0.7 in cells expressing unglycosylated β2 and in TUN-treated cells, in contrast with negligible overlap in untreated cells expressing β2 WT (Figure 5B). In the latter, β2 outlined the cell end, also displaying an obvious dotted pattern, which likely corresponds to β2 getting positioned at the developing apical surface, i.e. its final location in polarized cells (15). Because of its preponderant surface localization, no manifested overlap was observed between β2 WT and any of the markers tested (Supplementary Figure S3). Altogether, these data indicate that unglycosylated β2 becomes retained in the ER.

Unglycosylated β2 can reach the cell surface by bypassing the Golgi compartment

Even though unglycosylated β2 was seen retained in the ER, a small fraction reached the cell surface and, in polarized MDCK cells, even properly localized to the apical surface. We therefore tested whether blocking the ER-to-Golgi pathway with brefeldin A (BFA) would analogously prevent arrival of immature β2 to the plasma membrane. Here, transfected cells were treated o/n with BFA and both lysates and pulldowns were then deglycosylated with Endo H. As expected, mature, fully glycosylated β2 WT was not visible in cells treated with BFA, confirming lack of processing by Golgi enzymes (Figure 6A). Upon Endo H treatment, the faster-migrating band (immature β2) increased its mobility, coinciding with unglycosylated β2 (Figure 6A; see Figure 2A for comparison). Remarkably, this immature form was the only constituent of pulldowns from BFA-treated cells, indicating that it can reach the plasma membrane by bypassing Golgi glycosylation. Subsequently, pulldowns were also treated with Endo H, which again shifted a small fraction of immature β2 to the position of (faster-moving) unglycosylated β2 (Figure 6A).

Albeit to a lesser extent, β2 WT was still detected in pulldowns of BFA-treated cells. However, quantitation of western blots indicated that, similarly to unglycosylated β2, the ratio by which immature β2 can reach the cell surface in BFA-treated cells is only approximately one-third as compared with untreated cells (Figure 6B). Proper validation that the drug produced β2 accumulation in the ER was seen by its considerable overlap with calnexin (Figure 6D), whose pattern clearly differed from that of the cis-Golgi marker GM130, which became more tubulated and disperse in the presence of BFA (see also Supplementary Figure S4A). Thus, a large portion of β2 WT now appeared accumulated in enlarged calnexin-positive structures, often undistinguishable from buildup of unglycosylated β2 (arrowheads in Figure 6D). Moreover, in untreated cells expressing low levels of unglycosylated β2, the mutated protein largely overlapped with calnexin, further confirming its retention in the ER (Supplementary Figure S4B).
Dynamics of β2 in the plane of the membrane is not influenced by N-glycosylation

The data above provide strong evidence that N-glycosylation is required for β2 to reach the plasma membrane. It is plausible to contemplate that N-glycans ensure correct folding and oligomerization of β2 to exit the ER properly. Glycosylation may favor β2 clustering at the TGN, which in turn may increase affinity to lipid rafts, for subsequent inclusion into apical transport carriers (24). Thus, we hypothesized that β2 dynamics in the plane of the membrane may be influenced by its glycosylation, which could have important functional implications. Movement of fluorescently tagged β2 was monitored by fluorescence recovery after photobleaching (FRAP). The mobile fraction (MF), that is, the portion of molecules undergoing diffusion, differed depending on the cell’s location where the measurement was taken. Hence, we chose three representative regions for analysis, i.e. at the cell end, mostly representing cell surface β2 (Figure 7A); within the cytoplasm matrix, likely including the dispersed ER network as well as clusters of β2 already at the surface (Figure 7B); and in vesicular structures of unknown nature, which may represent large perinuclear ER elements with β2 in transit to the cell surface (Figure 7C). At the cell end, approximately 60 % of β2 WT molecules underwent diffusion 4-5 min after bleaching. MF at the cell end was slightly increased for unglycosylated β2, yet differences were not significant (Figure 7A and Supplementary Table S1). Similar data were found for cytoplasmic β2 (Figure 7B). However, when β2 found in large vesicles was bleached, mean fluorescence never recovered above 10 % of the total initial signal (Figure 7C). These differences in the portion of freely diffusible molecules suggest that the molecular environment of β2 seemingly accumulated in these large vesicles differed from that present in the other areas analyzed.

The FRAP data also showed that the mobility rate of WT and unglycosylated β2 is comparable, with a slight tendency of the mutant to move slower. Regardless of the location, half-time of recovery (τ/2, the time-point of half fluorescence recovery) was approximately 1 min in both β2 variants (Supplementary Table S1). Consequently, a diffusion coefficient (D) of ≈ 0.02 μm²/s was found in general, although β2 in large vesicles moved even slower, i.e. at ≈ one fourth of this speed (see Experimental procedures).

Unglycosylated β2 is defective in promoting surface localization of Naᵥ1.5

The comparable mobility of both WT and unglycosylated mutant β2 indicates that the sugar moiety does not influence β2 dynamics within the membrane bilayer. It is accepted that β subunits function in concert with the α subunit to promote channel trafficking to the plasma membrane, and in some cases to modulate its biophysical properties (5). In this regard, it has been shown that the major function of β2 in vivo is to chaperone α subunits to the plasma membrane, both in the heart ventricle (25) and in neurons (26). Therefore, we tested whether unglycosylated β2 was defective in promoting surface localization of Naᵥ1.5. As expected (15), a fraction of Naᵥ1.5 colocalized with β2 and the apical marker gp114 (Figure 8A). Even though Naᵥ1.5 distributed throughout the cell, calculation of the corrected total cell fluorescence (CTCF) along z-stacks showed its maximum fluorescence peak nearly overlapping to those of gp114 and β2, corresponding to the apical plasma membrane (Figure 8C). In the presence of unglycosylated β2, Naᵥ1.5 distribution was more widespread, mostly abounding at the nuclear level and right above the nucleus (Figure 8B and 8D). Moreover, a large portion of Naᵥ1.5 colocalized with accumulations of mutated β2 (arrowhead in Figure 8B).

By biochemical means, a small portion of Naᵥ1.5 can be effectively detected at the cell surface of MDCK cells in the presence of β2 (15). We thus biotinylated surface proteins to detect in pulldowns Naᵥ1.5, whose levels were visibly reduced in cells expressing unglycosylated β2 (Figure 8E and 8F), thus supporting the data obtained by immunofluorescence.

Since we could measure the presence of Naᵥ1.5 at the surface by biotinylation, we then wished to determine the magnitude of this defect over time. To this end, we first analyzed β2 function in promoting Naᵥ1.5 arrival to the surface early from transfection. We thus performed this analysis in cells growing non-polarized in wells. Here, we took advantage of our approach to quantify relative fluorescence
levels, i.e., mean fluorescence intensity (MFI), along a segment drawn from the cell end perpendicularly into the cytoplasm; by means of confocal microscopy, the cell end taken is a close approximation of the plasma membrane region (15). As expected, localization of Na$_{\text{v}}$1.5 to the plasma membrane was not promoted by unglycosylated β2 throughout time, and the bulk of Na$_{\text{v}}$1.5 label remained intracellular (Figure 9C and 9D), similarly as in cells not expressing β2 (Figure 9E and 9F). In contrast, the MFI of Na$_{\text{v}}$1.5 was concentrated at the cell end in the presence of β2 WT, also in parallel with Na/K-ATPase, especially at day 1, displaying a more widespread distribution at days 2 and 3 (Figure 9A and 9B); a general defect in promoting surface localization of Na$_{\text{v}}$1.5 at late time points was also verified by cell surface biotinylation, by which all β2 variants were ineffective, including the WT (Supplementary Figure S5).

We have shown that a single intact glycosylation site in β2 is sufficient for its proper surface localization (see Figure 3 and Figure 4). Now, we asked whether incomplete glycosylation would affect β2 in promoting surface localization of Na$_{\text{v}}$1.5. Interestingly, partial loss of glycosylation still allowed a positive effect, namely, only fully unglycosylated β2 is clearly defective in promoting surface localization of Na$_{\text{v}}$1.5. Thus, we found that single β2 mutants maintain effectiveness at day 1 from transfection (Figure 10), which we also verified by cell surface biotinylation (Figure 11 G-H). By biochemical means, we also observed a comparable behavior in double mutants, appearing similarly effective as the WT in promoting surface localization of Na$_{\text{v}}$1.5 (Figure 11 I-J). Moreover, single mutants were also effective to promote apical localization of Na$_{\text{v}}$1.5 in cells growing polarized in Transwells (Figure 11 A-F; compare with Figure 8 A-D).

In summary, glycosylation is required for β2 to reach efficiently the plasma membrane and is important for β2 to promote surface localization of Na$_{\text{v}}$1.5.

**Discussion**

In this work, we analyzed the mechanisms regulating β2 trafficking, and how this may be determinant for proper localization at the cell surface of Na$_{\text{v}}$1.5, the major cardiac Na$_{\text{v}}$ channel. We show that β2 is N-glycosylated in vivo and in vitro at residues 42, 66 and 74, becoming sialylated only at Asn-42, and that glycosylation is required for its efficient trafficking to the plasma membrane. We found that a comparatively small fraction of the fully unglycosylated mutant can reach the cell surface by bypassing the Golgi compartment, in fact, at only one-third the rate of the WT. In addition, it was defective in promoting surface localization of Na$_{\text{v}}$1.5. We therefore propose that N-linked glycosylation of β2 is required for Na$_{\text{v}}$1.5 trafficking to the surface.

Na$_{\text{v}}$1.5 is often mislocalized in inherited channelopathies triggering cardiac arrhythmias. Defective trafficking is often responsible (27), although proper organization of macromolecular complexes is also important (28). In addition, association with adaptor proteins should ensure proper sorting, targeting, anchoring, and stabilization of the channel complex to certain plasma membrane subdomains (29). Such proteins may include auxiliary β subunits. In this regard, β2 association with the α subunit – at least in neurons – is important for proper targeting and subcellular localization of the α/β complex (5,7).

By confocal microscopy and protein biotinylation, we observed that unglycosylated β2 was clearly defective in shifting the localization of Na$_{\text{v}}$1.5 from the ER to the cell surface. In fact, a considerable portion of both proteins appeared stuck in the ER; to avoid excessive β2 levels due to overexpression, in most of these experiments we used cells stably expressing β2-YFP in moderate levels transiently transfected to express Na$_{\text{v}}$1.5-FLAG. Virtually all exogenously expressed Na$_{\text{v}}$1.5 actually remains intracellular in MDCK cells, a large portion being in the ER. This fits with the classic idea that the ER may serve as a reservoir for cardiac (14) and neuronal Na$_{\text{v}}$ channels, generating a pool potentially essential to regulate export of the α/β complex to appropriate surface locations (13). Yet, even in MDCK cells, β2 can promote Na$_{\text{v}}$1.5 localization to the apical surface (15). The fully unglycosylated mutant was however defective, and therefore lacked the most relevant function described for the β2 subunit to date, at least within the context of the Na$_{\text{v}}$ channel (5,25). Remarkably, a single glycosylation site in β2 was sufficient to allow its trafficking to the apical surface and to promote surface localization of Na$_{\text{v}}$1.5.
The implication of β subunits in promoting trafficking of the α subunit to the plasma membrane is a common statement seen in the literature (5,30,31). For β2 in particular, it has long been believed that covalent assembly of α/β2 takes place right before their arrival to the plasma membrane (13), or at least after the subunits have left the Golgi apparatus (14). This is consistent with data from Scn2b deletion in mice, which causes, both in ventricular myocytes (25) and in primary hippocampal neuron cultures (26), approximately a 40% reduction of α subunits at the cell surface. Interestingly, β2 must associate with the α subunit for its targeting to nodes of Ranvier and to the axon initial segment (7). A similar scenario is seen for β4 (32). While it was concluded that trafficking to the plasma membrane of β1, but not β2, is altered by the α subunit (14), association of β2 to α actually determines β2 targeting to specialized neuron domains (7).

Regarding proper subcellular localization, this evidence may lead us to question whether β acts on the α subunit, or vice versa. Our data seem consistent with the notion that β2 plays an important role in ensuring efficient surface localization of Na\textsubscript{v}1.5; this process is seen only defective when β2 remains mainly retained in the ER as a result of no glycosylation. The data from the present work thus challenge the view that β2 acts on Na\textsubscript{v}1.5 at a later stage, such as at the cell surface, or in a post-Golgi compartment, as we also proposed previously (15). Indeed, the unglycosylated mutant seemed to drag along a large portion of Na\textsubscript{v}1.5 to intracellular compartments, likely in the very ER. According to this observation, it is plausible that the β2 mutant causes Na\textsubscript{v}1.5 retention early in the secretory pathway in an attempt to chaperone it for proper folding on its way to the cell surface.

A minor fraction of unglycosylated β2, estimated to be no more than 10%, was detected at the cell surface, contrasting with 25-30% of the WT; this reduction to approximately one-third of the rate by β2 WT was similarly seen at the apical domain of polarized cells. By blocking ER-to-Golgi transport with the fungal drug BFA, we demonstrated that even immature β2 WT, which is Endo H sensitive, could be detected at the plasma membrane. Based on this result, the most likely explanation by which a small fraction of unglycosylated β2 is detected at the cell surface is that it did so also by bypassing the Golgi compartment. At least for Na\textsubscript{v}1.5, there is evidence that the immature protein may follow such Golgi-independent, secretory pathway. For Na\textsubscript{v}1.5, the role of this alternative anterograde pathway is not clear, although it was proposed to be potentially useful for clearance of accumulating proteins in the ER as a constitutive response to relieve or prevent ER stress (33). In fact, it has been shown that a fraction of Na\textsubscript{v}1.5 remains Endo H sensitive and associates with K\textsubscript{v}2.1, the α subunit of the inward rectifying potassium channel, early in their biosynthetic pathway (34). In this regard, it has been hypothesized that Na\textsubscript{v}1.5 mutants described in BrS patients that are retained in the ER may still be delivered to the plasma membrane via an unconventional pathway (35).

We found that YFP-tagged, unglycosylated β2 displays similar kinetics of mobility in the plane of the membrane as β2 WT. Thus, N-glycosylation does not influence its lateral mobility as well as interactions with proteins and lipids within and across the membrane. Taking into account the relatively low D of around 0.02 µm\textsuperscript{2}/s, or even less, it would be conceivable to consider that β2 diffuses inside lipid rafts, fitting with reported D ≤ 0.05 µm\textsuperscript{2}/s (36). Yet, a considerably lower D would also agree with the possibility that the protein is tethered to cytoskeleton elements underlying the membrane (37).

We previously showed that proper localization of Na\textsubscript{v}1.5 to the cell surface is defective in the presence of β2 D211G (15), a missense mutation associated with BrS (10). In MDCK cells, β2 localizes in a polarized fashion, seen almost exclusively at the apical surface (15). In the present work, we found that fully unglycosylated β2 poorly reaches the apical plasma membrane. This agrees with previous data showing that N-glycans are required for polarized distribution of many apical membrane proteins in epithelia (24). As the WT, β2 D211G effectively localizes to the apical surface of MDCK cells, and similarly, to the plasma membrane of atria-derived HL-1 cells (15). Therefore, its defective action on Na\textsubscript{v}1.5 must be different from what we observed here for the fully unglycosylated mutant, and may be related to a potential effect on posttranslational modifications, such as phosphorylation of the
intracellular domain, as we suggested (15). However, previous work in heterologous systems actually showed that the cytoplasmic domain of β subunits does not have much influence on α/β interaction. Thus, a β1 chimera bearing the intracellular domain of β2 overlapped strongly with Na\textsubscript{V}1.5, supposedly in intracellular compartments (38), similarly as β1 does, but in contrast to β2 (14).

In summary, we found that β2 N-glycosylation is required for its efficient trafficking to the plasma membrane, although a small fraction of fully unglycosylated β2 can reach the cell surface by bypassing the Golgi compartment. Importantly, this mutant was defective in promoting surface localization of Na\textsubscript{V}1.5. These findings add to a better understanding of β2 function, which appears primarily relevant for proper Na\textsubscript{V}1.5 localization, thereby influencing cell excitability and electrical coupling in the heart, and in turn contributing to an improved knowledge on how arrhythmias develop.

**Experimental procedures**

**Plasmid vectors, cDNA cloning, and site directed mutagenesis**

The vector containing SCN2B-yfp, to express β2 with YFP fused to its C-terminus, has been described (15). Following the manufacturer’s instructions, the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) was used to change Asn for Gln at predicted N-glycosylation sites (22), thus preventing potential N-glycosylation of the expressed protein. Human SCN2B (designated in the Consensus Coding Sequence database (CCDS) with ID 8390.1), containing the desired mutation, was used as a template. Complementary primer pairs for PCR were designed with the QuikChange® Primer Design Program (Agilent) and synthetized by Metabion International AG. Sequences were sense: 5'-CCTTCAACGTCTTCAGGGCTCTGACGCC CG-3’ and antisense: 5'-CGGGCCGTACAGGCGCTTGGAGACGTGTA GGG-3’ (N42Q); sense: 5'-CACAAACAAGTTCCTCCCTGCACTGGACCTT ACCAGGAGTC-3’ and antisense: 5'-GCACTTCTGGTAAGTCCACGTGCAGGAG AACTTTTTGTTG-3’ (N66Q); sense: 5'-ACTTACCAGGAGTCAACACAGTGCCTCTG AGGAGATGTTC-3’ and antisense: 5'-GAACATCTCCTCAGAGCAGCATGGTTGCAC TCCTGGTAAAGT-3’ (N74Q). (Mutated bases are marked in bold and underlined.) All possible combinations of mutant β2 were generated: N42Q, N66Q, N74Q, N42Q / N66Q, N42Q / N74Q, N66Q / N74Q, and N42Q / N66Q / N74Q. The constructs were then verified by sequencing.

The FLAG-tagged human SCN5A cloned in pcDNA3.1 has been described; the tag is located in the extracellular loop (right after Pro154) between segments S1 and S2 of domain I (39).

**Cell culture and transient transfection**

MDCK cells II and transfectant derivatives were maintained in Minimum Essential Medium with Earl’s salts (MEME). To generate a fully polarized monolayer, cells were grown on polycarbonate Transwell filters of 12 mm diameter and 0.4 μm of pore size for at least 3 days (Corning-Costar), as described (40); the medium was supplemented with GlutaMAX™ (Gibco). Transfections were performed according to the manufacturer’s instructions. Cells to be split for transfection had been grown o/n until subconfluence. 400,000 cells/Transwell, 350,000 cells/22-mm well (12-well plates), or 1.2x10^6 cells/35-mm well (6-well plates), were seeded and immediately (co-)transfected – in suspension – with vector(s) to (co-)express Na\textsubscript{V}1.5, β2 and/or GFP, using Lipofectamine® 2000, at 1 μl reagent/μg DNA, in Gibco Opti-MEM™ I reduced-serum medium (Invitrogen). 2 μg SCN2B-yfp vector were used per transfection in Transwells and 22-mm wells, and 4 μg in 35-mm wells; 6.5 μg SCN5A-FLAG vector were transfected into β2 stable cells in Transwells; and 2 μg of the latter plus 3 μg SCN2B-yfp vector were transfected into cells in 35-mm wells. In cotransfections of Na\textsubscript{V}1.5 and β2, the pEGFP-N1 vector (Clontech) was used as a control for β2-YFP.

For experiments of Fluorescence recovery after photobleaching (FRAP), 180,000 cells were seeded in ibidi® µ-slides (with 4 wells Ph+ and a glass bottom) and transfected as above with 1.5 μg SCN2B-yfp vector.

**Generation of stable cell lines**

Transfections were performed by calcium phosphate coprecipitation, as described
(41), and single-cell clones then selected with 200 μg/ml G418 (Sigma). Positive clones for WT and unglycosylated β2-YFP mutants were identified visually using the appropriate filter under a fluorescence microscope, and then confirmed by anti-β2 western blot. Proper distribution of surface markers (gp114, apical; and p58, basolateral) and tight junctions (ZO-1) was then verified by immunofluorescence, ensuring normal cell polarity. The cell line expressing NaV1.5-YFP has been described (15).

**Pharmacological inhibition of glycosylation**

To block N-linked protein glycosylation, cells were treated with tunicamycin (TUN; Sigma T7765). TUN inhibits the initial events in glycosylation of Asn residues, resulting in the synthesis of totally unglycosylated proteins. TUN was first dissolved at 10 mg/ml in DMSO. Cells were treated 2 h after transfection with 0.3 µg/ml TUN for 24 h in complete medium; in untreated samples, an equivalent volume of solvent was added (0.003 %).

To inhibit O-linked protein glycosylation, cells were treated with benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (Gal-NAc-α-O-benzyl; Sigma B4894), a competitive inhibitor of O-glycan chain extension (42). Gal-NAc-α-O-benzyl was first dissolved at 100 mg/ml in DMSO. Cells were treated ~ 2 h after transfection with 2 mM Gal-NAc-α-O-benzyl for 24 h in complete medium; in untreated samples, the equivalent volume of solvent was added (0.6 %).

**Treatment with brefeldin A (BFA)**

To block transport from the ER to the Golgi, cells were treated with the fungal drug brefeldin A (BFA; Thermo Fisher Scientific 00-4506-51). BFA reversibly inhibits a GTPase activity necessary for coat formation on Golgi membranes, which ultimately induces a redistribution of Golgi components to the ER (43). Cells were treated ~ 2 h after transfection with 1.5 µg/ml BFA o/n in Opti-MEM (BFA was purchased already dissolved at 3 mg/ml in methanol); in untreated samples, an equivalent volume of solvent was added (0.05 %).

**In vitro deglycosylation**

Deglycosylation was performed in whole cell lysates. Reactions were stopped with Laemmli buffer. To remove completely N-glycans, we used peptide:N-glycosidase F (PNGase F; NEB P0708), which cleaves off the bond between Asn and the first N-acetylglucosamine (GlcNAc) moiety, liberating the entire N-glycan. The protocol by New England Biolabs (NEB) was used. Briefly, 10 µg protein were denatured for 10 min at 100°C in 10 µl Glycoprotein Denaturing Buffer (0.5 % SDS with 40 mM DTT). The reaction with 1 µl PNGase F (500 units) was then performed in 20 µl total volume, including GlycoBuffer 2 (50 mM sodium phosphate at pH 7.5) and containing 1 % Nonidet P-40 (NP-40), by o/n incubation at 37°C.

To discern between simple and complex N-glycosylation, we used endoglycosidase H (Endo H; NEB P0702), which cleaves N-glycans between the two GlcNAc moieties in the core region of the glycan chain on high-mannose and hybrid, but not complex, glycans. Similarly, 7.5 µg protein were denatured for 10 min at 100°C in 10 µl Glycoprotein Denaturing Buffer. The reaction with 1 µl Endo H (500 units) was then performed in 20 µl total volume, including GlycoBuffer 3 (50 mM sodium acetate at pH 6), by o/n incubation at 37°C.

To cleave terminal sialic acids, from N- and O-glycans, we used α2-3,6,8 neuraminidase (NA; NEB P0720), which hydrolyzes α2-3, α2-6, and α2-8-linked sialic acid residues from glycoproteins and oligosaccharides. Here, 2 µl NA (100 units) were added to 3.5 µg protein in GlycoBuffer 1 (5 mM CaCl2 in 50 mM sodium acetate at pH 5.5) and incubated o/n at 37°C. To ensure proper visibility in gels with samples from double mutants, twice the amount of protein and enzyme were used in digestions with Endo H and NA.

In experiments addressing the effect of BFA, material obtained by surface protein biotinylation (see below) was also digested with Endo H. Here, o/n NeutrAvidin pulldowns were resuspended in 20 µl Glycoprotein Denaturing Buffer and denatured as above to release the protein from beads. Beads were then spun down and 10 µl of supernatant was deglycosylated in GlycoBuffer 3 as above using 3-times as much enzyme.

**Antibodies**

Some antibodies were provided by other researchers, including the mouse monoclonal antibodies to gp114 (a cell adhesion molecule)
and to p58 (the Na/K-ATPase β subunit) (44), as well as the rat monoclonal antibody against ZO-1 (45). The following are commercially available mouse monoclonal antibodies: to the early endosome (EE) marker EEA1 and the Golgi matrix protein of 130 kDa (GM130) (BD Transduction Laboratories 610457 and 610822, respectively); and to the Na/K-ATPase α1 subunit and the trans-Golgi network (TGN) marker TGN46 (Abcam ab7671 and ab50595, respectively). Commercial rabbit polyclonal antibodies used were, from Alomone, ASC-013 to NaV1.5, and ASC-007 to anti-β2; from Abcam, anti-GFP (ab290) and anti-calnexin (ab75801); and from Sigma, anti-actin (A 2066). Secondary antibodies HRP-conjugated for western blot were from Jackson ImmunoResearch (codes 111-035-003 and 115-035-003), and Alexa Fluor®-labeled for immunofluorescence from Molecular Probes-Invitrogen (codes A11012 and A21050).

Sample preparation for western blot

Protein determination from cell lysates and preparation of samples for SDS-PAGE were done as previously (15,46), with the following remarks in samples analyzing NaV1.5. These were prepared in Laemmli buffer by heating at 70ºC for 10 min, and protein transferring to PVDF membranes was done for 30 h in the presence of 0.01 % SDS to optimize NaV1.5 solubilization and transfer.

Cell surface biotinylation

Surface protein biotinylation was done with EZ-Link™ Sulfo-NHS-SS-Biotin (Pierce 21331), a water-soluble and membrane impermeable reagent. The procedure followed has been described previously in detail (15,46). Unless otherwise specified, nine tenths of cell lysate was subjected to o/n pulldown with NeutrAvidin (Pierce 53150) and analyzed by western blot along with the remaining 10 % (referred to as lysate). Quantitation of blotted protein bands in lysates and pulldowns was performed as described (15) using the ImageJ program.

Confocal immunofluorescence microscopy and quantitative image analysis

MDCK cells were analyzed at subconfluence on glass coverslips or grown polarized in 12 mm Transwells. Cells were fixed with paraformaldehyde and immunostained, essentially as described (15,46).

High magnification images were taken on a Nikon A1R confocal microscope at a minimum pixel resolution of 1,024 x 1,024 using the NIS-Elements AR software, as described (47). Images were exported to TIFF format and 3D colocalization was done without image preprocessing using Fiji, the ImageJ-based package that includes the JACoP plugin for colocalization analysis. Manders’ colocalization coefficients were then calculated along apical-to-basal z-stacks to estimate the fraction of β2 present in compartments positive for a given subcellular marker, as described (15).

To measure cell fluorescence along z-stacks (optical slice thickness of 0.5 μm), confocal images were taken at 512 x 512 pixel resolution. As previously (47), we calculated along 3D reconstructions the corrected total cell fluorescence (CTCF), which integrates fluorescence intensity and area. In non-polarized cells, to measure relative fluorescence levels from the plasma membrane into the cytoplasm, we calculated for each channel the mean fluorescence intensity (MFI), which shows the percentage of fluorescence intensity/pixel over the pixel with maximum intensity, as described (15).

Fluorescence recovery after photobleaching (FRAP)

MDCK cells were transiently transfected and grown subconfluent (2 days) on ibidi® glass supports. Cells were placed in a live-cell imaging chamber at 37°C and 5 % CO₂, and imaged through a water-immersion objective (Plan-Apo 60X, 1.2 NA) on a Nikon A1R confocal microscope. Confocal images were taken at 512 x 512 pixel resolution. An argon laser with emission at 514 nm was used to image the YFP fluorescence and a 405 nm diode laser was used for photobleaching. The pinhole radius was set to 3 Airy Units, except when imaging perinuclear endoplasmic reticulum (ER) structures, when the pinhole was set to 1 Airy Unit. 3 regions-of-interest were drawn: a bleached area, in which fluorescence recovery was recorded along time; a background area, outside obvious fluorescence labeling; and a non-bleached (reference) area, in a different cell displaying similar fluorescence intensity as the
bleached cell. Both bleached and reference areas were circular regions with a nominal radius \( (r_n) \) of 2 μm, except when imaging perinuclear ER structures, where \( r_n \) was 1 μm.

Images were collected at a rate of 1 frame per second, as follows. First, 10 pre-bleaching images were taken, and then bleaching was done for 5 seconds at 100 % laser transmission. Immediately, post-bleaching images were captured until fluorescence recovery reached a plateau. Similarly as described (48), we used the NIS-Elements AR software to measure average fluorescence intensities and to correct for background and acquisition photobleaching, taking into account background and reference fluorescence values, respectively.

Next, data were normalized as follows. First, the lowest fluorescence value, obtained from the first post-bleaching recording, was subtracted from each time point value to set bleach depth to zero. Then, all values were divided by the value from the last pre-bleaching \( (10^{th}) \) frame, i.e. right before photobleaching. From each curve, we then obtained three parameters: [1] the mobile fraction (MF), determined by averaging the fluorescence values of the first 30 time-points throughout which the curve reaches a plateau (30 seconds), and expressing this value as a percentage of the maximum fluorescence at pre-bleaching, indicates the portion of molecules that can undergo diffusion during the experiment; [2] the half-time of recovery \( (\tau_{1/2}) \), i.e. the time-point in which half of total fluorescence recovery has occurred (this value inversely correlates to the rate of diffusion, and therefore to the speed of molecule movement in the area analyzed); and [3] the diffusion coefficient \( (D) \), indicating rate of diffusion, and calculated applying the simplified Soumpasis equation (49):

\[
D = 0.25 \cdot \left( \frac{r_n^2}{\tau_{1/2}} \right)
\]

Statistics
All experiments were performed a minimum of 3 times. Data are expressed as mean ± SD, as indicated in figure legends, and displayed as curves or bar graphs superimposed to scatter plots showing all the individual data points. Statistical significance was calculated by the two-tailed Student’s \( t \)-test, or by one-way ANOVA with Tukey’s honest significant difference (HSD) post hoc test by using the R software for statistical computing (50), when differences among groups needed to be tested. \( P \) values are also specified in figure legends.

Author contributions
E.C. carried out the experimental work, and contributed in designing the work and writing up the manuscript. R.B. gave advice and provided financial support to carry out the project. M.V. conceived the project, designed the work, supervised the experiments, and wrote the manuscript.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.
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**Abbreviations**

BrS, Brugada Syndrome; CTCF, corrected total cell fluorescence; D, diffusion coefficient; DAPI, 4’,6-diamidino-2-phenylindole; EE, early endosome; Endo H, endoglycosidase H; ER, endoplasmic reticulum; FRAP, fluorescence recovery after photobleaching; GalNAc-O-bn, benzyl-2-acetamido-2-deoxy-a-D-galactopyranoside; GlcNAc, N-acetylglucosamine; GM130, Golgi matrix protein of 130 kDa; τ_{1/2}, half-time of recovery; HSD, honest significant difference; LBPA, lysobisphosphatidic acid; LAMP, lysosomal-associated membrane protein; MDCK, Madin-Darby canine kidney; MF, mobile fraction; MFI, mean fluorescence intensity; NA, α2-3,6,8 neuraminidase; Na\(_v\), voltage-gated sodium; \(r_n\), nominal radius; PNGase F, peptide:N-glycosidase F; TGN, trans-Golgi network; TMD, transmembrane domain; TUN, tunicamycin; WT, wild-type.
**Figures**

**Figure 1. β2 is N-glycosylated at positions Asn-42, Asn-66 and Asn-74.**

MDCK cells were transiently transfected with the SCN2B-yfp vector to express WT, partially, or fully unglycosylated β2, or left untransfected (utf). (A, B) Cells were grown for 2 days in wells. Representative western blots are shown with the same amount of protein lysate loaded into each lane. (A) All glycosylation-defective mutants display increased electrophoretic mobility, with N42Q as the single mutant with the greatest change, and triple (fully) unglycosylated β2 showing complete shift. (B) Denatured protein from cell lysates was treated o/n at 37°C with PNGase F to cleave off all N-glycans. (C) Cells were treated with TUN, or with GalNAc-O-bn, to block N- or O-glycosylation, respectively, and grown for 1 day in wells; β2 WT remains unglycosylated only with TUN. DMSO: indicates cells with the equivalent volume of solvent added, and "-", untreated cells. Blots for Na/K-ATPase or actin are included as loading controls. Molecular weight markers are in kilodaltons (kDa).
Figure 2. β2 undergoes complex N-glycosylation and is sialylated at Asn-42.

MDCK cells were transiently transfected with the SCN2B-yfp vector to express WT, partially, or fully unglycosylated β2, and grown for 1 day in wells. Representative western blots are shown with the same amount of protein lysate loaded into each lane. Note that immature (unprocessed) β2 is clearly discernible from the slow-migrating mature form (compare with Figure 1). (A) Denatured protein from cell lysates was treated o/n at 37°C with Endo H to cleave off immature N-glycans (faster-migrating band) in β2. (B) Lysates were treated o/n at 37°C with NA to cleave off all terminal sialic acids. The upper band displays a slight increase in mobility in WT and single and double mutants not including the N42Q mutation (red fonts). Blots for actin are included as loading controls. Molecular weight markers are in kilodaltons (kDa). The division line in B separates different blots (taken from the same exposure) conveniently put together for clear display.
Figure 3. N-glycosylation is required for efficient cell surface localization of β2.

MDCK cells were transiently transfected with the SCN2B-yfp vector to express WT, partially, or fully unglycosylated β2. (A, B) Cells were grown for 2 days in wells, or (C, D) polarized in Transwells, and surface biotinylated at 4ºC. (A, C) Representative western blots and (B, D) band quantitation show that levels of fully unglycosylated β2 were reduced in comparison with the WT and partially glycosylated mutants in biotin-NeutrAvidin pulldowns, both in subconfluent and in polarized cells. One-way ANOVA with Tukey’s HSD post hoc test highlighted these differences (B: *P < 0.001; D: *P < 0.05). (A, C) Values underneath blots show the percentage of each β2 variant at the surface over total cellular β2 protein. (E, F) Cells were treated with TUN to block N-glycosylation, grown for 1 day in wells, and surface biotinylated at 4ºC (UT, untreated cells). (E) Representative western blots and (F) band quantitation show absence of unglycosylated β2 in pulldowns (two-tailed Student’s t-test shows significant differences; *P = 0.009). The same amount of protein was used to process each lysate (≈ 130 μg), and the corresponding portion (nine tenths) was subjected to overnight pulldown. Na/K-ATPase (B, E) or gp114 (C) were blotted as surface markers to correct for quantitations in pulldowns. All data are mean ± SD (n ≥ 3). Molecular weight markers are in
kilodaltons (kDa). The division line in E separates different parts of the same blot (taken from the same exposure) conveniently put together for clear display.
Figure 4. Defect over time in β2 surface localization due to lack of N-glycosylation.

MDCK cells were transiently transfected with the SCN2B-yfp vector to express WT, partially, or fully unglycosylated β2. Cells were grown in wells for the indicated number of days and surface biotinylated at 4°C. (A) Representative western blots and (B-E) band quantitation show that levels of fully unglycosylated β2 were reduced in comparison with the WT and partially glycosylated mutants in biotin-NeutrAvidin pulldowns (Membrane). One-way ANOVA with Tukey’s HSD post hoc test highlighted these differences, with a few exceptions (B: all $P < 0.05$, except 42 vs. UNG: $P = 0.054$; C: all $P < 0.05$ except 42 vs. UNG: $P = 0.052$, and 74 vs. UNG: $P = 0.189$; D: $P < 0.002$; and E: $P < 0.005$). The same amount of protein was used to process each lysate ($\approx 130 \mu$g), and the corresponding portion (nine tenths) was subjected to overnight pulldown. Na/K-ATPase was blotted as surface marker to correct for quantitations in pulldowns. Data are mean ± SD (n ≥ 3). Molecular weight markers are in kilodaltons (kDa).
Figure 5. Unglycosylated β2 is retained in the endoplasmic reticulum (ER).

MDCK cells were transiently transfected with the SCN2B-yfp vector to express WT or fully unglycosylated β2 (ung), and grown for 1 day in wells. Cells were treated with TUN 2 h after transfection, or left untreated (-), fixed, and immunostained with a rabbit polyclonal antibody against calnexin (red). (A) Representative XY sections show that unglycosylated β2 (green) is intracellular and overlaps with calnexin, as does the WT in TUN-treated cells. This contrasts with the localization of β2 WT at the cell end in untreated cells, also displaying a scattered pattern. To focus more accurately where β2 is found in each condition, sections were taken right above the nucleus (WT -) or at the nuclear level (for the rest). Nuclear staining by DAPI is in blue. Scale bar is 10 μm. (B) Line chart showing Manders’ coefficients calculated along the z-axis and indicating the fraction of β2 overlapping to compartments labeled with calnexin. The high overlap in TUN-treated cells and in those expressing unglycosylated β2 contrasts with negligible overlap in untreated cells expressing β2 WT. One-way ANOVA with Tukey’s HSD post hoc test revealed differences among means (*P < 0.0005). Data are mean ± SD (n ≥ 3).
Figure 6. Brefeldin A (BFA) prevents complex glycosylation of β2, a fraction of which can reach the cell surface.

MDCK cells were transiently transfected with the SCN2B-yfp vector to express WT or fully unglycosylated β2 (ung), then treated 2 h later with BFA (+), or left untreated (-), and grown o/n in wells. (A, C) Cells were surface biotinylated at 4°C. The same amount of protein was used to process each lysate (≈ 100 μg) and the corresponding portion (nine tenths) subjected to overnight pulldown. Denatured protein from cell lysates and pulldowns was treated o/n at 37°C with Endo H to cleave off immature N-glycans, or left untreated (-). Representative western blots show that the (lower) faster-migrating band of β2 WT is the only one visible in cells treated with BFA and increases its mobility with the Endo-H treatment; this band coincides with unglycosylated β2 (C; compare with Figure 2A). Note that Endo-H digestion in pulldowns is only partial, either due to saturation of the enzyme or to suboptimal conditions for enzyme action. Blots for Na/K-ATPase are included as loading controls. Molecular weight markers are in kilodaltons (kDa). (B) Band quantitation shows reduced levels of immature β2 in biotin-NeutrAvidin pulldowns (Membrane) of BFA-treated cells. Two-tailed Student’s t-test shows significant difference (*P < 0.05). Data are mean ± SD (n ≥ 3). (D) Cells were
fixed and immunostained with a rabbit polyclonal antibody against calnexin (red) and a mouse monoclonal to GM130 (blue). Representative XY sections show that, in BFA-treated cells, β2 WT displays an intracellular accumulation comparable to mutated β2 (green), grossly overlapping with calnexin in enlarged structures (arrowheads). This contrasts with its apparent localization in the plasma membrane in untreated cells, displaying also a scattered pattern that does not overlap with calnexin (sections were taken at the cell level where β2 is mainly found in each case). Nuclear staining by DAPI is in gray. Scale bar is 10 μm.
Figure 7. Dynamics of β2 is not influenced by N-glycosylation.

MDCK cells were transiently transfected with the SCN2B-yfp vector to express WT or fully unglycosylated (ung) β2, and grown for 2 days on glass supports. Mobility of β2-YFP on three different cellular locations was monitored by FRAP with a confocal microscope. Line charts of fluorescence intensity (mean ± SD) of ≥ 3 representative experiments show comparable mobile fraction between β2 WT (blue line) and mutant (red line) at the three regions analyzed, i.e., (A) the cell end, (B) the cytoplasm matrix, and (C) in large vesicular structures. For each, images on the right show a representative cell pre-bleached, right after bleaching, and after fluorescence recovery (arrowheads mark the bleached area); see the complete FRAP data in Supplementary Table S1. Scale bar is 10 μm.
Figure 8. Surface localization of Na\textsubscript{v}1.5 is reduced with unglycosylated β2.

(A, B) MDCK cells stably expressing WT or fully unglycosylated (ung) β2-YFP were transiently transfected with the vector SCN5A-FLAG and grown polarized in Transwells. Cells were fixed and immunostained with a rabbit polyclonal antibody against Na\textsubscript{v}1.5 (red), and with a mouse monoclonal antibody to gp114 (cyan). Images were obtained by confocal microscopy. In merged images, the YFP-emitted fluorescence is in green and DAPI is in blue. Representative XY sections taken at the apical (A), or nuclear (B) levels (sections taken at the cell level where Na\textsubscript{v}1.5 is mainly found in each case), and corresponding z-axis reconstruction (reciprocal XZ and XY sections marked by a yellow dashed line), show improved apical localization of Na\textsubscript{v}1.5 with β2 WT (A), which remains mostly intracellular in the presence of unglycosylated β2 (B); note the intracellular Na\textsubscript{v}1.5 accumulation with mutated β2 (arrowhead). Scale bars are 10 μm. (C, D) Line charts displaying the corrected total cell fluorescence (CTCF, mean percentage ± SD) along an apical-to-basal z-stack (section 1: most apical; 0.5 μm optical slice thickness) show the Na\textsubscript{v}1.5 curve peak close to those of apical gp114 and β2 WT (C). In contrast, Na\textsubscript{v}1.5 is displaced toward the nuclear section with mutated β2, which overlays with DAPI (D), included as reference for the nuclear level (≥ 6 cells were analyzed per condition). (E) MDCK cells stably expressing Na\textsubscript{v}1.5-YFP were transiently
cotransfected with the SCN2B-yfp vector to express β2, WT or fully unglycosylated (ung), plus additional SCN5A-FLAG vector to ensure extensive Na\textsubscript{v}1.5 overexpression, and grown o/n in wells; the pEGFP-N1 vector was used as a control. Cells were surface biotinylated at 4°C. The same amount of protein was used to process each lysate (≈ 600 μg), 97 % of which was subjected to overnight NeutrAvidin pulldown. Representative western blots and (F) band quantitation show reduced levels of Na\textsubscript{v}1.5 in biotin-NeutrAvidin pulldowns (Membrane) in the presence of unglycosylated β2, or without β2 (GFP), when comparing with the WT. One-way ANOVA with Tukey’s HSD post hoc test showed significant differences (*P < 0.002). The percentage of Na\textsubscript{v}1.5 at the cell surface over total cellular Na\textsubscript{v}1.5 protein varied from 1.42 ± 0.98 in the WT to 0.73 ± 0.50 % with unglycosylated β2. Data are mean ± SD (n ≥ 6). Na/K-ATPase was blotted as surface marker to correct for quantitations in pulldowns. Molecular weight markers are in kilodaltons (kDa). For clear display, the blot in E shows lysates and pulldowns separated by division lines, which indicate different exposure between lysates and pulldowns but equal exposure within each group.
Figure 9. Defect over time of unglycosylated β2 in promoting surface localization of Na\textsubscript{V}1.5.

MDCK cells stably expressing WT (A) or fully unglycosylated (ung) β2-YFP (C), or untransfected (parental) cells (E), were transiently transfected with the vector SCN5A-FLAG and grown in wells for the indicated number of days. Cells were fixed and immunostained with a rabbit polyclonal antibody against Na\textsubscript{V}1.5 (red), and with a mouse monoclonal antibody to Na/K-ATPase (blue). Images were obtained by confocal microscopy. In merged images, the YFP-emitted fluorescence is in green and DAPI is in gray. (A, C, E) Representative XY sections (sections taken at the cell level where Na\textsubscript{V}1.5 is mainly found in each case) show a general diffuse Na\textsubscript{V}1.5 pattern, intracellular and often perinuclear, except for a noticeable overlap with Na/K-ATPase, particularly at day 1, in the presence of β2 WT. Scale bars are 10 μm. Confocal images were analyzed by calculating the mean fluorescence intensity (MFI) along linear segments of 30 pixels in length (d, distance; 0.1 μm/pixel) drawn from the cell end perpendicularly into the cytoplasm. (B, D, F) Line charts show MFIs with the first 5 pixels of the segments, equivalent to the plasma membrane region (cell end), marked with a square bracket. The highest MFI levels are at the cell end for Na/K-ATPase and for β2 WT, which progressively decrease intracellularly. The profile for Na\textsubscript{V}1.5 increases at the cell end only in the presence of β2 WT and especially at day 1 (B), but remains comparatively low within this region with unglycosylated β2 (D) or in the absence of β2 (F). Data are mean ± SD (number of cells analyzed ≥ 3; 4 segments / cell).
Figure 10. Single β2 glycosylation mutants can promote surface localization of Na\(_{\text{v}}\)1.5 – analysis over time.

MDCK cells stably expressing the indicated single mutants for β2-YFP glycosylation were transiently transfected with the vector SCN5A-FLAG and grown in wells for the specified number of days. Cells were fixed and immunostained with a rabbit polyclonal antibody against Na\(_{\text{v}}\)1.5 (red), and with a mouse monoclonal antibody to Na/K-ATPase (blue). Images were obtained by confocal microscopy. In merged images, the YFP-emitted fluorescence is in green and DAPI is in gray. (A, C, E) Representative XY sections (taken at the level where Na\(_{\text{v}}\)1.5 is mainly found in each case) show some areas of overlap of Na\(_{\text{v}}\)1.5 with Na/K-ATPase at the cell end, particularly at day 1, in the presence of any of the mutants, while remaining mostly disperse throughout the cells in later time points. Scale bars are 10 μm. Confocal images were analyzed by calculating the MFI along linear segments of 30 pixels in length (d, distance; 0.1 μm/pixel) drawn from the cell end perpendicularly into the cytoplasm. (B, D, F) Line charts show MFIs with the first 5 pixels of the segments, equivalent to the plasma membrane region (cell end), marked with a square bracket. The highest MFI levels are at the cell end for Na/K-ATPase and for the different β2 single mutants, all progressively decreasing intracellularly. The profile for Na\(_{\text{v}}\)1.5 increases at the cell end at day 1 in all cases, remaining comparatively low within this region at later time points. Data are mean ± SD (number of cells analyzed ≥ 3, 4 segments / cell).
Figure 11
Figure 11. Single and double glycosylation mutants of β2 can promote surface localization of Na\(_{v}1.5\).

MDCK cells (A, C, E) stably expressing the indicated single mutants for β2-YFP glycosylation were transiently transfected with the vector SCN5A-FLAG and grown polarized in Transwells. Cells were fixed and immunostained with a rabbit polyclonal antibody against Na\(_{v}1.5\) (red), and with a mouse monoclonal antibody to gp114 (cyan). Images were obtained by confocal microscopy. In merged images, the YFP-emitted fluorescence is in green and DAPI is in gray. Representative XY sections taken at the apical level (section level chosen to assess presence of Na\(_{v}1.5\) at the apical surface), and corresponding z-axis reconstruction (reciprocal XZ and XY sections marked by a yellow dashed line), show noticeable apical localization of Na\(_{v}1.5\) with the different β2 variants. Scale bars are 10 μm. (B, D, F) Line charts displaying the CTCF (mean percentage ± SD) along an apical-to-basal z-stack (section 1: most apical; 0.5 μm optical slice thickness) show the Na\(_{v}1.5\) curve peak in close proximity to those of apical gp114 and any of the β2 mutants. DAPI is included as reference for the nuclear level (≥ 6 cells were analyzed per condition). (G, I) MDCK cells stably expressing Na\(_{v}1.5\)-YFP were transiently cotransfected with the SCN2B-yfp vector to express β2-YFP, WT, or any of the indicated single (G) or double (I) mutant, plus additional SCN5A-FLAG vector to ensure extensive Na\(_{v}1.5\) overexpression, and grown o/n in wells. Cells were surface biotinylated at 4ºC. The same amount of protein was used to process each lysate (≈ 600 μg), 97% of which was subjected to overnight NeutrAvidin pulldown. (G, I) Representative western blots and (H, J) band quantitation show comparable levels of Na\(_{v}1.5\) in biotin-NeutrAvidin pulldowns (Membrane) in the presence of any mutant variant of β2 as with the WT. One-way ANOVA revealed no differences among means. Data are mean ± SD (n ≥ 3). Na/K-ATPase was blotted as surface marker to correct for quantitations in pulldowns. Molecular weight markers are in kilodaltons (kDa). For clear display, the blots in G and I show lysates and pulldowns separated by division lines, which indicate different exposure between each.
N-glycosylation of the voltage-gated sodium channel β2 subunit is required for efficient trafficking of Na \( \beta_{1.5/\beta2} \) to the plasma membrane.

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