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N-glycans modulate $K_v1.5$ gating but have no effect on $K_v1.4$ gating

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ABSTRACT

Nerve and muscle action potential repolarization are produced and modulated by the regulated expression and activity of several types of voltage-gated K^+ (K_v) channels. Here, we show that sialylated N-glycans uniquely impact gating of a mammalian Shaker family K_v channel isoform, $K_v1.5$, but have no effect on gating of a second Shaker isoform, $K_v1.4$. Each isoform contains one potential N-glycosylation site located along the S1–S2 linker; immunoblot analyses verified that $K_v1.4$ and $K_v1.5$ were N-glycosylated. The conductance–voltage (G–V) relationships and channel activation rates for two glycosylation-site deficient $K_v1.5$ mutants, $K_v1.5^{N290Q}$ and $K_v1.5^{S292A}$, and for wild-type $K_v1.5$ expressed under conditions of reduced sialylation, were each shifted linearly by a depolarizing ~18 mV compared to wild-type $K_v1.5$ activation. External divalent cation screening experiments suggested that $K_v1.5$ sialic acids contribute to an external surface potential that modulates $K_v1.5$ activation. Channel availability was unaffected by changes in $K_v1.5$ glycosylation or sialylation. The data indicate that sialic acid residues attached to N-glycans act through electrostatic mechanisms to modulate $K_v1.5$ activation. The sialic acids fully account for effects of N-glycans on $K_v1.5$ gating. Conversely, $K_v1.4$ gating was unaffected by changes in channel sialylation or following mutagenesis to remove the N-glycosylation site. Each phenomenon is unique for K_v1 channel isoforms, indicating that sialylated N-glycans modulate gating of homologous K_v1 channels through isoform-specific mechanisms. Such modulation is relevant to changes in action potential repolarization that occur as ion channel expression and glycosylation are regulated.

1. Introduction

The voltage-gated potassium (K_v) channel family is comprised of a large and diverse set of ion channels that serve a variety of functions throughout the body [1,2]. Various sets of K_v channel isoforms are responsible for the repolarization phase of skeletal muscle, neuronal, and cardiomyocyte action potentials [3]. Slight changes in the type, relative density, or activity of K_v channel isoforms that occur following a remodeling process can lead to altered action potential repolarization. Remodeled action potential repolarization occurs through physiological processes such as development and aging, as well through pathologies such as Long QT Syndrome, deafness, and epilepsy [4–7].

Posttranslational modifications make up a significant portion of the mass of ion channels. Glycosylation contributes upwards of 30% of a channel's total mass [8]. Sialic acids are negatively charged residues terminally located on glycosylation structures [9]. Analyses of purified samples suggested that over 100 sialic acid residues per functional molecule are attached to some Na^+ channel [10,11] and Shaker K^+ channel proteins [12].

Previous studies showed that N-glycans can modulate voltage-gated Na^+ (Na_v) and K_v channel activity through isoform-specific mechanisms [13–15]. All reported data suggest that sialic acids contribute to the full effect of glycans on Na_v channel gating through electrostatic mechanisms [13,16–18]. Negatively charged sialic acid residues contribute to the external negative surface potential, thereby impacting channel gating. On the other hand, previous reports indicated that gating of Shaker K_v channel isoforms, $K_v1.1$ [14,19], $K_v1.2$ [15], and the Drosophila ShB channel [20], were modulated by N-glycans and/or sialic acids through at least two mechanisms per isoform: 1) an electrostatic mechanism (surface potential theory) and 2) glycan-dependent effects on the stability of channel proteins among functional states (gating stabilizing theory).

Here, we questioned whether N-glycans and/or sialic acids alter the gating of two homologous Shaker K^+ channel isoforms, $K_v1.4$ and $K_v1.5$. These two isoforms share a great deal of sequence homology, including a similar location for the sole N-glycosylation site positioned on the S1–S2 linkers. The data indicate that a reduction in sialylation caused a large, depolarizing shift in Kv1.5 voltage-dependent activation, while $K_v1.4$ gating was unaffected. Unlike previous studies of Shaker K_v channel isoforms [14,15,20], N-glycosylation did not exert an additional effect on the gating of either isoform. Further, sialic acids modulated $K_v1.5$ gating solely through electrostatic mechanisms.

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2. Materials and methods

2.1. CHO cell culture and transfection

Pro5 and Lec2 cells were grown in minimal media and transfected with channel cDNA as described previously [20,21]. Briefly, the cells were plated at 25–50% confluence on 35 mm dishes 24 h prior to transfection with 1 ml Opti-MEM (Invitrogen) containing 8 μl of lipofectamine (Invitrogen), 0.25 μg of eGFP, and 2.5 μg of plasmid DNA containing the human $K_v1.4$ (accession #: NM_002233) or $K_v1.5$ (accession #: NM_002234) cDNA inserted into vectors pRC/CMV and pcDNA3, respectively (clones were a gift from Dr. Stephen Korn). Cells were incubated with the liposomal/DNA mixture at 37 °C in a 5% CO_2 humidified incubator. Twenty-four hours post-transfection, the medium was replaced with growth medium, consisting of alpha-minimum essential medium (αMEM; Invitrogen) with (Pro5) and without (Lec2) ribo- and deoxyribonucleosides supplemented with 10% fetal bovine serum (FBS; Hyclone) and penicillin/streptomycin (Mediatech). Cells were incubated at 37 °C for another 48 h prior to commencing electrophysiological recordings.

2.2. Whole cell recordings in CHO cells

The Pro5/Lec2 expression system has been used successfully to determine the effects of sialic acids on channel gating [13–15,19,20,22]. We and others previously showed that Na_v channel and certain K_v channel gating as expressed in Lec2 cells mimicked channel gating following neuraminidase treatment to remove channel sialic acids [13–20,22]. The Pro5 cell line allows normal protein sialylation, while the Lec2 cell line produces essentially non-sialylated proteins due to a deficiency in the CMP-sialic acid transporter [23,24]. The Lec2 cell line used in this study serves as a model for CDG type IIf [25,26].

Whole cell current recordings were performed using pulse protocols, solutions, whole cell patch clamp techniques, and data analyses as described previously [13,20]. All experiments were conducted at room temperature, ~22 °C. Drummond capillary tubes were pulled into electrodes with a resistance of 1–2 MΩ using a Model P-97 Sutter electrode puller. Series resistance was compensated 95–98%. The extracellular solution was (mM): 65 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 155 sucrose, 5 glucose, 10 Hepes (pH 7.3), while the intracellular solution used was (mM): 70 KCl, 65 KF, 5 NaCl, 1 $MgCl_2$, 10 EGTA, 5 glucose, 10 Hepes (pH 7.3). The extracellular low divalent cation solution was identical to the extracellular solution listed above, with the exception of a 0.2 mM concentration of $CaCl_2$ and a 0.1 mM concentration of $MgCl_2$. To ensure complete dialysis of the intracellular solution, data were collected at least 10 min after attaining whole cell configuration.

2.3. Pulse protocols

2.3.1. Conductance–voltage (G–V) relationship

The steady-state and kinetic gating parameters were examined using standard pulse protocols and solutions described by our lab previously [13,20]. Current–voltage relationships were measured by stepping from the −120 mV holding potential to more depolarized potentials ranging from −100 mV to +40 mV in 10 mV increments. Consecutive pulses were initiated every 1.5 s and the data were leak subtracted using the P/4 method, stepping negatively from the holding potential. Steady-state whole cell peak conductance values (G) were determined by measuring the peak current (I) at each test potential (V_p) and the predicted K^+ Nernst equilibrium potential ($E_k = -84$ mV). The maximum conductance generated by each cell was used to normalize the data for each cell to its maximum conductance by fitting the data to a single Boltzmann distribution (Eq. (1), solving for maximal conductance). These single Boltzmann distributions were used to determine the average $V_a \pm$SEM and $K_a \pm$SEM values listed in Table 1. The normalized data were averaged with those from the other cells and the resulting average G–V curve was fitted via least squares using the Boltzmann relation below:

$$\text{Fraction of maximal conductance} = [1 + \exp-((V - V_a)/K_a)]^{-1} \quad (1)$$

where V is the membrane potential, V_a is the voltage of half-activation, and K_a is the slope factor.

2.3.2. Steady-state inactivation curves (h_{inf})

To discern the effects of glycosylation on $K_v1.5$ steady-state inactivation, cells were pre-pulsed from −110 mV to +40 mV (10 mV increments) for 5 s, stepped to +30 mV for 500 ms, and returned to the holding potential (−120 mV) for 15 s similar to that previously described [27]. Cells expressing $K_v1.4$ were pre-pulsed from −120 mV to +20 mV (10 mV increments) for 1 s, then stepped to +60 mV for 100 ms, and returned to the holding potential. The maximum current generated by each cell was used to normalize the data for each cell to its maximum current by fitting the data to a single Boltzmann distribution (Eq. (2), solving for maximal current), from which the mean $V_i \pm$SEM and $K_i \pm$SEM values listed in Table 1 were determined.

$$\text{Fraction of maximal current} = [1 + \exp-((V - V_i)/K_i)]^{-1} \quad (2)$$

where V is the membrane potential, V_i is the voltage of half-inactivation, and K_i is the slope factor.

Table 1
Steady-state gating parameters measured for $K_v1.4$ and $K_v1.5$.

Construct	n Activation	V_a mV	K_a mV	n Inactivation	V_i mV	K_i mV
$K_v1.5$ + SA	11	−27.1 ± 3.1	9.3 ± 0.7	7	−20.8 ± 2.4	−12.4 ± 0.2
$K_v1.5$ − SA	11	−8.9 ± 1.8*	10.4 ± 0.3**	5	−17.6 ± 1.3**	−12.2 ± 0.6**
$K_v1.5^{N290Q}$ + SA	5	−7.9 ± 3.0*	8.3 ± 0.3**	3	−21.1 ± 1.2**	−11.7 ± 0.4**
$K_v1.5^{N290Q}$ − SA	4	−8.9 ± 2.6*	9.5 ± 0.5**	5	−17.7 ± 3.6**	−12.6 ± 0.4**
$K_v1.5^{S292A}$ + SA	4	−7.1 ± 1.5*	9.8 ± 0.4**	3	−22.4 ± 2.6**	−11.6 ± 0.5**
$K_v1.4$ + SA	6	−29.3 ± 2.8	14.6 ± 0.8	6	−53.6 ± 1.1	−4.0 ± 0.5
$K_v1.4$ − SA	7	−26.3 ± 1.4**	15.3 ± 0.8**	7	−52.5 ± 1.6**	−4.3 ± 0.9**
$K_v1.4^{N354Q}$ + SA	3	−28.4 ± 3.5**	15.2 ± 1.3**	3	−52.7 ± 0.3**	−4.7 ± 0.1**

Data are mean ± SEM. Parameters as described in Eqs. (1) and (2). V_a: voltage of half-activation. K_a: Boltzmann activation slope factor. V_i: voltage of half-inactivation. K_i: Boltzmann inactivation slope factor. Significance was tested using a two-tailed Student's t-test to compare gating parameters as expressed under conditions of reduced glycosylation with control conditions.

* Significantly different from control ($p < 0.0005$).

** Not significantly different from control ($p > 0.05$).

2.3.3. Time constants of activation (τ_n)

Activation time constants were determined by fitting the current traces used to measure the G–V relationships. Whole cell current traces were fitted to a fourth power exponential function using the Hodgkin–Huxley function of the PulseFit software suit (HEKA) to determine τ_n.

2.4. Mutagenesis of K_v channel N-glycosylation sites

Vector construction and mutagenesis were performed similar to that described previously [20]. For $K_v1.4$ and $K_v1.5$, the asparagine residues (N) located at sites 354 and 290, respectively, were mutated to glutamine (Q). Additionally, the serine residue (S) at site 292 was mutated to an alanine (A) to generate another $K_v1.5$ N-glycosylation mutant (Fig. 1). The mutations were performed to eliminate the single N-glycosylation site of each isoform (by mutating either the N or S of the consensus N-glycosylation sequence) with a minimal effect on channel structure and function. Mutagenesis was completed using the Stratagene Quickchange IIXL site-directed mutagenesis kit and mutant constructs were verified through sequencing.

2.5. K_v channel V5/His epitope tagging

For immunoblot analyses, $K_v1.4$ and $K_v1.5$ isoforms were tagged with the V5/His epitope attached to the C-terminus. Channel cDNA open reading frames (ORFs) minus the stop codons were amplified using PCR and subcloned into pcDNA3.1/V5-His TOPO TA expression vector (Invitrogen), similar to that previously described by us [20].

2.6. Whole cell homogenization

Cells were rinsed with cold PBS and incubated for 5 min in ice cold sodium pyrophosphate buffer with protease inhibitors (PI; 20 mM tetrasodium pyrophosphate, 20 mM Na_2PO_4, 1 mM $MgCl_2$, 0.5 mM EDTA, 300 mM sucrose, 0.8 mM benzamidine, 1 mM iodacetamide, 1.1 μM leupeptin, 0.7 μM pepstatin, 76.8 nM aprotinin). Cells were then homogenized using manual tissue grinders. The homogenates were centrifuged for 10 min at 1000 ×*g* in a Beckman bench-top centrifuge. The supernatant was centrifuged in a Beckman ultracentrifuge for 1 h at 100,000 ×*g* after which, the pellet was resuspended in an appropriate volume of sodium pyrophosphate buffer containing PI's. The lysates were then stored at −80 °C [20,28]. Protein levels were determined using the Pierce BCA Protein Assay kit.

2.7. Immunoblots

Immunoblot gel shift analysis was performed as described previously [13,20]. Briefly, whole cell homogenates (5–15 μg/lane) were combined with one volume of 2× sample buffer (10% glycerol, 5% 2-mercaptoethanol, 3% sodium dodecyl sulfate, and 12.5% upper Tris buffer) and denatured for 3 min in boiling water. Samples were then run on 6.5% acrylamide gels (SDS-PAGE) for 90 min at 75–110 mV and transferred onto nitrocellulose membranes using a semi-dry transfer cell (BioRad). Some homogenates were treated with 5 U/10 μg protein PNGase-F (Sigma) or 2 μl neuraminidase/sample (Sialidase A, Prozyme) for 3 h at 37 °C. A monoclonal anti-V5-HRP antibody (Invitrogen) was used at a 1:5000 dilution followed by enhanced chemiluminescence (Pierce) to visualize each isoform on the blot.

2.8. Data analysis

Electrophysiological data were analyzed using Pulse/PulseFit (HEKA) and Sigma Plot (SSPS Inc.).

3. Results

The aim of this study was to investigate whether and how N-glycosylation and sialic acids modulate the function of two homologous K_v1 channel isoforms, $K_v1.4$ and $K_v1.5$. Each isoform has one potential N-glycosylation site located in a similar position along the S1–S2 linker (Fig. 1) [30]. Two methods for deglycosylation were used: 1) mutagenesis was utilized to remove the putative N-glycosylation sites and, 2) channel sialylation was prevented using the previously validated method of comparing channel function in CHO cells (Pro5 versus Lec2) with differing abilities to sialylate proteins [13–20,22]. We predicted that $K_v1.4$ and $K_v1.5$ gating would be similarly impacted by the N-glycans attached to homologous positions along the channel structure. However, the data indicate that sialylated N-glycans attached to $K_v1.5$ modulate channel activation through electrostatic mechanisms, while N-glycans have no measurable effect on $K_v1.4$ gating.

3.1. Sialylated N-glycans account for the full effect of N-glycans on $K_v1.5$ gating

3.1.1. N-glycans impact $K_v1.5$ gating

$K_v1.5$ is partially responsible for the production of a slowly inactivating current involved in action potential repolarization in cardiomyocytes and neurons [29,31,32]. To determine whether

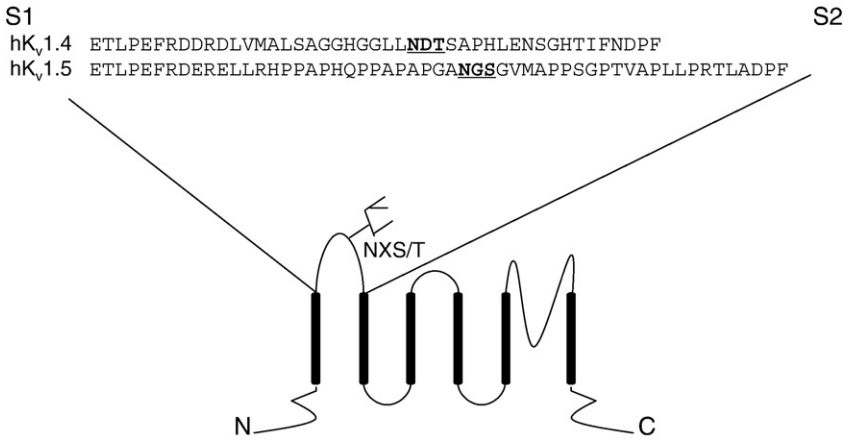

Fig. 1. $K_v1.4$ and $K_v1.5$ contain one putative N-glycosylation site located in the S1–S2 linker region. Schematic of a K_v1 channel α-subunit. The amino acid sequences of the S1–S2 linker for human $K_v1.4$ and $K_v1.5$ are shown above. The N-glycosylation consensus sequences (NXS) are emboldened and underlined.

N-glycans impact $K_v1.5$ gating, a $K_v1.5$ N-glycosylation mutant ($K_v1.5^{N290Q}$) was generated by mutating the asparagine residue (N) that initiates the potential N-glycosylation consensus sequence to a glutamine (Q). $K_v1.5^{N290Q}$ expressed currents similar to that produced by the wild-type channel. However, the conductance–voltage (G–V) relationship for $K_v1.5^{N290Q}$ was shifted linearly along the voltage axis by a depolarizing ~18 mV compared to the wild-type $K_v1.5$ G–V relationship, suggesting that N-glycosylation modulates $K_v1.5$ channel activation (Fig. 2B, Table 1). Mutagenesis of the $K_v1.5$ N-glycosylation site had no measurable effect on steady-state inactivation (Fig. 2D, Table 1). To confirm that lack of N-glycosylation caused by the N to Q mutation was responsible for the observed effect on channel gating, the serine (S) residue of the same N-glycosylation sequence was mutated to an alanine (A) in order to generate a second N-glycosylation-site deficient $K_v1.5$ channel ($K_v1.5^{S292A}$). The G–V relationship for the $K_v1.5^{S292A}$ channel was not significantly different from that measured for the $K_v1.5^{N290Q}$ channel, but was, like $K_v1.5^{N290Q}$, shifted by ~18 mV to more depolarized potentials compared to the G–V relationship for wild-type $K_v1.5$ (Fig. 2B, Table 1). As with $K_v1.5^{N290Q}$, the $K_v1.5^{S292A}$ steady-state inactivation curve was nearly identical to the wild-type $K_v1.5$ inactivation curve (Fig. 2D). These data indicate that mutation of the asparagine and the serine residues that comprise the N-glycosylation consensus sequence impacted channel gating identically. It can be inferred that the changes in amino acid structure made through mutagenesis had little to no effect on $K_v1.5$ gating, but rather the lack of N-glycosylation was likely to be primarily responsible for the observed modulation of $K_v1.5$ gating. The data indicate that N-glycosylation caused a significant hyperpolarizing and linear shift in the voltage at which $K_v1.5$ activates.

3.1.2. Sialic acids attached through N-linkages account for the full effect of sugars on $K_v1.5$ gating

To determine whether $K_v1.5$ sialic acids contribute to the effect of N-glycans on channel gating, $K_v1.5$ activation was studied under conditions of full and reduced sialylation by expressing the channel in Pro5 (full sialylation) and Lec2 (reduced sialylation) cell lines. This method previously was used by our lab and others to characterize the effect of sialic acids on gating of Na_v and some K_v channels [13–20,22]. As shown in Fig. 2, wild-type $K_v1.5$ under conditions of reduced sialylation activated at more depolarized potentials than the fully sialylated channel. The G–V relationship for the less sialylated wild-type $K_v1.5$ was shifted linearly by a depolarizing ~18 mV from that observed for the fully sialylated channel, indicating that a stronger depolarization is required to activate wild-type $K_v1.5$ in the absence of sialic acids (Fig. 2B, Table 1). As described, the glycosylation-site deficient mutant channels, $K_v1.5^{N290Q}$ and $K_v1.5^{S292A}$, activated at potentials ~18 mV more depolarized than fully sialylated wild-type $K_v1.5$. The corresponding G–V relationships were nearly identical to wild-type $K_v1.5$ under conditions of reduced sialylation. Further, the G–V curve for $K_v1.5^{N290Q}$ was not altered when studied under conditions of reduced sialylation and was nearly identical to the G–V curves measured for wild-type $K_v1.5$ under conditions of reduced sialylation (Fig. 2B). The slope factors of the Boltzmann relationships were not significantly different among all compared G–V relation-

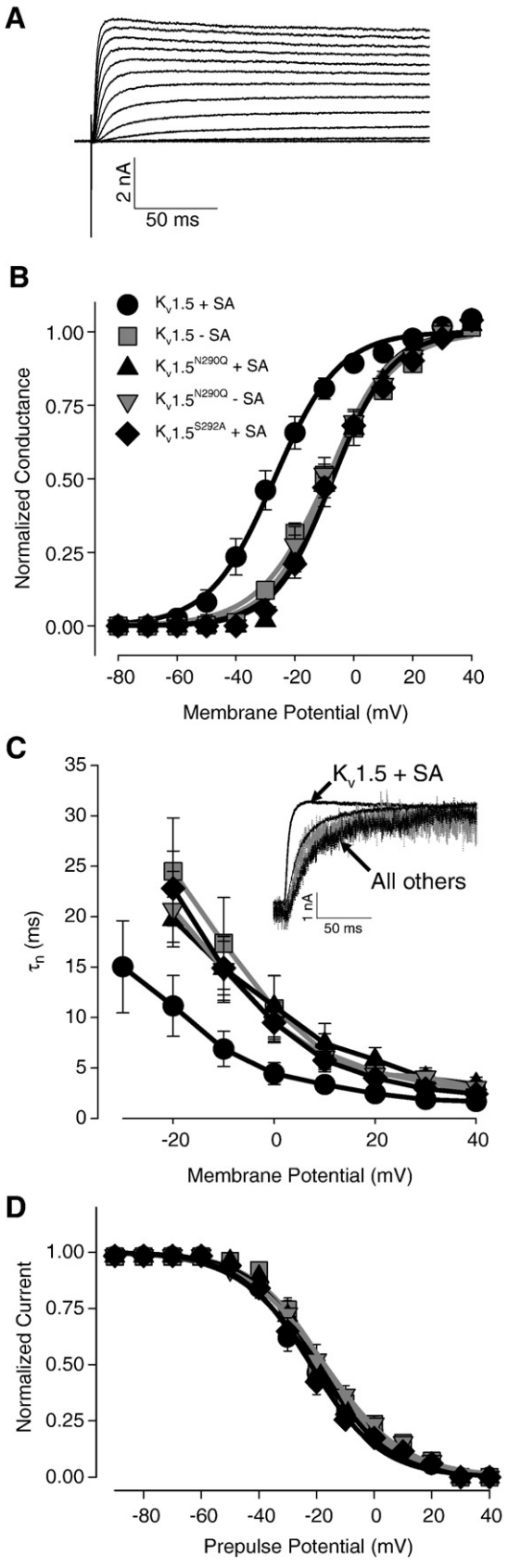

Fig. 2. N-linked sialic acids account for the full effect of glycans on $K_v1.5$ activation. (A) Typical wild-type $K_v1.5$ whole Pro5 cell current traces. (B–D), Voltage-dependence of activation and inactivation for wild-type $K_v1.5 \pm$ sialic acid (SA; + SA in Pro5 cells; −SA in Lec2 cells), $K_v1.5^{N290Q} \pm$SA, and $K_v1.5^{S292A}$ +SA. (B) Conductance–voltage (G–V) relationships. Data are the mean normalized peak conductance ± SEM and are fit to single Boltzmann relationships (lines). $n = 3$–11. (see Table 1). (C) Activation time constants (τ_n). Data are mean ± SEM. Lines are non-theoretical point to point. Symbols are as listed in panel B. Inset: typical current traces measured during a 0 mV test potential. Peak currents were normalized for comparison. The scaling factors used to normalize the currents were 2.63 to 23.75. Solid black: wild-type $K_v1.5$ +SA; solid gray: wild-type $K_v1.5$ −SA; dotted black: $K_v1.5^{N290Q}$ +SA; dotted gray: $K_v1.5^{N290Q}$ −SA; dashed black: $K_v1.5^{S292A}$ +SA. (D) Steady-state channel availability (inactivation). Data are the mean normalized peak current ± SEM measured during a 500 ms pulse to +30 mV, following a 5 s pre-pulse to the plotted potentials. Curves are single Boltzmann distribution fits to the data. Symbols (for B, C, and D) as listed in panel B. $n = 3$–11 (see Table 1).

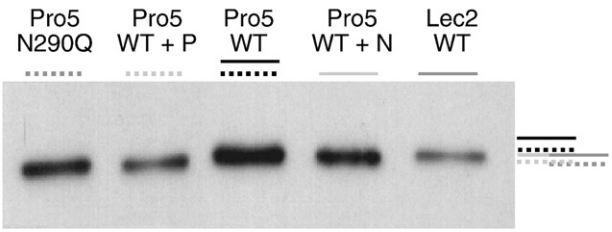

Fig. 3. $K_v1.5$ is N-glycosylated. Immunoblot of whole Pro5 and Lec2 cell homogenates expressing wild-type $K_v1.5$ (WT) and $K_v1.5^{N290Q}$ (N290Q) under variable conditions of glycosylation and sialylation. Samples labeled were treated with PNGase-F (P) to remove full N-glycan structures or neuraminidase (N) to remove sialic acids. Lines to the right of the blot indicate the relative mobility of the blot bands as labeled above each lane. 5–12 μg protein loaded/lane.

ships, suggesting that the effect of glycans on $K_v1.5$ activation caused a linear shift in G–V relationships (Table 1). Together, these data indicate that sialic acid residues attached to $K_v1.5$ N-glycans fully account for the effect of N-glycans on $K_v1.5$ gating—a novel finding among Shaker family K_v channel isoforms (see Discussion).

Sialic acids similarly modulated $K_v1.5$ activation rate, with channels activating more slowly under conditions of reduced sialylation (Fig. 2C). Note the time constants of activation were shifted along the voltage axis by approximately 18 mV, consistent with the magnitude of the shift in voltage of half-activation (V_a) observed (Table 1, Fig. 2). The activation rates for the $K_v1.5^{N290Q}$ and $K_v1.5^{S292A}$ mutant channels when studied under conditions of full or reduced sialylation were nearly identical and did not significantly deviate from the activation rates for wild-type $K_v1.5$ under conditions of reduced sialylation (Fig. 2C). Thus, the data indicate that sialic acids impact $K_v1.5$ gating, causing the channel to activate at more hyperpolarized potentials.

Unlike $K_v1.5$ activation, the voltage dependence of steady-state inactivation (channel availability) was not significantly altered under conditions of reduced sialylation. As previously described, the glycosylation-site deficient mutant channels, $K_v1.5^{N290Q}$ and $K_v1.5^{S292A}$ inactivated at potentials not significantly different than that of wild-type $K_v1.5$ expressed under conditions of full and reduced sialylation. Thus, the data indicate that sialic acids modulate $K_v1.5$ activation, but have no effect on $K_v1.5$ inactivation—another characteristic of the effects of glycans on $K_v1.5$ gating that is unique among K_v1 isoforms.

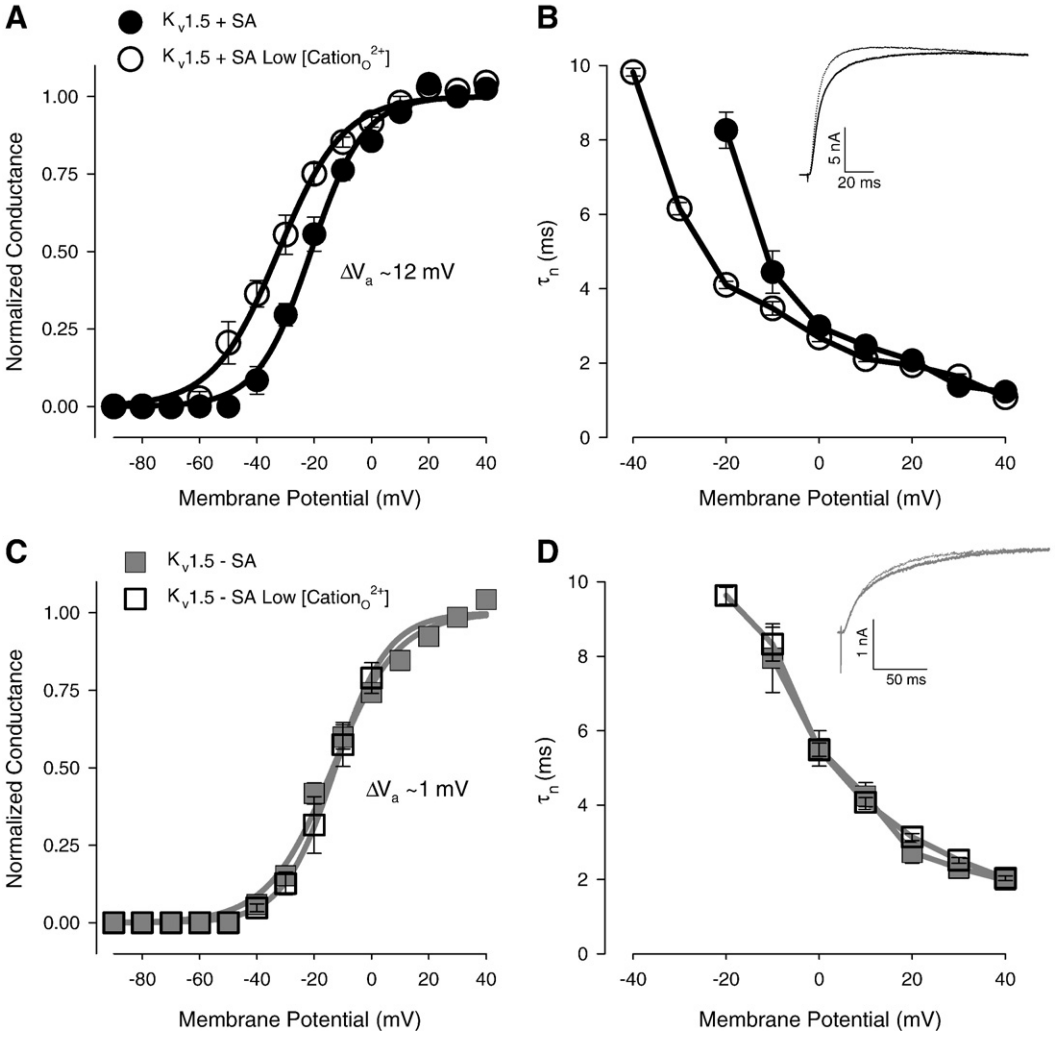

Fig. 4. Sialic acids modulate $K_v1.5$ gating through apparent electrostatic mechanisms. Wild-type $K_v1.5$ expressed under conditions of full and reduced sialylation at 3 mM (normal) and 0.3 mM (low) external divalent cation concentrations ($n = 3$–4). Symbols as listed in panels A and C. (A and C) Conductance–voltage (G–V) relationships of wild-type $K_v1.5$ expressed in fully sialylating (Pro5, A) and reduced sialylating (Lec2, C) cells at normal and low external divalent cation concentrations. Data are the mean normalized peak conductance ± SEM and are fit to single Boltzmann relationships (lines). (B and D) Activation time constants (τ_n) for wild-type $K_v1.5$ under conditions of full (Pro5, B) and reduced (Lec2, D) sialylation in the presence of normal and low external divalent cation concentrations. Insets: current traces measured during a −20 mV test pulse. Peak currents were normalized for comparison. The scaling factors used to normalize the currents were 1.02 to 1.13. Solid: normal [divalent cation$_o$]; dotted: low [divalent cation$_o$]. Data are mean ± SEM. Lines are non-theoretical point to point.

3.1.3. $K_v1.5$ is N-glycosylated

Immunoblot analysis verified that $K_v1.5$ expressed in Pro5 cells is likely N-glycosylated and sialylated (Fig. 3). To test whether wild-type $K_v1.5$ is N-glycosylated and sialylated, whole cell homogenates expressing wild-type $K_v1.5$ and the glycosylation-site deficient channel, $K_v1.5^{N290Q}$, under varying conditions of glycosylation and sialylation were run on 6.5% acrylamide gels (SDS-PAGE). The predicted molecular weight (MW) of wild-type $K_v1.5$ was compared to that of wild-type $K_v1.5$ treated with PNGase-F (to remove N-glycans attached to $K_v1.5$) to determine whether wild-type $K_v1.5$ is N-glycosylated. The data show that wild-type $K_v1.5$ bands are the most diffuse, and that the $K_v1.5$ Pro5 lysate is the only sample that runs as two bands, with the upper band of the Pro5 $K_v1.5$ lysate the largest MW of any band on the blot. The deglycosylated (PNGase-F treated) wild-type $K_v1.5$ runs as a single band of lower MW than untreated wild-type $K_v1.5$, suggesting that $K_v1.5$ is N-glycosylated. Confirmation that wild-type $K_v1.5$ is N-glycosylated was indicated by comparing the MW of deglycosylated wild-type $K_v1.5$ to that of a glycosylation-site deficient mutant that cannot be N-glycosylated, $K_v1.5^{N290Q}$. Both samples ran as single bands of nearly identical MW, confirming that wild-type $K_v1.5$ is N-glycosylated. Note also that wild-type $K_v1.5$ expressed in Lec2 cells and desialylated wild-type $K_v1.5$ (expressed in Pro5 cells and treated with neuraminidase) produced single bands of intermediate MW, suggesting that wild-type $K_v1.5$ is sialylated. Together, the data suggest that wild-type $K_v1.5$ is N-glycosylated and sialylated as expressed in Pro5 cells.

3.1.4. $K_v1.5$ sialic acids modulate channel gating by contributing to the external negative surface potential

Negatively charged sialic acids are typically the terminal residue attached to external glycans [9]. These negative charges may contribute to the external surface potential that impacts channel gating, as outlined by the surface potential theory [33]. If so, the fully sialylated channel should activate at more hyperpolarized potentials than the less sialylated channel, as observed for $K_v1.5$ (Fig. 2B). Further, the presence of external divalent cations would screen the surface potential and reduce the impact of the negatively charged sialic acid residues on the voltage dependence of channel gating [33,34]. Here, the G–V relationships for wild-type $K_v1.5$ expressed in Pro5 and Lec2 cells were studied at two different external divalent cation concentrations (Fig. 4A and C). The data show that wild-type $K_v1.5$ under fully sialylating conditions (in Pro5 cells) was sensitive to changes in extracellular divalent cations; the G–V relationship at the lower [divalent cation$_o$] shifted by a hyperpolarizing ~12 mV (Fig. 4A). Changes in [divalent cation$_o$] had no significant effect on wild-type $K_v1.5$ gating when expressed in the non-sialylating (Lec2) cell line (Fig. 4C). Extracellular divalent cations similarly modulated wild-type $K_v1.5$ activation rate, with fully sialylated channels activating more rapidly under conditions of reduced extracellular [divalent cation$_o$] (Fig. 4B). Wild-type $K_v1.5$ activation rate was not affected by changes in external divalent concentration when expressed in Lec2 cells (Fig. 4D). These data suggest that sialic acids attached to $K_v1.5$ N-glycans contribute to the external negative surface potential and thereby modulate $K_v1.5$ activation through electrostatic mechanisms.

3.2. $K_v1.4$ gating is not modulated by N-glycosylation or sialylation

3.2.1. Sialic acids do not affect $K_v1.4$ gating

$K_v1.4$ is an A-type K_v channel involved in action potential repolarization and is expressed in many regions of the brain and heart [30,35–38]. Fig. 5 A shows the normalized, average G–V relationships for wild-type $K_v1.4$ expressed in Pro5 and Lec2 cells. Note that the G–V relationships for wild-type $K_v1.4$ under conditions of full and reduced sialylation were nearly identical (Fig. 5A, Table 1). Further, there was no significant difference in the voltage dependence of steady-state inactivation for wild-type $K_v1.4$ as expressed in Pro5 and Lec2 cells (Fig. 5B). Therefore, sialylation (in CHO cells) does not alter $K_v1.4$ channel gating.

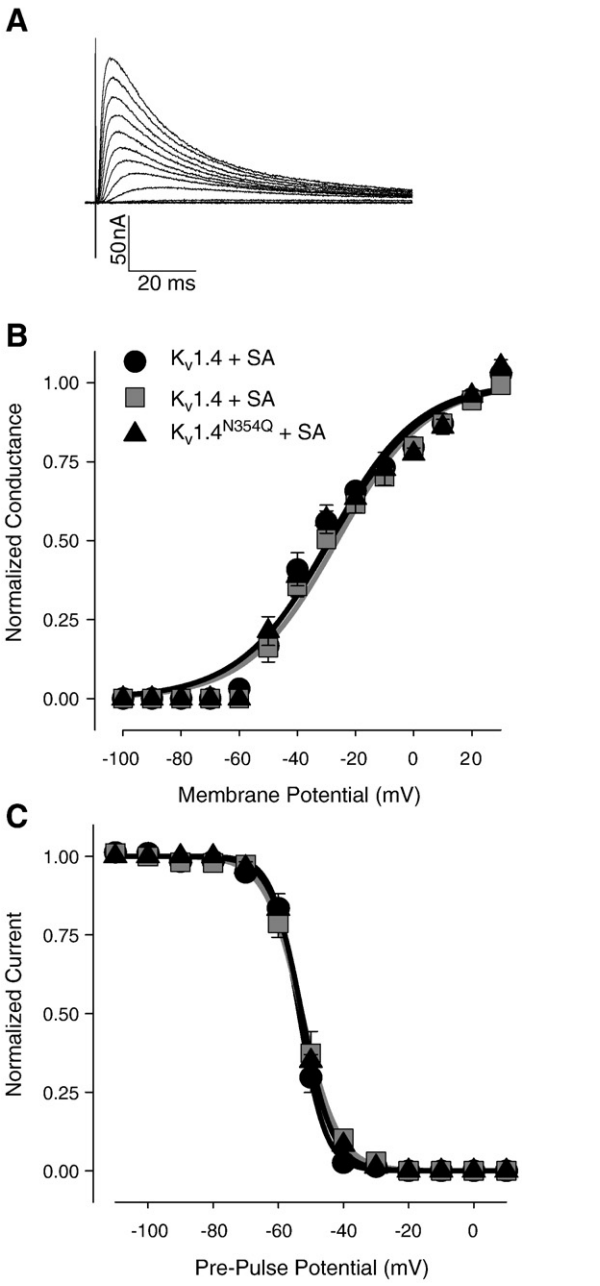

Fig. 5. $K_v1.4$ gating is not modulated by sialylation or N-glycosylation. (A) Typical wild-type $K_v1.4$ whole Pro5 cell current traces. (B) G–V relationships for wild-type $K_v1.4 \pm$ SA and $K_v1.4^{N354Q} +$ SA. Data are the mean normalized peak conductance ± SEM and are fit to single Boltzmann relationships (lines). (C) Steady-state channel availability (inactivation). Data are the mean normalized peak current ± SEM measured during a 100 ms pulse to +60 mV, following a 1000 ms pre-pulse to the plotted potentials. Curves are single Boltzmann distribution fits to the data. Symbols (for B and C) as listed in panel B. $n = 3–7$ (see Table 1).

3.2.2. N-glycosylation does not alter gating of $K_v1.4$

Like most K_v1 channels, $K_v1.4$ contains an N-glycosylation consensus sequence along the S1-S2 linker [30]. To determine whether N-glycans other than sialic acids modulate $K_v1.4$ gating, a mutant $K_v1.4$ ($K_v1.4^{N354Q}$) was generated by replacing the asparagine residue (N) of the N-glycosylation consensus site with a glutamine residue (Q). $K_v1.4^{N354Q}$ produced currents similar to those of the wild-

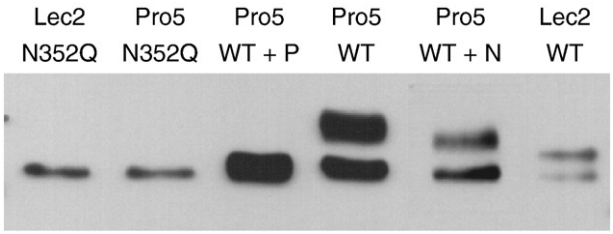

Fig. 6. $K_v1.4$ is N-glycosylated and sialylated. Immunoblot of whole cell homogenates expressing wild-type $K_v1.4$ (WT) and $K_v1.4^{N354Q}$ (N354Q) under variable conditions of glycosylation and sialylation. Samples labeled were treated with PNGase-F (P) to remove full N-glycan structures or neuraminidase (N) to remove sialic acids. 7.5–15 μg protein loaded per lane.

type channel. Gating of wild-type $K_v1.4$ and the mutant $K_v1.4^{N354Q}$ was nearly identical, with no significant difference observed for steady-state activation or inactivation (Fig. 5, Table 1). Thus, neither N-glycans nor sialic acids modulate $K_v1.4$ gating.

3.2.3. $K_v1.4$ is N-glycosylated and sialylated

To determine whether $K_v1.4$ expressed in CHO cells is N-glycosylated and sialylated, immunoblot gel shift analysis of whole cell homogenates expressing wild-type $K_v1.4$ and the glycosylation-site deficient $K_v1.4^{N354Q}$ was performed under varying conditions of glycosylation and sialylation (Fig. 6). Note that $K_v1.4^{N354Q}$ expressed in Pro5 or Lec2 cells and the deglycosylated (PNGase-F treated) wild-type $K_v1.4$ lysate ran as single bands of lower predicted MW. However, wild-type $K_v1.4$ ran as a doublet, with a lower band of similar MW to the mutant and deglycosylated samples and an upper band of much higher MW. Further, wild-type $K_v1.4$ homogenates under conditions of reduced sialylation (expressed in Lec2 cells or treated with neuraminidase) also ran as doublets. The lower band was of similar MW to the mutant and deglycosylated samples; however, the upper band was a lower MW than that of the wild-type $K_v1.4$ Pro5 cell homogenate, indicating that $K_v1.4$ as expressed in Pro5 cells is sialylated, but is not sialylated as expressed in Lec2 cells. Further, the data indicate that $K_v1.4$ expressed in CHO cells is N-glycosylated.

4. Discussion

As indicated by Fig. 1, $K_v1.4$ and $K_v1.5$ each contain an N-glycosylation consensus site along the S1–S2 linker [30]. Alignment (starting from the S1 segment, see Fig. 1) of $K_v1.4$ and $K_v1.5$ S1–S2 linkers indicate that the $K_v1.5$ linker is ten amino acids longer than the $K_v1.4$ linker. In addition, the $K_v1.5$ consensus N-glycosylation site is located four amino acids C-terminal to the location of the $K_v1.4$ N-glycosylation consensus site. Figs. 3 and 6 confirm that $K_v1.4$ and $K_v1.5$ are N-glycosylated. The data presented here indicate that sialic acids attached to channel N-glycans contribute to the voltage-dependence of channel gating for $K_v1.5$, but have no effect on $K_v1.4$ gating. As will be described, the impact of sialic acids on $K_v1.5$ gating is unique among K_v1 isoforms. Further, $K_v1.4$ is the first K_v1 isoform tested in which neither sialic acids nor N-glycans modulated channel gating.

4.1. Sialylated N-glycans account for the full effect of glycosylation on $K_v1.5$ gating—a novel finding for modulation of Shaker potassium channel function

Sialic acids have a large impact on $K_v1.5$ gating. The G–V relationship for $K_v1.5$ was shifted by a linear ~18 mV under conditions of reduced sialylation and following mutagenesis used to prevent N-glycosylation (Fig. 2B; Table 1). This ~18 mV shift in voltage-dependence of channel activation is the largest effect of N-glycans on a K_v1 isoform reported to date. The time constants of activation were shifted in the same manner as the G–V relationships and were nearly identical for wild-type $K_v1.5$ under conditions of reduced sialylation and for the non-glycosylated mutant channels, suggesting that sialic acids modulate $K_v1.5$ gating through electrostatic interactions (Fig. 2C). This was confirmed by the charge screening experiment outlined in Fig. 4. Together, these data indicate that the full effect of N-glycans on $K_v1.5$ gating is imposed by sialylated N-glycans.

The surface potential theory predicts that the electric field experienced by the ion channel voltage sensor(s) is altered by external negative charges that contribute to a surface potential. External divalent cations act to screen these negative charges, effectively neutralizing their impact on the voltage sensed by the channel gating mechanism(s). Thus, a greater depolarization is required to activate the channel, producing a positive shift in the voltage at which the channel activates. Previous studies that described an electrostatic mechanism reported all voltage-dependent gating parameters (e.g., steady-state activation and inactivation) were nearly equally shifted along the voltage axis by changes in channel sialylation, with no impact on the Boltzmann slope factors [13,17,18]. However, with each gating step and between forward and reverse gating kinetics, the contribution of local surface potentials can vary (as reviewed by Hille [3]). Here, for $K_v1.5$ under conditions of reduced sialylation and of no N-glycosylation, we observed an ~18 mV linear shift in G–V relationship and a similar ~18 mV shift in the activation time constant (Fig. 2A–C). We confirmed that this shift in $K_v1.5$ activation gating was likely produced through electrostatic mechanisms (Fig. 4). However, no shift in steady-state channel availability (inactivation) was observed (Fig. 2D), suggesting that sialic acids do not confer a uniform effect on all voltage-dependent gating parameters. Consistent with the data, sialic acids may variably impact the voltage sensed by $K_v1.5$ gating mechanisms, resulting in a non-homogenous effect of sialic acids on channel gating.

In addition to an impact on surface potential, previous reports suggested that glycosylation can alter channel state stability [14,20]. The gating stabilizing theory (summarized by Hille [3] and Watanabe et al. [14]), predicts a change in G–V relationship slope for any change in state stability, as reported previously [14]. Such an effect was not observed in this study. The G–V slope factor (K_a) measured for the two non-glycosylated mutant channels and wild-type $K_v1.5$ expressed under conditions of reduced sialylation were not significantly different than the K_a measured for the fully sialylated wild-type $K_v1.5$ channel (Fig. 2B, Table 1). Thus, the effect of sialic acids on $K_v1.5$ activation is likely achieved solely through electrostatic mechanisms (surface potential theory). The data do not support an additional mechanism by which sialic acids or other N-glycans contribute to $K_v1.5$ gating (gating stabilizing theory). This finding is unique among Shaker-like potassium channel isoforms. Previous studies of the Drosophila Shaker K^+ channel, ShB, and the mammalian $K_v1.1$ and $K_v1.2$ channels reported additional effects of N-glycans and/or sialic acids on K_v1 channel function, consistent with a contribution of N-glycans to channel state stability [14,19,20]. Further, reports indicated an effect of sialic acids and N-glycans on K_v channel inactivation [14,15,39]. The data shown here provide the first evidence that gating of a Shaker family potassium channel isoform, $K_v1.5$, is modulated by sialylated N-glycans acting through an electrostatic mechanism that does not measurably impact channel inactivation, but is responsible for the full effect of N-glycans on $K_v1.5$ gating.

4.2. N-glycosylation does not affect $K_v1.4$ voltage-dependent gating

In this study, reduced $K_v1.4$ glycosylation, achieved through a reduction in channel sialylation or mutagenesis used to remove the full N-glycan structure, had no effect on $K_v1.4$ gating (Fig. 5). In particular, gating of the glycosylation-site deficient mutant channel, $K_v1.4^{N354Q}$, mimicked the gating of the fully sialylated wild-type $K_v1.4$, which strongly suggests that N-glycosylation does not modulate $K_v1.4$ gating as expressed in CHO cells.

Interestingly, a previous study by Johnson and Bennett examined the impact of sialylation and N-glycosylation on a similar channel, ShB [20]. K_v1.4 and ShB are the only two Shaker family K^+ channel isoforms that undergo N-type inactivation. Both are N-glycosylated, but ShB has two potential N-glycosylation sites located within the S1–S2 linker, while K_v1.4 has only one site [40,41]. Unlike K_v1.4, a reduction in ShB sialylation resulted in depolarizing shifts in steady-state and kinetic activation and inactivation of ShB. Further, N-linked sugars other than sialic acids were shown to exert an additional, state stabilizing, effect on channel gating [20]. The distinct effects of N-glycans on K_v1.4 and ShB gating may be due to the number and location of putative N-glycosylation sites. That is, 1) the presence of two N-glycosylation sites on the ShB S1–S2 linker may vary the position of the glycans and glycan structures relative to the voltage sensor and/or 2) two glycan structures per subunit could lead to a mature K^+ channel that is potentially twice as sialylated. Both possible outcomes could lead to a greater effect of N-glycans on channel gating. Further, the ShB S1–S2 linker is significantly shorter than are any of the mammalian K_v1 isoforms, and the ShB N-glycosylation sites are located near the middle of the linker. The K_v1.4 N-glycosylation site can be found closer to the S2 transmembrane segment (Fig. 1). These slight alterations in linker length and position of the N-glycosylation site(s) may account for the differing effects of glycosylation on rapidly inactivating Shaker family K^+ channel gating.

Why does sialylation of one K_v1 channel isoform (K_v1.5) modulate channel gating, but has no measurable effect on gating of another member of the K_v1 channel subfamily (K_v1.4)? There are several possible mechanisms that could explain our data that include: 1) the slight difference in S1–S2 linker size, with the K_v1.4 linker 10 amino acids shorter, would place the N-glycans in different locations relative to the channel gating mechanisms. If, for example, sialic acid residues are too distant from the electric field to alter it, then sialic acids could not impose an electrostatic effect on channel gating, and 2) the relatively lengthy N-terminus of K_v1.4 required for N-type inactivation might impact channel and glycan structure such that the glycans no longer modulate channel gating, and 3) the distinct characteristics of the amino acid residues in the middle of the N-glycosylation consensus sequence or residues surrounding the N-glycosylation site could be responsible for the different phenomena observed relative to N-glycans. For K_v1.5, the middle N-glycosylation site residue is glycine (G), while the middle K_v1.4 residue is aspartate (D) (Fig. 1). Also, there are many structurally important proline residues in the K_v1.5 S1–S2 linker with only one in the K_v1.4 linker. The difference in side chain length, in charge, or in impact of the S1–S2 amino acid side chains on the channel's secondary and tertiary structure, might have a direct effect on the ability of N-glycans to modulate channel gating. In summary, the lack of a sialic acid or N-glycosylation-dependent effect on K_v1.4 gating parameters may be caused by a slight change in channel or glycan structure and/or location relative to the electric field sensed by the channel gating mechanism. Further study is required to determine the exact difference (if any) in location of the glycan structure relative to the channel gating mechanisms of K_v1.4 and K_v1.5.

4.3. Physiological relevance

Voltage-gated ion channels contribute to action potential initiation and propagation in skeletal muscle, neuronal, and cardiac cells. K_v channels produce currents that are responsible for AP repolarization [3]. Often, the activities of several K_v channel isoforms are responsible for AP repolarization. The AP waveform is modulated (remodeled) through changes in the expression, density, and/or distribution of specific K_v isoforms. Changes in posttranslational modifications, such as glycosylation, can potentially impact gating of K_v channels, leading to changes in AP waveform [15,22,39]. Thus, K_v channel activity would be modulated by changes in glycosylation and/or sialylation that result from remodeling of K_v isoforms (subunit-specific differential glycosylation). For example, as K_v1.5 expression and distribution are regulated, AP repolarization would be altered by the changes in K_v1.5 activity. Because sialylation modulates K_v1.5 function uniquely, regulated expression of K_v1.5 would lead to isoform-specific changes in the contribution of sialic acid to AP repolarization.

In addition to the relevant isoform-specific effects of glycosylation on channel function, AP repolarization may be modulated through a cell-specific process of regulated glycosylation in which the glycosylation structures attached to a single K_v channel type might vary, thereby altering K_v channel activity. The data presented here indicate that K_v1.5 activity is modulated by sialylated N-glycans. Our lab previously showed that the cardiac voltage-gated sodium channel, Na_v1.5, is less sialylated in neonatal ventricles than in neonatal atria. This difference in Na_v1.5 sialylation is responsible for the differences in channel gating observed [28]. Recently, we showed that regulated and aberrant changes in the ability of cardiomyocytes to glycosylate cardiac Na_v channels led to altered Na_v channel gating and to consistent changes in action potential waveforms [42]. K_v channels, including K_v1.5, may follow a similar sialylation pattern in atria and ventricles. If so, based upon these studies we would predict that action potential duration (APD) would be shortened in the neonatal atria compared to ventricles because of a sialic acid-dependent increase in K_v channel activity in the atria (as observed, personal observation). Therefore, as cardiac glycosylation is regulated, K_v1.5 sialylation levels would change, modulating K_v1.5 gating. This cell-specific change in K_v1.5 gating would have a direct effect on AP repolarization.

K_v1.5 encodes the ultra rapid delayed rectifier current, I_{Kur}, in human atria [43]. Due to the specificity of I_{Kur}, as it is not expressed in human ventricular myocytes or Purkinje fibers, modulation of I_{Kur} and K_v1.5 is considered a potential target for the treatment of atrial arrhythmias [44]. Considering the results from this study, altering sialylation of K_v1.5 could be explored as a potential therapy to control atrial fibrillation.

Alterations in normal K_v channel function can have severe pathological consequences. Diseases such as Long QT Syndrome, deafness, and epilepsy can be due to changes in the AP waveform that result from variations in K_v channel gating [5–7,45,46]. At least two major disorders of aberrant glycosylation are known to result in decreased glycoprotein sialylation—Congenital Disorders of Glycosylation (CDGs; ~30 known diseases) and Chagas' disease; the latter of which is caused by parasitic infection. Nearly 20 million people worldwide are afflicted with these diseases and suffer symptoms consistent with altered excitability of neurons and muscle [25,26,47,48]. Because reduced glycoprotein sialylation is a consistent outcome of these diseases, it is likely that K_v1.5 sialylation and activity would be decreased in such patients, leading to prolonged AP repolarization.

4.4. Summary

Here, we show that N-glycosylation, specifically sialic acids, impact gating of one K_v1 subfamily member, K_v1.5, but has no effect on gating of K_v1.4. Our data are novel in several respects including: 1) K_v1.4 is the only member of the K_v1 subfamily in which the attached N-glycans have no measurable effect on channel gating, 2) with reduced sialylation/glycosylation, K_v1.5 exhibited the largest depolarizing shift in voltage-dependence of activation of any member of the K_v1 subfamily, 3) K_v1.5 is the first K_v isoform in which channel inactivation is not modulated by N-glycans, and 4) K_v1.5 is the only member (to date) of the Shaker K_v channel family for which sialic acids attached through N-linkages account fully for the impact of N-glycans on channel activation, produced solely by an electrostatic effect. Because K_v1.5 produces an important repolarizing current in the atria, I_{Kur}, altered K_v1.5 channel function, whether through regulated expression or glycosylation, could lead to significant changes in AP repolarization [49]. Together, these data demonstrate that members of

the K_v1 channel subfamily are affected by glycosylation through distinct mechanisms. The unique effects of glycans on K_v1 family isoforms are relevant to changes in AP repolarization that occur with regulated expression and glycosylation of each K_v1 isoform. Further, the implications of this study are broad given the extent of regulated glycosylation throughout the body and the potential for pathological consequences due to aberrant glycosylation.

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