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COMMUNICATION

Deglycosylation of Shaker K_V channels affects voltage sensing and the open–closed transition

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Most membrane proteins are subject to posttranslational glycosylation, which influences protein function, folding, solubility, stability, and trafficking. This modification has been proposed to protect proteins from proteolysis and modify protein–protein interactions. Voltage-activated ion channels are heavily glycosylated, which can result in up to 30% of the mature molecular mass being contributed by glycans. Normally, the functional consequences of glycosylation are assessed by comparing the function of fully glycosylated proteins with those in which glycosylation sites have been mutated or by expressing proteins in model cells lacking glycosylation enzymes. Here, we study the functional consequences of deglycosylation by PNGase F within the same population of voltage-activated potassium (K_V) channels. We find that removal of sugar moieties has a small, but direct, influence on the voltage-sensing properties and final opening–closing transition of Shaker K_V channels. Yet, we observe that the interactions of various ligands with different domains of the protein are not affected by deglycosylation. These results imply that the sugar mass attached to the voltage sensor neither represents a cargo for the dynamics of this domain nor imposes obstacles to the access of interacting molecules.

Introduction

N-glycoproteins are highly abundant within the proteome (Apweiler et al., 1999). Most membrane proteins, as they travel along the secretory pathway, are exposed to a diverse and complex set of sequential glycosylation reactions that have important roles in protein folding and other biological functions (Varki, 1993; Moremen et al., 2012). A wide range of ion channels, including voltage-activated Na^+, Ca^{2+}, and K^+ (K_V); acetylcholine receptors; transient receptor potential channels; and cyclic nucleotide–gated channels, have been shown to be glycosylated (Covarrubias et al., 1989; Recio-Pinto et al., 1990; Deal et al., 1994; Santacruz-Toloza et al., 1994; Schwalbe et al., 1995; Gurnett et al., 1996; Thornhill et al., 1996; Castillo et al., 1997; Petrecca et al., 1999; Shi and Trimmer, 1999; Zhang et al., 1999; Freeman et al., 2000; Pabon et al., 2000; Ufret-Vincenty et al., 2001a; Zhu et al., 2001, 2003; Bennett, 2002; Much et al., 2003; Faillace et al., 2004; Sutachan et al., 2005; Wirkner et al., 2005; Meighan et al., 2013; Watanabe et al., 2015).

Voltage-activated K^+ (K_V) channels are involved in shaping action potentials as well as tuning the pattern of electrical signals in neurons (Hille, 2001). The membrane core of these channels is formed by four identical or homologous α subunits that assemble as a complex that contains a central ion permeation pathway and four peripheral voltage-sensor domains (VSDs; Long et al., 2005). Each α subunit comprises six transmembrane segments from which the first four (S1–S4) constitute the VSD and the last two (S5–S6) constitute the pore. Diversity of this family of ion channels derives from the large number of members (Southan et al., 2016) as well as posttranscriptional (Holmgren and Rosenthal, 2015) and posttranslational modifications, such as phosphorylation (Drain et al., 1994; Ivanina et al., 1994; Levin et al., 1996) and glycosylation (Deal et al., 1994; Santacruz-Toloza et al., 1994; Schwalbe et al., 1995; Thornhill et al., 1996; Petrecca et al., 1999; Shi and Trimmer, 1999; Freeman et al., 2000; Khanna et al., 2001; Ufret-Vincenty et al., 2001b; Zhu et al., 2001, 2003; Watanabe et al., 2003, 2004, 2007, 2015; Napp et al., 2005; Sutachan et al., 2005; Fujita et al., 2006; Johnson and Bennett, 2008; Hall et al., 2015).

Glycosylation in K_V channels influences cell surface expression and stability (Petrecca et al., 1999; Khanna et al., 2001; Watanabe et al., 2004, 2015; Fujita et al., 2006; Hall et al., 2015) and in some instances changes the functional properties of the channel (Thornhill et al., 1996; Freeman et al., 2000; Ufret-Vincenty et al., 2001b; Watanabe et al., 2003, 2007; Napp et al., 2005; Sutachan et al., 2005; Johnson and Bennett, 2008). Many members of the K_V1 channel family are glycosylated within the VSD. Because the movements of this domain are coupled to opening of the channel,

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its glycosylation has been shown to influence the speed of channel opening and the steady-state voltage dependence of activation (Thornhill et al., 1996; Watanabe et al., 2003, 2007; Johnson and Bennett, 2008). Here, we set to determine the direct influence of sugar moieties attached to the VSD on the structural dynamics of this domain. Because the functional consequences of glycosylation in ion channels have commonly been assessed by comparing different populations of ion channels (e.g., glycosylated channels with N→Q mutant channels or expression of channels in cell lines with distinct restrictions in the glycosylation process), we implemented an approach that directly assesses the role of glycosylation on the VSD function within the same population of channels. We studied the consequences of deglycosylation by PNGase F on the gating currents (Bezanilla, 2000) and the binding/unbinding of hanatoxin, a voltage-sensor toxin (Swartz, 2007). We found that deglycosylation of Shaker K_V channels occurs in minutes, giving rise to ion channels with rightward displaced charge distribution on the voltage axis. This shift was not accompanied by changes in the kinetics of the gating currents, consistent with the notion that sugars attached to the VSD do not represent a cargo to the dynamics of this domain. Nonetheless, as previously reported (Thornhill et al., 1996; Watanabe et al., 2003, 2007; Johnson and Bennett, 2008), a reduction in the speed of activation at positive potentials and deactivation at negative potentials was observed from macroscopic ionic currents after PNGase F treatment, indicating that sugars might influence the C↔O transition. To isolate this transition, we introduced the triple-mutant ILT into our Shaker K_V construct (Smith-Maxwell et al., 1998a,b). Deglycosylation by PNGase F to these channels also slowed the opening of these channels. These results combined suggest that glycosylation has a direct influence on the opening and closing of K_V channels. Finally, we found that glycosylation does not alter the interactions of either gating-modifier toxins or pore blockers with the Shaker K_V channels.

Materials and methods

Protein extraction and Western blotting

Xenopus laevis oocytes were injected with cRNA of noninactivating (Δ6–46) Shaker K_V channels (Hoshi et al., 1990) that contained a c-myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) inserted at the C terminus (after Val638) and incubated in ND96 solution (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 5 HEPES, pH 7.6) at 17°C. 24 h after injection, oocytes were exposed to 10 mg/ml PNGase F diluted 1:10 in ND96. The enzyme activity was monitored by removing 10 oocytes at different times, supplemented with 100 mM glycine and lysed in 200 µl buffer H (1% Triton X-100, 100 mM NaCl, and 20 mM Tris-HCl, pH 7.4). Lysates were rocked at room temperature for 15 min and then centrifuged at 13,000 rpm for 3 min. The pellet was discarded, and the supernatant was analyzed by Western blot by using anti–Myc mouse monoclonal antibody at a dilution of 1:5,000 (Clontech) and detected with a secondary, goat anti–mouse antibody conjugated to horseradish peroxidase at a dilution of 1:10,000 (Pierce). Membranes were developed by SuperSignal WestFemto (Thermo Fisher Scientific) and visualized by chemiluminescence by using a FluorChem E Imager (Cell Biosciences).

Electrophysiological recordings

Xenopus oocytes were injected with in vitro transcripts of three different Shaker K_V channel constructs: noninactivating (Δ6–46) Shaker K_V channels (Hoshi et al., 1990) to determine the voltage dependence of the relative probability of opening, hanatoxin-sensitive Shaker K_V channels (Milescu et al., 2013) to assess toxin binding, and nonconductive Shaker K_V channels W434F (Perozo et al., 1993) to measure gating currents. Oocytes were incubated at 17°C for 24–48 h in ND96 solution before recordings. Currents were acquired before treatment, then PNGase F was applied for 5 min in the recording chamber and washed out thoroughly before subsequent recordings.

Two-microelectrode voltage-clamp currents were acquired with an OC-725C oocyte amplifier (Warner Instruments) and digitized by using a Digidata 1321A interface and pCLAMP 10.3 software (Axon Instruments). Data were filtered at 1 kHz and digitized at 10 kHz. Microelectrode resistances were between 0.1 and 1.2 MΩ when filled with 3 M KCl. Solutions for recording contained 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, and 5 mM HEPES, pH 7.4. Voltage steps to +40 mV in 10-mV increments were given from a holding potential of −80 mV, which were returned to −50 mV by using a P/−4 subtracting protocol. Toxins were applied for 5 min before acquisitions were initiated.

For currents acquired by using a cut-open voltage-clamp oocyte technique (Taglialatela et al., 1992), we exclusively used the animal pole for recording. Oocytes were clamped with a Dagan CA-1B high-performance oocyte clamp. Data were acquired at 10 kHz and filtered at 5 kHz. A 0.2- to 0.3-MΩ pipette and groundpool were filled with 3M Tris HCl, while bridges were filled with 3M Na-MES in 3% agarose. Oocytes were permeabilized with 0.4% saponin in internal solution. For ionic current experiments, the internal solution contained (in mM): 110 potassium glutamate, 10 HEPES-NMG, and 10 EGTA-NMG, pH 7.3, and the external solution (in mM): 2.5 KCl, 120 NaMES, 1.8 $CaCl_2$, and 10 HEPES-NMG, pH 7.6. Gating-current experiments were performed with the same external solution, but the internal solution contained (in mM): 120 NMG-glutamate, 10 HEPES-NMG, and 10 EGTA-NMG, pH 7.3. A P/−4 subtracting protocol was used. Toxins used in this study were provided by K. Swartz (National Institutes of Health, Bethesda, MD).

Data analyzing

A weighted time constant was calculated when a double-exponential fit was used. The time constant of the activating or deactivating phases of ionic currents was determined by using distinct exponential fits.

PNGase F extraction

Origami (DE3) *Escherichia coli* competent cells transformed with pET22b (PNGase F) construct were grown at 37°C until absorbance at 600 nm reached 1.0 in Lysogeny broth medium supplemented with 15 mg/ml kanamycin, 50 mg/ml ampicillin, and 12.5 mg/ml tetracycline. The culture was then induced with 0.5 mM isopropyl β-D-1 thiogalactopyranoside and grown in a shaker incubator overnight at 20°C. Cells were harvested, washed with Tris buffered saline, and resuspended in lysis buffer (50 mM Tris, pH 8.0,

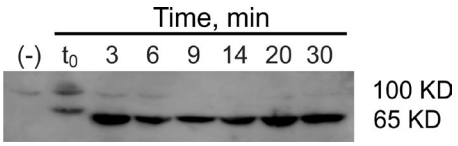

Figure 1. **Temporal course of PNGase F action on Shaker K_V channel.** Western blot showing the reduction of molecular weight after enzymatic digestion of the sugar moiety with PNGase F (1 mg/ml; *n* = 5).

150 mM NaCl, 1 mM PMSF, 0.5 mM $MgCl_2$, 0.5 mg/ml lysozyme, and 25 µg/ml deoxyribonuclease I). Cells were lysed by using EmulsiFlex-C5 (Avestin). The cell lysate was ultracentrifuged at 40,000 rpm for 1 h by using a TL45 rotor (Beckman). The soluble fraction was loaded on a 5-ml Ni-NTA prepacked column equilibrated with 150 mM NaCl, 10 mM imidazole, and 50 mM HEPES, pH 8.0, and eluted with a gradient from 10 to 500 mM imidazole. Peak fractions were dialyzed with two changes by using a storage buffer containing 150 mM NaCl, 50 mM HEPES, pH 8.0, and 10% glycerol. The purified protein was concentrated and flash frozen until use. Preliminary experiments were performed with a PNGase F gift from M. Mayer (National Institutes of Health, Bethesda, MD).

Results

PNGase F enzymatic activity in *Xenopus* oocytes

To assess the temporal course of PNGase F enzymatic activity, we incubated 70 *Xenopus* oocytes expressing Shaker K_V channels with 1 mg/ml PNGase F and proceeded to remove 10 oocytes at different times (Fig. 1). The WT Shaker K_V channel expressed in oocytes has two predominant glycosylation products (Santacruz-Toloza et al., 1994): a small (~70-kD) form and a large (~100-kD) form (Fig. 1, line t_0). Even within 3 min of PNGase F exposure, most of the Shaker K_V channel protein is deglycosylated, migrating as a smaller (~65-kD) band (Fig. 1, lines 3–30), similar to Shaker K_V channels in which their glycosylation sites have been mutated (Santacruz-Toloza et al., 1994). These results show that PNGase F acts rapidly, providing an amenable opportunity to study the role of glycosylation on the function of the channels in the same population of proteins.

Fig. 2 A (control) shows a family of K^+ currents in response to voltage protocol pulses from −80 mV to +40 mV, every 20 mV. Immediately after acquiring these data, the oocyte was exposed to PNGase F for 5 min to allow complete enzymatic digestion. After removal of the enzyme from the recording chamber, the oocyte was subjected to the same voltage protocol (Fig. 2 B). Shaker K_V channels clearly activated and deactivated more slowly after PNGase F exposure. The time the currents took to rise from 10% to 90% increased by approximately two- to threefold after deglycosylation (Fig. 2 C). Deglycosylation also slowed deactivation, with the time the tail currents took to decline to 10% increased from 14.7 ± 1.8 ms to 20.7 ± 2.6 ms before and after deglycosylation, respectively. These results are comparable to previous observations obtained from Shaker K_V or rat K_V1.1 channels expressed in mammalian cell lines (Thornhill et al., 1996; Watanabe et al., 2003; Johnson and Bennett, 2008). In those instances, however, functional comparisons were performed with different

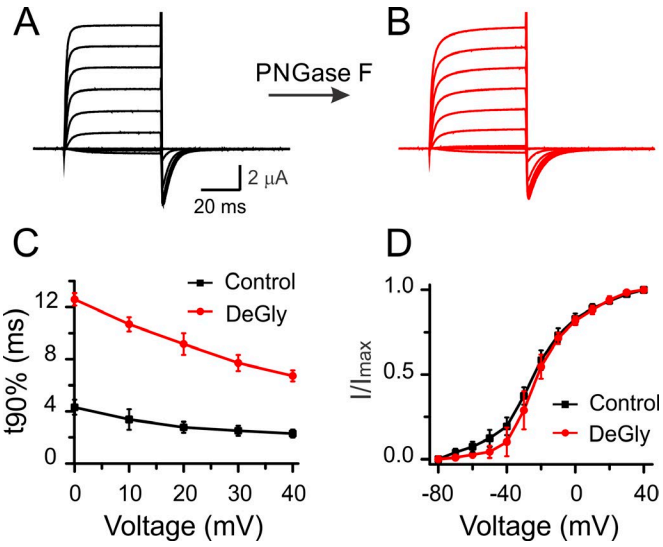

Figure 2. **Effects of deglycosylation on the ionic currents carried by Shaker K_V channels. (A and B)** Ionic currents from an oocyte expressing Shaker K_V channels before (A) and after (B) 5-min exposure to PNGase F. Ionic currents were recorded by using the two-microelectrode voltage-clamp technique. These currents were elicited by 50-ms voltage steps from a holding potential of −80 to +40 mV, every 10 mV. **(C)** Time rise from 10% to 90% current activation. **(D)** Relative conductance plots before (black) and after (red) PNGase F treatment. Error bars in C and D represent mean ± SEM, (*n* = 5). DeGly, deglycosylation.

populations of channels. For example, the population of deglycosylated channels derived from channels in which the glycosylated sites have been mutated or from WT channels expressed in mammalian cell lines in which glycosylation has been impaired. Even though the activation and deactivation kinetics of deglycosylated channels changed, the relative probability of opening at different voltage is similar (Fig. 2 D).

Because we externally applied PNGase F to the oocytes, deglycosylation should have occurred in all glycoproteins at the cell surface. To assess whether the removal of sugars from the Shaker K_V channels has a direct influence on function of the protein, we developed a Shaker K_V construct with both glycosylation sites mutated to aspartate (N259D–N263D), the end product at the site of cleavage by the enzyme (Tarentino and Plummer, 1994). Expressing this Shaker K_V construct in untreated oocytes would imply that all native glycoproteins remain intact while the N259D–N263D Shaker K_V mutant channels lack sugar moieties. Fig. 3 shows that the rise times from 10% to 90% of the current activation of these channels are comparable to those from WT channels treated with PNGase F. As a comparison, we also plotted the rise times of a Shaker K_V construct with the traditional mutations (N259Q–N263Q) to study glycosylation and protein function (Fig. 3, blue triangles). PNGase F treatment to oocytes expressing these glycosylation-deficient Shaker K_V channels did not alter the kinetics of activation or deactivation (Fig. 4, B and C). These results combined provide support to the idea that the functional consequences of deglycosylation of WT K_V channels are specific to the removal of sugars from the channels.

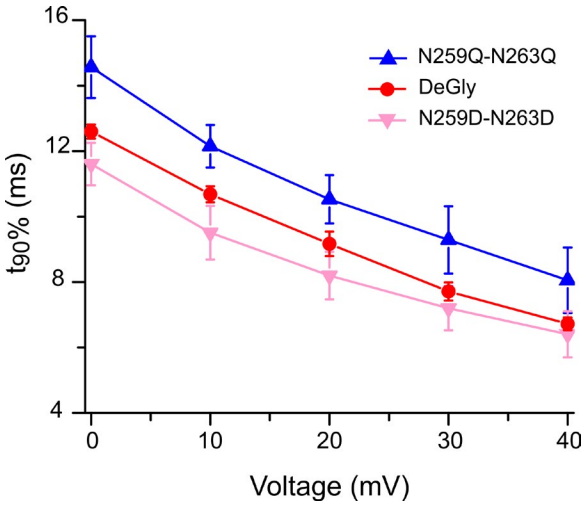

Figure 3. **Activation from glycosylation-deficient mutant Shaker K_V channels.** Rise time from 10% to 90% current activation from N259D–N263D mutant Shaker K_V channels (n = 3) and N259Q–N263Q mutant Shaker K_V channels (n = 3) and, for comparative purposes, those after PNGase F treatment. These data derived from ionic currents recorded using the two-microelectrode voltage clamp technique. Error bars represent mean ± SEM. DeGly, deglycosylation.

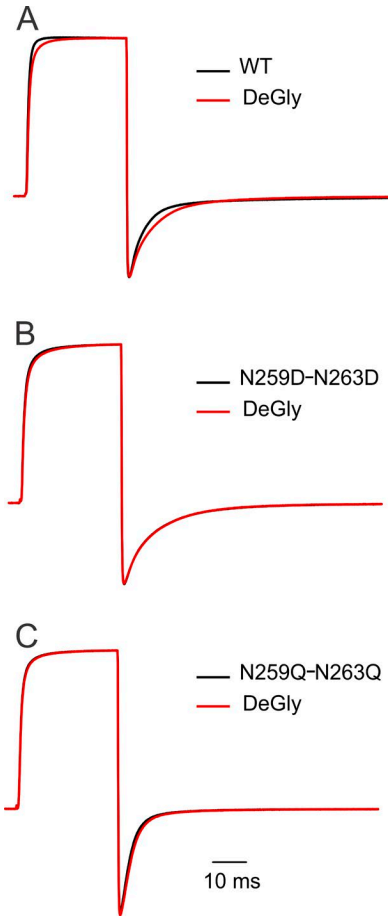

Figure 4. **PNGase F treatment of glycosylation-deficient mutant Shaker K_V channels. (A)** Normalized and superimposed ionic current traces from WT Shaker K_V channels before and after PNGase F treatment, shown for comparative purposes. **(B and C)** Normalized and superimposed ionic current recordings before and after PNGase F treatment of oocytes expressing glycosylation-deficient N259D–N263D Shaker K_V channels (B, n = 3) and N259Q–N263Q Shaker K_V channels (C, n = 3). These experiments were performed with the same batch of PNGase F enzyme. These ionic currents were elicited in response to a voltage step to +60 mV from a holding potential of −80 mV and were recorded by using the cut-open oocyte voltage-clamp technique. DeGly, deglycosylation.

Effect of deglycosylation on gating currents

We next asked whether sugar removal affects the motions of the VSD. The VSD is formed by the first four transmembrane segments of the channel (S1–S4). Within the S4, there are several arginines that are responsible for sensing voltage across the membrane (Aggarwal and MacKinnon, 1996; Seoh et al., 1996). In response to a voltage step, these charges rearrange resulting in nonlinear currents known as the gating currents (Bezanilla, 2000). Because the two glycosylation sites in Shaker K_V channels are within the VSD (Santacruz-Toloza et al., 1994), it is conceivable that the sugar mass attached to the VSD might influence its motions. We measured gating currents using a nonconductive (W434F) Shaker K_V channel construct (Perozo et al., 1993) using the cut-open voltage-clamp technique (Taglialatela et al., 1992). Fig. 5 A shows superimposed gating current traces in response to four voltage steps acquired from the same oocyte before (black) and after (red) PNGase F treatment. Two effects of deglycosylation were observed: (1) a reduction of 18 ± 4% (n = 7) of the total amount of charge (Fig. 5 B) and (2) a small shift of ∼6 mV toward positive potentials of the steady-state charge distribution (Fig. 5, B–D). Interestingly, this shift was not accompanied by changes in the kinetics of the gating currents (Fig. 5 A), as can be appreciated by the relaxations of the "on" gating currents. Further, the early development of the rising phase in the "off" currents, which is observed at −48 mV before glycosylation, is apparent after deglycosylation at about −44 mV.

Effect of deglycosylation on the activation and deactivation of ILT mutant channels

That PNGase F treatment produced little functional consequences in the movement of the voltage sensor suggests that the effects of deglycosylation on the activation and deactivation of the WT Shaker K_V channels might originate from changes in the opening and closing events. To isolate this transition, we used the ILT mutant Shaker K_V channels, which shift both the relative probability of opening by ∼80 mV toward positive potentials (Smith-Maxwell et al., 1998a,b) and the steady-state charge distribution of the gating currents toward negative potentials by ∼40 mV (Ledwell and Aldrich, 1999). Fig. 6 A shows ionic currents from ILT mutant channels before and after PNGase F treatment at activation voltages of +100 and +120 mV from a holding potential of −100 mV. Clearly, deglycosylation slowed the opening of these channels. The rise times from 10% to 90% of the current activation changed by 5.4 ± 1.0 ms at +100 mV and 4.7 ± 1.1 ms at +120 mV (n = 4). ILT channels close fast at −100 mV (Fig. 6), requiring nearly perfect linear capacity subtraction to have an appreciation of the effect of deglycosylation on closing. Like WT channels, the changes in deactivation are small, yet observed in all four experiments, two of them shown in Fig. 6 B.

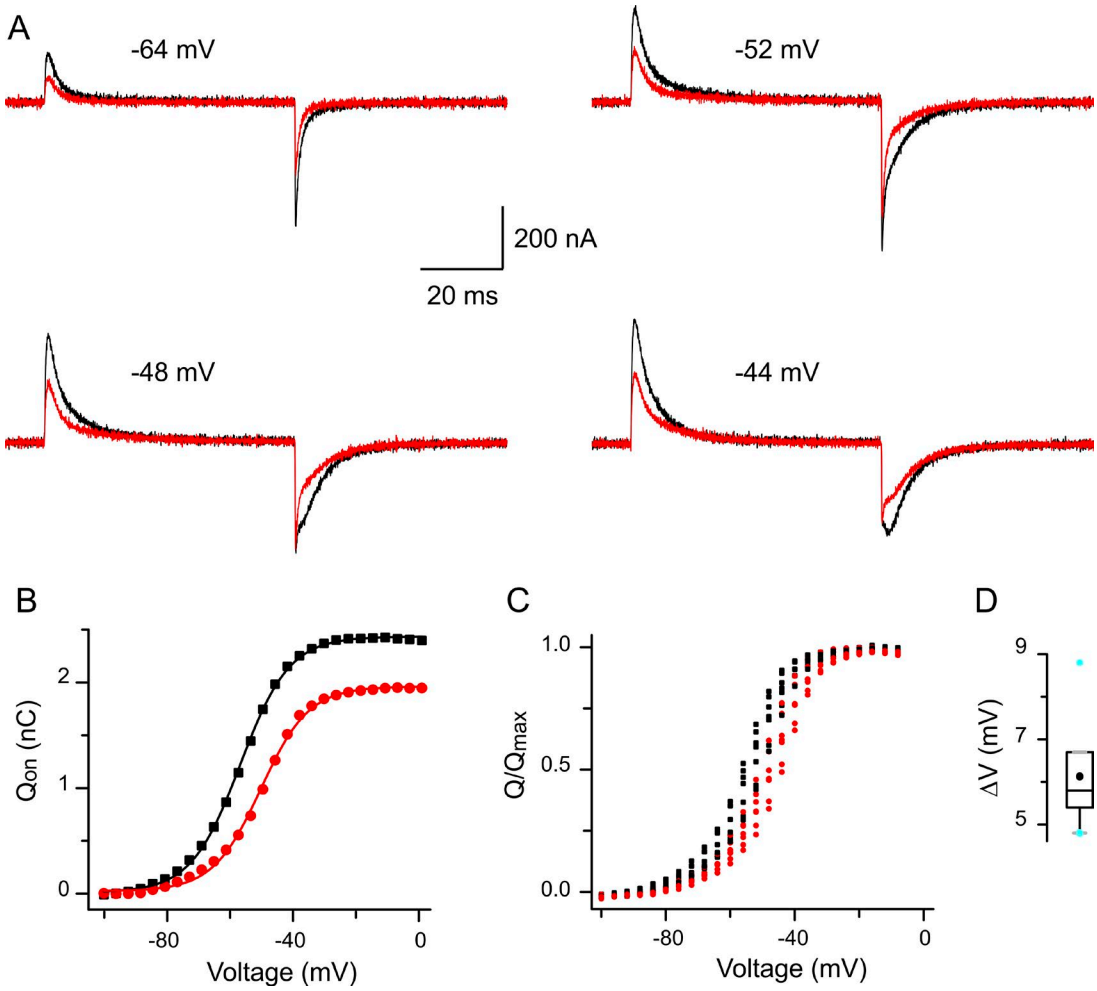

Figure 5. **Effect of deglycosylation on gating currents from the nonconducting (W434F) mutant Shaker K_V channel. (A)** Gating currents from an oocyte expressing nonconductive Shaker K_V channels before (black) and after (red) 5-min exposure to PNGase F. Gating currents were elicited in response to voltage steps from −100 mV to the voltage shown on the traces by using the cut-open oocyte voltage clamp. **(B)** Voltage dependence of the gating charge before and after PNGase F treatment from the experiment shown in A. Solid lines represent Boltzmann fits. The best parameter values for the total amount of charge (Q_{tot}) and the midpoint voltage ($V_{1/2}$) were 2.42 nC and −55.4 mV before treatment and 1.94 nC and −48.7 mV after PNGase F treatment, respectively. The reduction in Q_{tot} was observed in all experiments. It progresses with a slow time constant (~60 min) to ~30% of the initial value of Q_{tot}. The reason for this response is unknown presently, but it appears to be specific for the nonconducting W434F mutant channel. **(C)** Normalized voltage dependence of the steady-state charge distribution. The plot shows the charge distribution before (black symbols) and after (red symbols) PNGase F treatment from seven experiments. In all experiments, the $V_{1/2}$ shifted to the right in the voltage axes. **(D)** Box plot of the changes in $V_{1/2}$ from the seven experiments.

Effect of deglycosylation on the binding of ligands

In addition to changes in protein function, glycosylation might interfere directly with the binding and unbinding of ligands that interact with the protein in question. We tested the consequences of deglycosylation of Shaker K_V channels with three ligands: hanatoxin (HaTx), agitoxin (AgTx), and 4-amino pyridine (4AP). HaTx interacts with the VSD (Swartz and MacKinnon, 1997a,b; Li-Smerin and Swartz, 2000, 2001; Phillips et al., 2005; Herrington et al., 2006; Milescu et al., 2007, 2009, 2013), whereas the remaining two bind to the pore domain: AgTx to the exterior pore, and 4AP to the internal pore (Kirsch and Drewe, 1993; Kirsch et al., 1993; Garcia et al., 1994; Gross and MacKinnon, 1996).

To test HaTx, we used a Shaker K_V construct in which five residues in the third transmembrane segment were mutated to the corresponding amino acids of K_V2.1 (Shaker Δ5; Milescu et al., 2013) to make the Shaker K_V channel sensitive to HaTx (Milescu et al., 2013). Fig. 7 A shows current recordings before and after exposure to 200 nM HaTx in the same oocyte. The voltage steps shown are −50, −40, 0, and +40 mV from a holding potential of −80 mV. In the presence of HaTx, ionic currents could be elicited at more negative potentials compared with the control, consistent with the toxin stabilizing the open state (Milescu et al., 2013). Fig. 7 B shows a comparable experiment with a different oocyte, except that before HaTx exposure, the oocyte was treated with PNGase F. Similar to untreated K_V Shaker channels, HaTx increased the open probability at negative potentials so ionic current could be detected at −50 and −40 mV. The changes by HaTX on the relative probability of opening are also comparable for untreated and PNGase F–treated channels (Fig. 7 C).

Fig. 8 shows the consequences of deglycosylation on the blockade of Shaker K_V channels by either AgTx (100 nM; Fig. 8 A) or

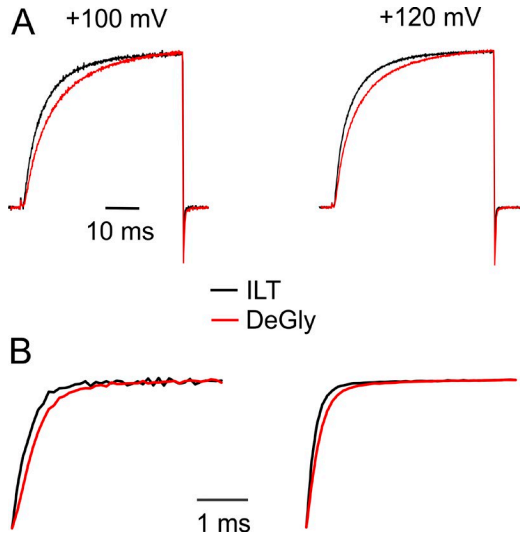

Figure 6. **Effect of deglycosylation on ionic currents from the ILT mutant Shaker K_V channel. (A)** Normalized ionic current traces elicited from a holding potential of −100 mV to +100 mV (left) and +120 mV (right), before (black) and after (red) PNGase F treatment. **(B)** Normalized tail currents before (black) and after (red) PNGase F treatment upon return from the voltage step to +120 mV. Left: Zoomed-in traces depicted in A (+120 mV). Right: Traces from a different oocyte. Ionic currents from ILT channels were P/6 subtracted with subpulses of opposite polarity from the holding potential. These experiments were performed by using the cut-open oocyte voltage clamp. DeGly, deglycosylation.

4AP (0.1 mM; Fig. 8 B). In both cases, these pore blockers inhibited similarly untreated and PNGase F–treated channels.

Discussion

Many K_V channels are known to be subject to glycosylation as they traffic toward the cell membrane. In some instances, glycosylation influences cell surface expression (Petrecca et al., 1999; Khanna et al., 2001; Watanabe et al., 2004, 2007, 2015; Fujita et al., 2006; Hall et al., 2015), whereas in others it might also impact protein function (Schwalbe et al., 1995; Thornhill et al., 1996; Freeman et al., 2000; Ufret-Vincenty et al., 2001b; Watanabe et al., 2003, 2007; Napp et al., 2005; Sutachan et al., 2005; Johnson and Bennett, 2008). Shaker K_V channel contains two glycosylation sites within the VSD of the protein (Santacruz-Toloza et al., 1994). In a previous study (Johnson and Bennett, 2008), the kinetics of Shaker K_V channels expressed in CHO cell lines with either normal or deficient glycosylation were shown to be altered by the extent of sugar incorporation into the channel. Here, we aimed to assess whether glycosylation has a direct influence on the function of Shaker K_V channels' VSD, the domain where the glycans are attached.

We studied the role of sugars on the channel's function by assessing their presence and absence within the same population of ion channels. We chose PNGase F to remove sugars because it acts within minutes (Fig. 1), and the Asn at the cleavage site becomes an Asp, which has the potential to restore the electrostatic nature of the sugars removed, and its influence can be directly assessed by site-directed mutagenesis. There are some advantages of this approach. For example, (a) in some instances, mutation of glycosylation sites might not be a viable alternative because of failures in cell membrane expression; (b) the small functional consequences observed in the gating currents (Fig. 5, C and D) would have been extremely difficult to discern comparing two populations of channels; and (c) the effect on the Q_{tot} from the gating currents would have remained undetected by analyzing two populations of channels. Yet, the method needs to be carefully controlled because PNGase F treatment is expected to act on many native glycosylated proteins that might indirectly influence the protein under study. Further, this approach is limited to the study of the presence or not of sugar moieties; therefore, the role of partial glycosylation is inaccessible.

We found that deglycosylation by PNGase F slows down channel activation and deactivation with an undetected shift in the relative probability of opening, suggesting that the effect of deglycosylation on the rates of opening and closing is similar. This is similar to previous observations in which K_V channels were expressed in cell lines with different degrees of glycosylation capabilities (Thornhill et al., 1996; Johnson and Bennett, 2008) or when functional comparisons were made between WT and glycosylation-deficient N→Q mutant channels expressed in the same cell line (Watanabe et al., 2003, 2007). To obtain direct evidence of the effect of glycans on the VSDs of K_V channels, we assessed the influence of deglycosylation by PNGase F on the gating currents. The steady-state distribution of the gating currents is shifted by 6 mV toward more depolarized potentials after deglycosylation (Fig. 5). This shift appears to be a pure voltage bias to the charge moving transitions carrying no changes in the kinetics of the gating currents. This implies that the mass of the sugar attached to the VSD does not influence the sensors' movements in response to voltage steps.

The functional consequences observed by PNGase F treatment of WT Shaker K_V channels likely originated from the direct removal of sugar moieties from the channels. First, the activation kinetics of glycosylation-deficient mutant Shaker K_V (N259Q–N263Q) and Shaker K_V (N259D–N263D) are slower than WT channels (Fig. 3), comparable to those measured after WT channels were deglycosylated. And second, PNGase F treatment of glycosylation-deficient mutant Shaker K_V channels produces no changes in the opening and closing of the channels (Fig. 4), indicating that the functional consequences observed with WT channels are not mediated by the deglycosylation of a native membrane protein that could have been interacting with Shaker K_V channels.

The changes in channels' opening and closing by deglycosylation cannot be explained by the shift in the steady-state distribution of the VSD transitions, suggesting they should originate from a direct effect on the C↔O transition. We isolated this transition by introducing the triple ILT mutations in the VSD. This channel construct has a distribution of the relative probability of opening that is shifted by ∼80 mV toward positive potentials, and a charge distribution of the gating currents shifted ∼40 mV in the opposite direction (Smith-Maxwell et al., 1998a,b; Ledwell and Aldrich, 1999). Deglycosylation by PNGase F had qualitatively similar consequences on the opening and closing of ILT channels (Fig. 6), like those observed in WT channels. These results lead to the conclusion that sugar moieties attached to the

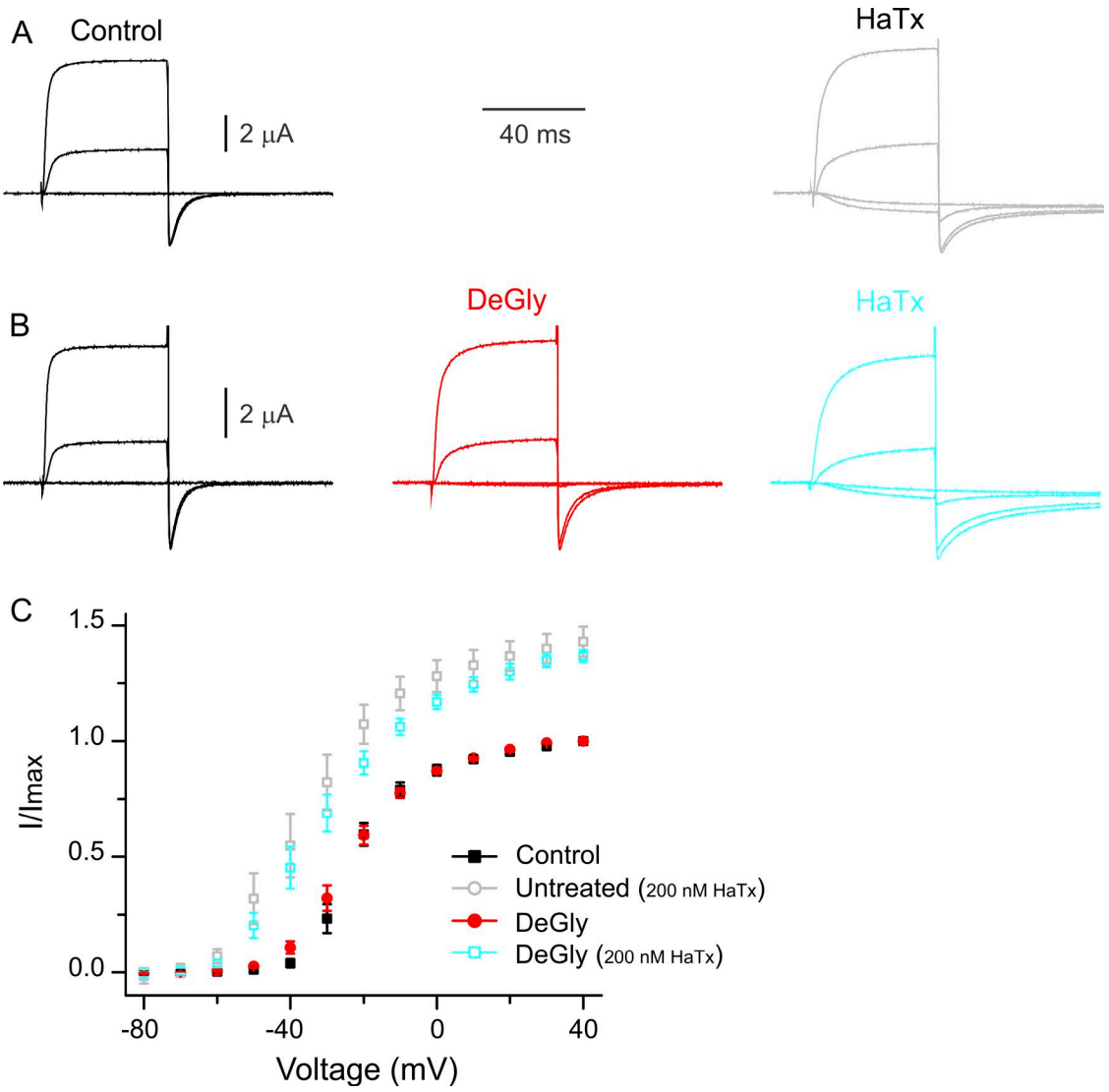

Figure 7. **Effect of deglycosylation on the binding of Hanatoxin. (A and B)** Current traces represent ionic currents in response to voltage steps to −50, −40, 0, and +40 mV from a holding potential of −80 mV. Tail currents shown were in response to voltage of −50 mV. In A, 200 nM HaTx was tested on unmodified channels (n = 5) and in B after deglycosylation by PNGase F (n = 4). **(C)** Relative probability of opening estimated from the tail currents. Data in the presence of HaTx were normalized with respect to the control (black and gray symbols) or immediately after PNGase F treatment (red and cyan symbols). These experiments were performed by using the two-microelectrode voltage-clamp technique. Error bars represent mean ± SEM. DeGly, deglycosylation.

S1–S2 linker influence the motions of the pore domain accompanying the opening and closing of the channels. By using kinetic modeling of ionic currents derived from mammalian K_V1.1 WT and the glycosylation-deficient mutant channel K_V1.1 (N207Q), the opening transition C→O had to be reduced approximately threefold to account for the slowdown in activation (Watanabe et al., 2003). As in Shaker K_V channels, mammalian K_V1.1 are glycosylated within the VSD at the linker between transmembrane segments S1–S2.

The association between the VSDs and the C↔O transition in Shaker K_V channels had initially been focused to regions of the VSD directly involved in charge movement steps. For example, the ILT mutations in the transmembrane segment S4 produced a substantial shift (~40 mV) of the charge movement distribution, which allowed a practical separation between the VSD movements and the C↔O transition (Smith-Maxwell et al., 1998a,b; Ledwell and Aldrich, 1999). This ILT mutant construct has also been used to show that the dynamics of the S4 are directly linked to the movements of the S6 that determine the C↔O transition (Pathak et al., 2005) as well as to establish that HaTx binding to the VSDs can directly stabilize the open state of the channel (Milescu et al., 2013). Additionally, by bridging the S4 and S5 transmembrane segments with metal ions (Lainé et al., 2003) and studying the state dependence of bridge formation, it was demonstrated they formed in the open state (Phillips and Swartz, 2010). However, by using statistical coupling analysis, it has also been proposed that the external end of transmembrane segment S1 forms an interface with the pore helix of K_V channels (Lee et al., 2009). Therefore, it is conceivable that sugars bound to the S1–S2 linker might influence the state of this interface to determine the kinetics of the C↔O transition. In fact, mutations of some residues at the external end of transmembrane segment

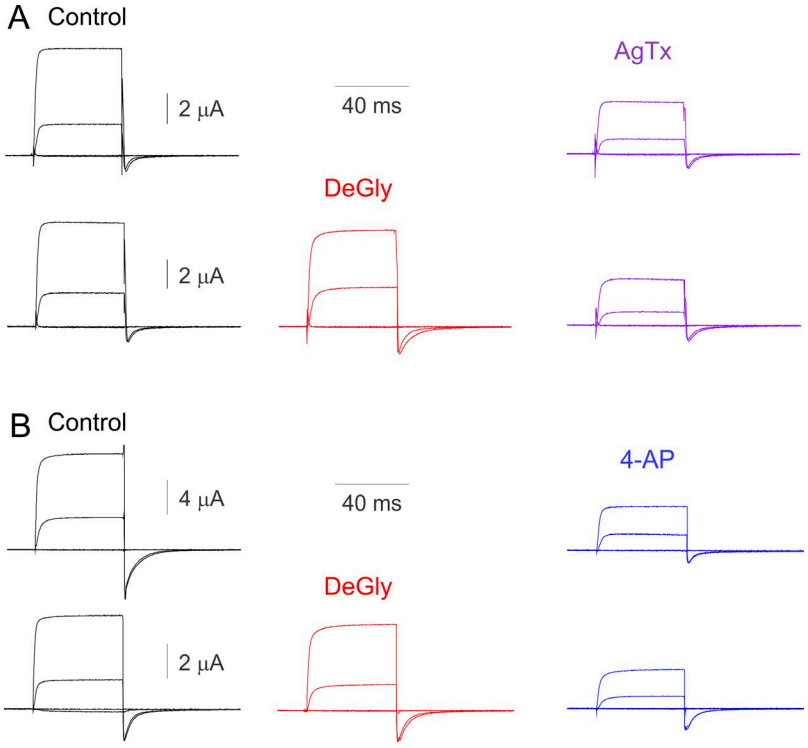

Figure 8. **Effect of deglycosylation on the binding of pore blockers. (A and B)** Current traces represent ionic currents in response to voltage steps to −50, −40, 0, and +40 mV from a holding potential of −80 mV. Tail currents shown were in response to voltage of −50 mV. A compares the inhibition by 100 nM AgTx before (top) and after (bottom) PNGase F treatment (n = 2). At +40 mV, AgTx blocked 48% of the ionic current before and 62% after treatment, respectively. At 0 mV, it inhibited 48% of the ionic current before and 63% treatment, respectively. B compares the inhibition by 0.1 mM 4AP before (top) and after (bottom) PNGase F treatment (n = 2). At +40 mV, 4AP blocked 55% of the ionic current before and 53% after treatment, respectively. At 0 mV, it inhibited 48% of the ionic current before and 50% after treatment, respectively. These experiments were performed by using the two-microelectrode voltage-clamp technique. DeGly, deglycosylation.

S5, the pore helix, and their connecting linker have shown substantial changes in the C↔O transition (Yifrach and MacKinnon, 2002). Further, it has recently been reported noncanonical interactions between adjacent VSDs and pore domains indicate a far more extensive functional relationship of these domains than structures predict (Carvalho-de-Souza and Bezanilla, 2017).

The presence of sugars at the external side of Shaker K_V channels does not appear to possess a stearic hindrance to access and binding of interacting molecules such as toxins and blockers of both VSD and the pore region (Figs. 7 and 8). The lack of changes in the interactions of HaTx-targeting VSDs by deglycosylation is compatible with the general mechanism of action of this toxin. On the one hand, HaTx access their binding site through the membrane (Lee and MacKinnon, 2004; Phillips et al., 2005; Milescu et al., 2007), so sugars at the external side of the protein are not in the path of voltage-sensor toxins. On the other hand, HaTx interacts with the surface of the VSD delineated by S3 and S4 transmembrane segments (Milescu et al., 2007, 2009), which are facing the membrane at the opposite direction as S1 and S3. Even though it is not surprising that the interactions of pore-blocking molecules such as AgTx and 4AP are not influenced by glycans, these results indicate that the ∼20-kD sugar moiety has a rather confined surface of interaction with the channel.

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