The glycosylation of the extracellular loop of β2 subunits diversifies functional phenotypes of BK Channels

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ABSTRACT

Large-conductance Ca^{2+}- and voltage-activated potassium (MaxiK or BK) channels are composed of a pore-forming α subunit (Slo) and 4 types of auxiliary β subunits or just a pore-forming α subunit. Although multiple N-linked glycosylation sites in the extracellular loop of β subunits have been identified, very little is known about how glycosylation influences the structure and function of BK channels. Using a combination of site-directed mutagenesis, western blot and patch-clamp recordings, we demonstrated that 3 sites in the extracellular loop of β2 subunit are N-glycosylated (N-X-T/S at N88, N96 and N119). Glycosylation of these sites strongly and differentially regulate gating kinetics, outward rectification, toxin sensitivity and physical association between the α and β2 subunits. We constructed a model and used molecular dynamics (MD) to simulate how the glycosylation facilitates the association of α/β2 subunits and modulates the dimension of the extracellular cavum above the pore of the channel, ultimately to modify biophysical and pharmacological properties of BK channels. Our results suggest that N-glycosylation of β2 subunits plays crucial roles in imparting functional heterogeneity of BK channels, and is potentially involved in the pathological phenotypes of carbohydrate metabolic diseases.

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Introduction

Large-conductance Ca^{2+}- and voltage-activated potassium (MaxiK or BK) channels, composed of either pore-forming α subunit (Slo) in combination with auxiliary β subunits or a pore-forming α subunits themselves, are ubiquitously expressed in many tissues, and contribute to the cell excitability, action potential waveform and patterns, and secretion. Native BK channels exhibit a variety of physiological properties, largely due to tissue-specific auxiliary β1—β4 subunits that associate with the pore-forming α subunit. These β subunits contain the intracellular N- and C-termini with 2 transmembrane (TM) helices, TM1 and TM2, which are connected via an extracellular loop of ~100 residues with several potential N-glycosylation sites.

Glycosylation is an important form of post-translational modification implicated in regulating the activity, intracellular trafficking and targeting of many membrane proteins including ion channels. Glycosylation of the auxiliary subunits of ion channels also regulates the channel function. For example, the glycosylation of the β4 subunits of Na_{v} channels is developmentally involved in neuritic degeneration. Deglycosylation of the β1 subunit of BK channels with N-glycosidase F affects the channel mean open time, open probability and voltage-dependence without changing single channel conductance, suggesting the native β1-subunit exists in glycosylated form in myocytes, albeit precise sites of glycosylation have not been identified. The glycosylation of the β4 subunits of BK channels precludes the channel sensitivity to...
iberiotoxin, yet whether or how this structural modification affects gating has not been studied.²

Among the 4 β members, the β2 subunit has a more profound impact than other subunits, and diversely influences the kinetics of BK channels such as activation, inactivation, sensitivity to toxins, and other modulators.² More importantly the β2 subunit cannot be targeted to cytoplasmic membrane without associating the α subunit.¹⁷⁻¹⁹ This subunit contains 3 N-linked glycosylation consensus sequences in its extracellular loop (N-X-T/S at N88, N96 and N119), but little is known whether some or all of these sites can be glycosylated to impact the functional properties of BK channels.

Given the unique roles of β2 subunit in BK channels, we have investigated the potential glycosylation sites and functional outcome in HEK293 cells co-expressing mSlo1 (α subunit) and wild-type (WT) β2 subunits. We found that all 3 sites (i.e. N88, N96 and N119) in the extracellular loop of β2 are glycosylated and removal of the central glycosylation site (i.e., N96Q mutant) generated the strongest impact on the rectification and toxin sensitivity of BK channels. We thus proposed a structural model to explain changes in their biophysical and pharmacological properties as a consequence of glycosylation. This work provides new mechanistic and structural insights into how glycosylation potentially diversifies the functional features of BK channels under physiological and pathophysiological conditions.

Results

Three N-linked glycosylation sites in the extracellular loop of β2

Although there are 3 consensus sequences for N-linked glycosylation (N-X-T/S) at positions N88, N96 and N119 in the extracellular loop of β2 (Fig. 1a), it is not known if any of them can be glycosylated. To address this question, we studied a series of mutants, in which the asparagine residues (N) of 3 potential N-glycosylation sites in the β2 loop were replaced by glutamine (Q) so as to yield single, double and triple un-glycosylated constructs. Using protein gel blot assay, we found that the WT β2 is presented as a large molecular weight (MW) of ~27 kDa band (Fig. 1b lane 1), the 3 un-glycosylated single mutants (N88Q, N96Q, N119Q) presented as ~30 kDa bands (Fig. 1b lane 2, 3, 4), any 2 sites of un-glycosylation mutants (N88/96Q, N88/119Q, N96/119Q) exhibited as an intermediate ~27 kDa single band (Fig. 1b lane 5, 6, 7), and the un-glycosylation triple mutant (N88/96/119Q, or 3N3Q) presented as the single band with the least molecular weight (about 24 kDa) (Fig. 1b lane 8). The ~24 kDa band likely represented an un-glycosylated β2, the ~27 kDa and ~30 kDa bands represented immature, intermediate glycosylated types of β2 whereas the ~33 kDa band represented a mature, fully glycosylated β2. These results indicated that β2 is endogenously glycosylated under physiological condition, and that all 3 sites can be glycosylated but may vary in the level of glycosylation.

To confirm that the ~24 kDa band represented the un-glycosylated β2, a glycosylation inhibitor tunicamycin ²⁰ was added to the cell culture for 20 hrs when β2 transfected to HEK293 cells. As shown in Fig. 1d, cell lysate from the culture treated with a high concentration of 10 μg/ml tunicamycin contained a single band of about 24 kDa, the same as normal cultured β2(3N3Q). In contrast, when treated with lowed concentration of tunicamycin 5 μg/ml, WT β2 showed 3 bands: an un-glycosylated band, an immature glycosylation band and a mature glycosylation band, respectively (Fig. 1d). These data suggested that WT β2 can be glycosylated to different levels by targeting different glycosylation sites, as indicated by their distinct molecular weight.

Un-glycosylation of the β2 at N96 enhances the outward rectification of BK(β2) channels

One BK channel associating with up to 4 β2 subunits typically shows a rapid inactivation behavior and strong outward rectification.¹⁷⁻²¹ To specifically examine the effect of glycosylation of individual sites on BK (β2) channels, we first examined their rectification properties with equal intracellular and extracellular K⁺ concentration. Consistent to the previous work,¹⁸ the normalized instantaneous current-voltage (I-V) curve of mSlo1/β2 channels showed nonlinearity, significantly different from that of the mSlo1 α channel alone. To quantitatively describe the nonlinearity behavior, we calculated the rectification ratio of the amplitude of instantaneous tail currents at +100 and −100 mV(R = |I₁₀₀/I⁻₁₀₀|) for wide-type and all mutants as illustrated in Fig. 2a (red traces). Among all 3 single-site mutants, we found that the β2(N96Q) mutant dramatically increased the rectification ratio R, but the mutants β2(N88Q) and β2(N119Q) almost had no effect on the R value (Fig. 2c).
To further establish the role of glycosylation at multiple sites we measured the R’s values for 3 double-mutants \( \beta_2(N88/96Q), \beta_2(N96/119Q), \beta_2(N88/119Q) \) and a triple-mutant \( \beta_2(3N3Q) \). However, all the mutants containing N96Q exhibited increased rectification ratio R just as \( \beta_2(N96Q) \) did alone, indicating that the N96-glycosylation site is the dominant site for altering the charge distribution around the center of channel pore and hence influenced the extent of outward rectification of BK channels.\(^{18}\)

Glycosylation facilitates the association of mSlo1/\( \beta_2 \) subunits

It is well-known that one BK channel can associate with up to 4 \( \beta_2 \) subunits, and that the number of \( \beta_2 \) is strongly correlated with the extent of inactivation, ranging from minimal inactivation in the absence of \( \beta_2 \) to a complete inactivation with all 4 \( \beta_2 \) subunits incorporated in a single BK(\( \beta_2 \)) channel.\(^{17}\) Under our experimental conditions that mSlo1:\( \beta_2 \) or its mutants was co-transfected with 1:2 ratio in mass, the amount of \( \beta_2 \) co-expressed with mSlo1 was about 20-fold more than necessary to maintain a saturating stoichiometry of \( \beta_2 \) per channel for all experiments.\(^{7,9}\) It is thus likely that one WT mSlo should associate with 4 \( \beta_2 \) subunits and exhibit full inactivation as expected if the association of \( \alpha/\beta_2 \) is normal. Interestingly, together with the abnormal rectification and ChTX toxin sensitive results, we found an incomplete inactivating current mediated by BK(\( \beta_2(3N3Q) \)) in most cases (Fig. 3a). In line with this observation,
pretreatment of cells expressing WT BK(b2) channels with 10 μg/ml tunicamycin for 20 hrs showed the same incomplete inactivating current with BK(b2(3N3Q)) (Fig. 3a). These observations raised the possibility that the stoichiometry of the mSlo1/b2 combination may be altered such that less than 4 b2 subunits may be associated with one BK channel, when all the 3 N-glycosylation sites were mutated.

Assuming that the binding probability between the mSlo1 and b2 subunit is p, the unbinding probability should be q = 1−p. Given the peak amplitude of the currents traces as A, we can calculate the unbinding probability from the formula q = (c/A)^1/4 (Fig. 3a–3b), in which c (>0) denotes the steady-state current reflecting the non-inactivating component of the null-b2 BK(mSlo1) channels.9 With this simplified mathematic model, we simulated inactivation time constants (τi), and found that simulated results (empty circles) on τi for b2(3N3Q) overlap well with the experimental data in a voltage-dependent manner (pink circle), indicating that slowed inactivation time is strongly correlated with the increasing unbinding probability q due to un-glycosylation (Fig. 3b–3c). Together, these results suggested that glycosylation of the extracellular loop of b2 is directly engaged in tightening its association with the pore-forming α subunit of BK channels.

Un-glycosylation of the β2 at N96 decreases the sensitivity of BK(β2) channels to Charybdotoxin (ChTX)

Knowing that the glycosylation of the extracellular loop of another β subunit (i.e. β4) regulates the
sensitivity of BK-type channels to iberiotoxin, we postulated that the toxin sensitivity of BK(β2) channels may be affected by the glycosylation. To test this, we conducted a series of outside-out patch experiments with the application of 1 μM ChTX to WT BK(β2) and all the β2 mutants deprived of glycosylation sites (Fig. 4a–4b). We found that the mutant N96Q showed significantly less sensitivity and slower time course of use-dependent block (t_{on} = 303.5 ± 20.4, n = 7) than that of the WT β2 (t_{on} = 151.5 ± 16.1, n = 5), while other mutants without N96Q were not significantly different (Fig. 4c). These results reinforced the critical role of glycosylation site at the β2 (N96) not only in regulating voltage-dependent gating of BK channels (i.e., rectification), but also their pharmacological properties as exemplified by the toxin sensitivity of BK channels.

A model was constructed by molecular dynamics (MD) to predict how the glycosylation facilitates the association of α/β2 subunits

To understand the functional impact of β2 glycosylations on BK channels, we constructed a model of mSlo1/β2 (glycosylated) complex via ab initio prediction method (Fig. 5) to simulate how the N-glycosylation of β2 may potentially regulate its association with α subunit. Because interactions between carbohydrate–carbohydrate
or carbohydrate–protein extensively exist in cells, we modeled the interactions between carbohydrate chains in glycosylated β2 tetramer, and found that central oligosaccharide chains at N96 are positioned toward the center while other chains form inter-subunit hydrogen bonds between β2 subunits (Fig. 5d). This suggested that interactions generated by these carbohydrate chains facilitate β2 association into a tetramer to stabilize the alignment of α/β2 subunits. Because the outward rectification of BK channel is known to be caused by the positively charged rings of the β2 extracellular loop located at the outer entrance of the channel pore, this model further predicted a shrinkage of the ring diameter from ~60 Å to ~30 Å for N96 glycosylated and unglycosylated β2, respectively. The N96 mutant appears to form more tightened tetramer than that of WT β2 (Fig. 5b–c), providing structural basis for enhanced outward rectification and decreased sensitivity to ChTX in N96 un-glycosylated β2 mutant. Such modification in the ring size likely constrains the access of ChTX to its binding site and attenuates its effectiveness in blocking the BK channel. These glycosylation-dependent extracellular inter-subunit interactions may work in concert with intracellular binding of the α/β in the cytosolic domain of BK(β2) to form functional channels with diverse properties.

Figure 4. The effect of unglycosylation on the sensitivity of ChTX to BK(β2) channels. (a) The time courses of blockade of mSlo1 + β2(N88Q), mSlo1 + β2(N96Q), mSlo1 + β2(N119Q), mSlo1 + β2 and mSlo1 by 1 μM ChTX. Each patch was perfused with 1 μM ChTX as indicated by the horizontal bars. (b) Traces show the representative currents recorded from outside-out patches. Currents were elicited by a 100-ms voltage step from −180 mV to 120 mV, in the presence of 10 μM Ca2+. The current traces are for control, 1 μM ChTX, and wash as indicated. The on-time constant t on of blockade by 1 μM ChTX are 161.7 ± 18.2 (n = 5) for mSlo1 + β2(N88Q), 303.5 ± 20.4 (n = 7) for mSlo1 + β2(N96Q), 145.7 ± 16.2 (n = 5) for mSlo1 + β2(N119Q), 151.5 ± 16.1 (n = 5) for mSlo1 + β2, and t on = 5.4 ± 3.2 (n = 4) for mSlo1, respectively. (c) The blockage by 1 μM ChTX normalized to control are plotted. They are 0.78 ± 0.03 (n = 5) for mSlo1 + β2(N88Q), 0.19 ± 0.02 (n = 7) for mSlo1 + β2(N96Q), 0.68 ± 0.04 (n = 5) for mSlo1 + β2(N119Q), 0.15 ± 0.03 (n = 5) for mSlo1 + β2(N88Q/96Q), 0.21 ± 0.03 (n = 6) for mSlo1 + β2(N96/119Q), 0.81 ± 0.06 (n = 6) for mSlo1 + β2(N88/119Q), 0.37 ± 0.04 (n = 7) for mSlo1 + β2(3N3Q), 0.70 ± 0.03 (n = 5) for mSlo1 + β2 and 1 ± 0.02 (n = 4) for mSlo1, respectively. Statistical significance for the data is determined with Student’s t-test (**P < 0.01).
Discussion

N-linked glycosylation was reported to regulate the activity, trafficking and pharmacological property of ion channels, including BK-type channels. Previous studies have reported that the N-glycosylation of β1 and β4 subunits affect the BK channel activity and toxin sensitivity. In this study, we discovered 3 glycosylation sites on the extracellular loop of β2 and systematically examined the impact of each of 3 glycosylation sites by single, double and triple site mutants. Our results indicated that an un-glycosylation mutant at the site β2(N96Q) showed the highest rectification ratio with the lowest ChTX sensitivity, suggesting that glycosylation of this site is critical for imparting the BK channel with unique biophysical and pharmacological characteristics.

As shown in Fig. 2, we found that the triple mutant (i.e. 3N3Q) behaved differently as compared to the single mutant N96Q. Because the triple mutant (3N3Q) contained the mutation...
N96Q, the channels are expected to show similar electrophysiological phenotypes, namely enhanced outward rectification and decreased ChTX sensitivity. Such an inconsistency may be potentially reconciled by the fact that the triple mutant (3N3Q) has a defective association with α tetramer (Fig. 3). Hence the channel properties may be similar to BK (α) channels (i.e., weaker outward rectification and higher sensitivity to ChTX). As a consequence, the triple mutants showed different characteristics from that of single mutant N96Q. It is entirely possible that there is a reduction in the density of β2 in the plasma membrane as a consequence of a decreased trafficking or stability when β2 is not properly glycosylated. New antibodies against the extracellular domain of β2 subunit or tags are needed to address this possibility in future experiments.

To understand the functional impact of β2 glycosylations on BK channels, we constructed a structure model of mSlo1/β2(glycosylated) complex via ab initio prediction method (Fig. 5) to describe how the N-glycosylation of β2 may regulate its association with α subunit. Our simulations suggest that interactions generated by the carbohydrate chains facilitate β2 coupled into a tetramer in advance, and then stabilize the alignment of α/β2 subunits. Our model provides a 3-dimensional structure for the extracellular cavum potentially important for underlying the regulation of rectification, gating and sensitivity to ChTX of BK channels by glycosylation.

Among the 4 auxiliary β subunits of BK channels, β2, β3 and β4 contain 8 conservative cysteine residues in their extracellular loops. The eight cysteine residues in the extracellular loop of β3 subunits have been shown to form 4 pairs of disulfide bonds critical for stabilizing their extracellular structure.28 It is reasonable to extrapolate that similar disulfide bonds are formed by the 8 cysteine residues in the extracellular loop of β2 subunits to interplay with N-glycosylation sites identified in this study. Previous studies have shown such interactions are important for stabilizing the topography of other membrane protein.29 In this context, we postulate that failure to form glycan side chains in N96Q may also destabilize the formation of the neighboring disulfide bonds (e.g. at C97), leading to a rearrangement of charge distribution surrounding the pore18(i.e. the positive electrical charged ring in the extracellular loop of β2), ultimately yielding the most robust phenotype in rectification and toxin sensitivity.

Conclusion

We identified the 3 glycosylation sites of β2, and delineated the influences of glycosylation on the rectification and toxin sensitivity of the BK channel. Most importantly, we showed that glycosylation of β2, particularly at N96 site, help tighten the association of α/β2 and possibly β2-β2 subunits to stabilize the extracellular space above the pore and control the channel rectification properties and toxin accessibility to its binding sites. Therefore, glycosylation-dependent regulation of the β subunits likely imparts BK channels with expanded functional heterogeneity in excitable cells under physiological and pathophysiological conditions associated with glycan metabolism.

Materials and methods

Cloning

For electrophysiological experiments, mouse Slo1 (KCMA1, GenBank™ accession number ADO63674.1), β2 (KCMB2, GenBank™ accession number AF099137.1), were cloned into a pcDNA3.1 (+) vector (Invitrogen). For Western Blots experiments, an epitope HA tag (hemagglutinin, YPYDVPDYA) was added at the carboxyl terminus of β2(C-HA tagged β2). The QuikChange protocol (Stratagene) was used to make all point mutations. All constructs were verified by sequencing.

Cell culture and transient transfections

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DME) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin in incubators with 37 °C and 5% CO2. One day before transfection, cells were transferred to 24-well plates. At 80–90% confluence, transfections can be performed with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. When co-transfected, mSlo1:β2 (or its mutants) was 1:2 in mass, giving the amount of β2 about 20-fold more than that of mSlo1 to promote a saturating stoichiometry of 4 β2 per channel for all experiments.7 9 About 5 hours after transfection, cells were transferred to poly-D-lysine (Sigma) coated
chambers for following slides for electrophysiological experiment.

**Western blot**

C-HA tagged β2 and its mutants in pcDNA3.1 were expressed in HEK293 cells, 24 hrs after transfection, the cells were lysed (lysis buffer contained 20 mM Tris-HCl/pH 7.5, 150 mM NaCl, 1 % NP-40, 0.1 % Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride and protease inhibitors). After vertical rotation at 4°C for 1 h, the lysed cells were then high-speed centrifuged (12,000 rpm) at 4°C for 30 min. Proteins in the lysate were separated on polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with 5 % nonfat milk in 0.1 % Tween 20 in Tris-buffered saline, the blots were probed with mouse monoclonal anti-human HA antibody (Millipore). Horseradish peroxidase-coupled rabbit anti-mouse IgG was used as the secondary antibody for β2 blots. The membrane was washed with 0.1 % Tween 20 in Tris-buffered saline, and proteins were visualized with an enhanced chemiluminescence detection system.

**Patch clamp recording**

For the recordings, one day after transfection, HEK293 cells were transferred to either 160 K+ solution (containing 160 mM MeSO3K (potassium methanesulfonate), 2 mM MgCl2, 10 mM HEPES, pH = 7.0) or 10 Ca2+ solution (containing 160 mM MeSO3K, 10 mM HEPES, 5 mM N-hydroxyethylene-diaminetriacetic acid (HEDTA) with added Ca2+ to make 10 μM free Ca2+, as defined by the EGTAETC program (McCleskey, Vollum Institute, Portland, OR), pH = 7.0). All experiments were carried out with excised patches, including inside-out and outside-out recording configuration. Patch pipettes were pulled from borosilicate glass capillaries with resistances of 2–3 MΩ when filled with pipette solution (containing 160 K+ solution for inside-out recordings or 10 Ca2+ solution for outside-out recordings). Experiments were performed using an AXON-200B patchclamp amplifier with its software (AXON, USA). Currents were typically digitized at 20 kHz and filtered at 8.5 kHz. During inside-out recording, 10 μM Ca2+ solution was applied onto membrane patches via a perfusion pipette containing 8 solution channels. And for outside-out patches, 1 μM Charybdotoxin (ChTX) was added to the 160 K+ solution and then applied onto membrane patches via a perfusion pipette containing 8 solution channels. All experiments were performed at room temperature (22~24°C).

**Structure prediction of the glycosylated β2 and assembly of the BK/β2 complex**

The well-known Rosetta (version 2.3.1) suite was used to predict 3D structure of extracellular β2 loop. To acquire the structure of extracellular β2 loop, 10 k decoys were produced during the ab initio protein folding task based on its sequence information. After cluster analysis, the top models were selected for further modeling. Based on the results from Western Blot, we deduced the molecular weight of a carbohydrate chain which is about 3,000 Da, and hypothesized the stucture of carbohydrate chain (LinucsisID 3961) from glycosciences.de (http://glycosciences.de, a web databases and bioinformatics tools for glycobiology and glycomics). The full model of β2 subunit was then constructed by connecting the predicted extracellular β2 loop, 2 transmembrane helices, N-terminal (PDB ID: 1JO6) and the carbohydrate chains. The full models of BK channels were built as previous. The final model was extracted from the trajectory of simulation and analyzed in more details as general.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

J.P.D conceived the project. Z.G.H., H.W.L., L.Y.W. and J.P.D. designed all the experiments. Z.G.H., H.W.L., Z.Y.Y., performed the experiments. S.W. provided technical guidance. All authors analyzed the data and contributed to manuscript preparation. Z.G.H., H.W.L., L.Y.W. and J.P.D. wrote the manuscript. All authors have given approval to the final version of the manuscript.
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