Modulation of Cav3.2 T-type calcium channel permeability by asparagine-linked glycosylation

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ABSTRACT
Low-voltage-gated T-type calcium channels are expressed throughout the nervous system where they play an essential role in shaping neuronal excitability. Defects in T-type channel expression have been linked to various neuronal disorders including neuropathic pain and epilepsy. Currently, little is known about the cellular mechanisms controlling the expression and function of T-type channels. Asparagine-linked glycosylation has recently emerged as an essential signaling pathway by which the cellular environment can control expression of T-type channels. However, the role of N-glycans in the conducting function of T-type channels remains elusive. In the present study, we used human Cav3.2 glycosylation-deficient channels to assess the role of N-glycosylation on the gating of the channel. Patch-clamp recordings of gating currents revealed that N-glycans attached to hCav3.2 channels have a minimal effect on the functioning of the channel voltage-sensor. In contrast, N-glycosylation on specific asparagine residues may have an essential role in the conducting function of the channel by enhancing the channel permeability and/or the pore opening of the channel. Our data suggest that modulation of N-linked glycosylation of hCav3.2 channels may play an important physiological role, and could also support the alteration of T-type currents observed in disease states.

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Introduction
Low-voltage-activated T-type calcium (Ca^{2+}) channels consist of a pore-forming Ca_{3,1}, Ca_{3,2}, and Ca_{3,3}) embedded in the plasma membrane of most excitable cells including neurons. One of the key features of T-type channels arises from their low-threshold of activation that makes these channels perfectly suited to operate near the resting membrane potential of cells. Although T-type channels may require a preceding period of hyperpolarization that removes inactivation when cells are partially depolarized at rest, they are typically recruited by subthreshold membrane depolarizations, and generate a transient low-threshold Ca^{2+} current (so-called T-type current) as well as low-threshold Ca^{2+} spikes, which support high frequency bursts of action potentials essential to maintain various forms of neuronal rhythogenesis. In addition, because of their unique gating properties, a small population of T-type channels remains open at rest, providing an incentive for Ca^{2+} to flow insight of the cell near typical neuronal resting membrane potentials ("window current"). Besides regulating neuronal excitability, T-type channels also contribute to low-threshold exocytosis by virtue of their functional coupling with the vesicular release machinery.

Over the last few years, various pathways and mechanisms underlying the modulation of T-type channels have been identified. Besides numerous small molecules that acutely modulate channel activity, T-type channels are subject to regulation at the levels of transcription, alternative splicing, protein interaction, and post-translational modification including ubiquitination and phosphorylation. In addition, asparagine (N)-linked glycosylation has emerged as an essential level of control of T-type channel expression. We have previously reported that N-glycosylation of human Ca_{3,2} channels (hCa_{3,2}) is critical for the proper maturation, surface trafficking and stability of the channel.
addition, acute enzymatic removal of N-glycans attached at the surface of the channel altered recombinant and native T-type currents, suggesting that N-glycosylation can also modulate the activity of the channel while at the plasma membrane. However, the detailed mechanism by which glycosylation modulates T-type channel activity besides controlling surface expression of the channel remains elusive.

In the present study, we have specifically analyzed the role of N-linked glycosylation on the functioning of hCav3.2 T-type channels. Using recombinant glycosylation-deficient hCav3.2 channels, we compared charge movements and ionic currents measured from HEK-293 cell-expressing hCav3.2 channels and revealed that N-glycosylation on specific loci modulates channel conductance, by enhancing channel permeability and/or opening probability.

Results

Effect of N-glycosylation on the voltage-dependence of T-type currents

The human Ca₃.2 channel (hCav3.2) presents 4 N-linked glycosylation loci, located in the extracellular loops of domains I, III and IV (Fig. 1). We and others have previously reported that acute enzymatic deglycosylation of hCav3.2 channels resulted in a significant decrease of the T-type current density. Because alteration of T-type currents may have resulted from an alteration of the negative surface potential, we assessed the voltage-dependence of T-type currents from cells expressing wild-type (WT) or glycosylation-deficient hCav3.2 channels where the potential N-glycosylation motifs (N-X-S/T) have been disrupted by replacing the asparagine residue (N) with glutamine (Q). Representative Ba²⁺ current traces in response to 30 ms depolarizing steps to values ranging between −80 mV and +70 mV, from a holding potential of −100 mV, are shown in Figure 2A for hCav3.2WT, N192Q, N1466Q, N192Q/N1466Q, and N1710Q channels. Figure 2B shows the corresponding mean normalized peak Ba²⁺ current density as a function of membrane voltage. The voltage-dependence of the T-type current activation was determined by fitting the values with a modified Boltzmann equation (see Methods). The mean half-activation potential remained unaltered in cells expressing glycosylation-deficient hCav3.2 channels (−38.3 ± 0.8 mV, n = 31 for N192Q; −39.2 ± 1.3 mV, n = 13 for N1466Q; and −38.0 ± 1.7 mV, n = 12 for N192Q/N1466Q) compared to cells expressing the wild-type channel (−36.1 ± 0.8 mV, n = 29). In contrast, we observed a significant alteration of the reversal potential of Ba²⁺ currents in cells expressing glycosylation-deficient channels. For instance, the reversal potential was shifted by −9.3 mV (p < 0.01) in N1466Q-expressing cells (48.2 ± 2.9 mV, n = 13), and by −9.0 mV (p < 0.01) in N192Q/N1466Q-expressing cells (29.9 ± 2.1 mV, n = 12) as compared to cells expressing the wild-type hCav3.2 channel (38.9 ± 1.6 mV, n = 29). Consistent with our previous observation that preventing glycosylation at asparagine N1710 gives rise to a non-functional channel, we did not observe any measurable current from cells expressing the N1710Q mutant channel.

Effect of N-glycosylation on gating currents of hCav3.2 channels

To determine whether N-glycosylation affects the functioning of the channel voltage-sensor, we analyzed charge movements that refer to the movement of the elements of the voltage-sensor following depolarization of the plasma membrane and produce gating currents (Q). Because glycosylation-deficient hCav3.2 channels usually are not sufficiently abundant at the plasma membrane to accurately assess charge movements over a large range of potentials, we measured the total charges (Qmax) at the reversal potential around +40 mV.
(Q_{rev}), where we can consider Q_{rev} to be equal to Q_{max} (Fig. 3A–B). Representative gating current traces recorded from cells expressing the wild-type hCa_{3.2} channel, or the various N-glycosylation mutants are shown in Figure 3C (top panels). No gating currents were detected in cells expressing N1710Q mutant channels (Fig. 3C; n = 54 cells from 5 independent transfections). Kinetics of charge movements were assessed by measuring the 10–90 % rise time of the gating current integral (Fig. 3C, bottom panels). The 10–90 % rise time of the gating current remained unaltered in cells expressing hCa_{3.2} channels (1.63 ± 0.20 ms, n = 24 for N192Q; 1.36 ± 0.11 ms, n = 12 for N1466Q; and 1.58 ± 0.24 ms, n = 12 for N192Q/N1466Q) compared to cells expressing the wild-type channel (1.41 ± 0.13 ms, n = 29) (Fig. 3D).

**N-glycosylation controls the permeability of hCa_{3.2} channels**

Activation of voltage-gated Ca^{2+} channels requires the initial mobilization of the channel voltage-sensor that generates charge movements (Q), followed by the opening of the pore evidenced by the occurrence of a Ca^{2+} conductance (G). Hence, comparing Q over G provide valuable insights into the functioning of the channel. Figure 4A shows the scatter plot of G_{max} as a function of Q_{max} for wild type and glycosylation-deficient hCa_{3.2} channels, and fitted with the following linear regression:

\[ G_{max} = k.Q_{max} \]

with k being the steepness factor. G_{max} was calculated from the Boltzmann-Ohm fit of the voltage-dependence
of the peak Ca$^{2+}$ current (see Fig. 2 and Methods). Whereas disrupting N-glycosylation at asparagines N192 and N1466 had no significant effect on the $Q_{\max} - G_{\max}$ dependency, the steepness factor was reduced by $\approx 1.7$ fold ($p < 0.05$) in N192Q/N1466Q-expressing cells ($0.034 \pm 0.006$, $n = 12$) as compared to cells expressing...
Figure 4. N-glycosylation modulates the permeability of hCa\textsubscript{3.2} channels. (A) Scatter plots of the maximal conductance ($G_{\text{max}}$) as a function of the maximal ON-gating charge ($Q_{\text{max}}$) for wild-type hCa\textsubscript{3.2} ($n = 29$) and glycosylation-deficient N192Q ($n = 24$), N1466Q ($n = 12$), and N192Q/N1466Q ($n = 12$) channels. Data were fitted with a linear regression (continuous line). The dotted lines represent the fit of the wild-type channel. (B) Corresponding mean values for $G_{\text{max}}$ over $Q_{\text{max}}$. 
the wild-type hCaV3.2 channel (0.057 ± 0.05, n = 29) (Fig. 4B). $Q_{\text{max}}$ and $G_{\text{max}}$ can be described by the following equations (1) and (2), respectively:

$$Q_{\text{max}} \simeq n.q$$

$$G_{\text{max}} \simeq n.g.Po$$

with $n$ being the number of functional channels, $q$ the unitary charge per channel, $g$ the unitary conductance, and $Po$ the opening probability. Hence, dividing $G_{\text{max}}$ by $Q_{\text{max}}$ as shown in equation (3) provides information on the single channel properties:

$$\frac{G_{\text{max}}}{Q_{\text{max}}} = \frac{n.g.Po}{n.q} = \frac{g}{q} .Po$$

Considering that the charge movements were found unaltered, this result is in support of a decreased channel conductance and / or opening probability of the glycosylation-deficient N192Q/N1466Q channel.

**Discussion**

In the present study, we provide evidence for an essential role of N-glycosylation in the control of human CaV3.2 T-type channel gating. Whereas glycosylation has minimal influence on the functioning of the channel voltage-sensor, N-glycans attached to the hCaV3.2 subunit are essential to regulate the permeability of the channel.

Glycosylation has been shown to play an essential role in the expression and functioning of a wide variety of ion channels. To assess the role of N-glycosylation in T-type channel function, we used N-glycosylation-deficient hCaV3.2 channels where glycosylation motifs have been disrupted by mutagenesis. It was previously proposed that sialic acid residues attached to the outermost ends of glycan chains may contribute to the negative surface potential, and contributing to the gating modulation of some voltage-gated Na\(^{+}\) and K\(^{+}\) channels by an electrostatic mechanism. In contrast, we showed that disruption of N-glycosylation had no influence on the mean half-activation potential of hCaV3.2 currents. This result is consistent with previous reports showing that enzymatic deglycosylation of recombinant and native CaV3.2 channels with PNGase F or neuraminidase did not alter the voltage-dependence of T-type currents.

In contrast, we showed that N-glycosylation plays an essential role in modulating the permeability of hCaV3.2 channels. Whereas disruption of N-glycosylation at asparagine N192 and N1466 had no influence on single channel properties, simultaneous disruption of the two N-glycosylation sites resulted in a decreased single channel conductance and / or the opening probability. This result supports initial observations that acute deglycosylation of hCaV3.2 with PNGase F, or desialylation with neuraminidase, attenuates the Ca\(^{2+}\) conductance of CaV3.2 channels. In addition, the alteration of the channel permeability is further supported by our observation that cells expressing glycosylation-deficient channels had altered reversal potential of the T-type current. As the potential for half-maximal current activation was unaffected, a shift of the reversal potential can be attributed to an altered outward current flowing through CaV3.2 glycosylation-deficient channels. However, this alteration was observed in non-physiological conditions with Cs\(^{+}\) as a main intracellular cation and with intracellular Cl\(^{-}\) replaced by methansulfonate (see Methods). As one could expect, an outward current through T-type channels depends considerably on the type of intracellular cation. It is unlikely that the alteration of the reversal potential has any physiological relevance, but strongly supports the notion that N-glycans attached to the CaV3.2 subunit influences channel selectivity.

Asparagine N1466 is located in the third transmembrane domain of hCaV3.2 in the pore-forming loop between segments S5 and S6 that project into the pore of the channel to form the selectivity filter. Thus, glycosylation of asparagine N1466 may provide a local negative environment that could affect the Ca\(^{2+}\) permeability. However, our observation that deglycosylation of asparagine N1466 is not sufficient to alter the channel conductance and requires the concomitant deglycosylation of asparagine N192 suggests the implication of other channel gating domains. In contrast, the asparagine N192 is located in the first domain of the channel in the short extracellular loop linking segments S3 and S4. It is thus conceivable that glycosylation of asparagine N192, by providing a negative charge in the vicinity of the S4 voltage-sensor may have an effect on the gating of the channel. Consistent with this idea, it was reported that the open state of the inwardly rectifying K\(^{+}\) channel Kir1.1 was destabilized in the absence of N-glycosylation. Although we
have not observed any change in the ON kinetics of the gating currents of glycosylation-deficient channels, it is possible that the OFF kinetics may be affected. This aspect was not possible to investigate in our experimental conditions, as it would have required a full block of the ionic current. Nevertheless, our data clearly established N-glycosylation as an essential determinant of hCav3.2 channel gating.

Repeatedly, no gating current was measured in cells expressing N1710Q mutant channels. While previous experiments detected channel expression at the cell surface, lack of measurable charge movement suggests that either the channel is not properly inserted in the plasma membrane, or its voltage sensors are not functional.

Taken together, our data extend our understanding of the role of N-glycosylation in the functioning of T-type channels. Our observation that the conducting properties of hCav3.2 channels can be altered by the amount of occupied N-glycosylation sites in the protein may have important pathophysiological implications, as a defect in ion channel glycosylation has been reported in numerous disease states. Interestingly, a causal increased activity of T-type channels has been documented in various animal models of chronic pain including painful diabetic neuropathy. And consistent with the idea that the increased T-type channel activity may be caused by a defect in the glycosylation of the channel, in vivo desialylation by injection of neuraminidase in an animal model of diabetes restored normal T-type currents and pain behavior. In addition, an increased T-type channel activity has also been documented in animal model of epilepsy. Interestingly, a missense mutation (D1463N) in the CACNA1H gene encoding for Cav3.2 channels and located near to the asparagine N1466 was identified in patients with childhood absence epilepsy. Whether this mutation affects N-glycosylation of the channel remains to be determined.

Material and methods

Plasmid cDNA constructs

The human wild type and glycosylation-deficient hCav3.2 channels used in this study were previously described. Briefly, the wild-type hCav3.2 construct (hCav3.2WT) was used as a template for mutation of the consensus asparagine (N)-linked glycosylation sites by substituting the asparagine residues N192, N1466, and N1710 with glutamine (Q) residues. Glutamine was chosen because of its structural similarity with asparagine residues, differing only by one methyl group in the amino acid side chains, which is consequently expected to preserve the local charge distribution within the protein and the secondary structure of the channel. The N to Q substitution was introduced by site-directed mutagenesis using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies Inc.), and the final constructs were verified by sequencing of the full-length cDNAs.

Heterologous expression

Human embryonic kidney HEK-293 cells (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, German Collection of Microorganisms and Cell Cultures) were grown in MEM with Earle’s salts containing 10% fetal calf serum and 100 U/mL penicillin-streptomycin and maintained under standard conditions at 37°C in a humidified atmosphere containing 5% CO2 and 95 % air. Cells were harvested from their culture flasks by trypsinization, plated out onto 25 cm2 culture flasks (Sigma Aldrich), and transiently transfected using the calcium/phosphate method with the human hCav3.2 channel, along with a green fluorescent protein. For calcium/phosphate transfection we used Solution A (250 mM CaCl2) and Solution B (2x HEPES buffer containing in mM; NaCl, 250; HEPES, 40 Dextrose 12; Na2HPO4, 1.4; pH 7.05 with NaOH) to form a calcium phosphate precipitate that was directly layered onto the cells and 3 μg of plasmid cDNA.

Electrophysiology and data analysis

Patch-clamp recordings were performed 48–72 h after transfection at room temperature (22–24°C) using an EPC-10 patch clamp amplifier (HEKA Electronic, Lambrecht, Germany). The extracellular solution contained (in mM): CsCl, 95; HEPES, 10; glucose, 10; TEA-Cl, 40; BaCl2, 5; MgCl2, 1; pH 7.4 (CsOH). Background Cl- current in HEK-293 cells was minimized by use of cesium methansulfonate in the pipette solution. The composition of the intracellular solution was (in mM): CH3SO3Cs, 130; EGTA, 10; MgCl2, 5; TEA-Cl, 10; Na-ATP, 5; and HEPES, 10; pH 7.4 (CsOH). Patch pipettes were made out of borosilicate glass (Sutter Instrument, Novato, CA). When filled with the
in intracellular solution, the input resistance ranged between 1.6 and 2.0 MΩ. The capacitance of individual cells ranged between 10 and 40 pF. Series resistance reached values between 2.5 to 6 MΩ and was compensated by built-in circuits of the EPC 10 amplifier. Data were recorded with HEKA Patchmaster and analyzed offline using HEKA Fitmaster v2×73.1 and Origin 8.1 software. The holding potential (HP) in all experiments was −100 mV. Ionic currents were measured by 30-ms-long depolarizing pulses from the HP to membrane potentials between −80 mV and +70 mV applied with a frequency of 0.33 Hz. The linear components of leak current were subtracted offline. The voltage-dependence of the peak Ca²⁺ current was fitted with the following modified Boltzman equation:

\[ I(V) = G_{\text{max}} \frac{(V - V_{\text{rev}})}{1 + \exp \left(\frac{V_0.5 - V}{k}\right)} \]

with \(I(V)\) being the peak current amplitude at the command potential \(V\), \(G_{\text{max}}\) the maximum conductance, \(V_{\text{rev}}\) the reversal potential, \(V_0.5\) the half activation potential, and \(k\) the steepness factor. Opening currents were measured by a series of 5 identical depolarizing pulse to the reversal potential of each respective cell. The linear components of leak current and capacitive transients were subtracted using the -P/8 procedure. The total QON charge was evaluated by integrating the area below averaged gating current traces at the beginning of the depolarizing pulse.

**Statistical analysis**

Data values are presented as mean ± S.E.M. for \(n\) recorded cells. Statistical significance was determined using ANOVA test. \(^*\) \(p < 0.05\), \(\ast \ast \ast \) \(p < 0.001\), and NS, statistically not different.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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