Kir7.1 is an inwardly rectifying potassium channel with important roles in the regulation of the membrane potential in retinal pigment epithelium, uterine smooth muscle, and hypothalamic neurons. Regulation of G protein–coupled inwardly rectifying potassium (GIRK) channels by G protein–coupled receptors (GPCRs) via the G protein βγ subunits has been well characterized. However, how Kir channels are regulated is incompletely understood. We report here that Kir7.1 is also regulated by GPCRs, but through a different mechanism. Using Western blotting analysis, we observed that multiple GPCRs tested caused a striking reduction in the complex glycosylation of Kir7.1. Further, GPCR-mediated reduction of Kir7.1 glycosylation in HEK293T cells did not alter its expression at the cell surface but decreased channel activity. Of note, mutagenesis of the sole Kir7.1 glycosylation site reduced conductance and open probability, as indicated by single-channel recording. Additionally, we report that the L241P mutation of Kir7.1 associated with Lebers congenital amaurosis (LCA), an inherited retinal degenerative disease, has significantly reduced complex glycosylation. Collectively, these results suggest that Kir7.1 channel glycosylation is essential for function, and this activity within cells is suppressed by most GPCRs. The melanocortin-4 receptor (MC4R), a GPCR previously reported to induce ligand-regulated activity of this channel, is the only GPCR tested that does not have this effect on Kir7.1.

Kir7.1, encoded by the *Kcnj13* gene, is a two-transmembrane domain potassium channel, closer in homology to Kir channels associated with potassium transport such as Kir1.1, 4.x, and 5.1 (1). Compared with other channels, Kir7.1 exhibits a small unitary conductance and low dependence on external potassium (2). This was shown to be due to the presence of a methionine at position 125 in the pore, where other Kir channels have an arginine. Mutation of this residue to arginine was found to mimic/restore the conductance and potassium dependence observed in like-family channels. Kir7.1 is widely expressed, but particularly high expression has been reported in the retinal pigment epithelium (RPE), thyroid, uterine smooth muscle, small intestine, and choroid plexus of the brain (3, 4).

Until recently, Kir7.1 was primarily studied within the RPE. In RPE cells, Kir7.1 is found in the apical membrane close to photoreceptor neurons, where it is thought to contribute to ion homeostasis (5). Mutations in Kir7.1 lead to snowflake vitreoretinal degeneration and Leber congenital amaurosis (LCA), which are both retinal dystrophies (6–10). In jaguar/obelix zebrafish, mutations in Kir7.1 affect pigment patterns, specifically as a result of the failure of melanosomes to respond to changes in light (11). These functional mutations of Kir7.1 in RPE cells and melanocytes have been characterized and shown to cause alterations in channel currents resulting in the described pathologies.

Recent studies have also identified a role for Kir7.1 in the regulation of uterine smooth muscle excitability (12). Specifically, Kir7.1 expression is elevated by 30-fold midgestation, hyperpolarizing muscle cells and reducing uterine excitability. This expression falls off toward the end of pregnancy, leading to parturition. In the mouse, Kir7.1-null mutations cause perinatal lethality, and this was determined to be due to a role for Kir7.1 in tracheal tubulogenesis (13). Kir7.1 was also revealed to be a potential regulator of neuronal excitability in the paraventricular nucleus of the hypothalamus, through modulation of channel activity by the G protein–coupled melanocortin-4 receptor (MC4R) (14). Activation of MC4R led to robust neuronal depolarization, which was
found to be mediated by Kir7.1 channel closure. Conversely, inhibition of MC4R by an endogenous inverse agonist, AgRP, led to channel opening and hyperpolarization of the neuron. These results are interesting in that Kir7.1 is not a canonical G protein–coupled inwardly rectifying potassium (GIRK) channel such as Kir3.x. Furthermore, in HEK293T cells, Kir7.1 was found to exist in a complex with MC4R (14).

Mechanisms by which non-GIRK channels interact with GPCRs vary, with some channels behaving like GIRKs, such as Kir2.x channels. Specifically, unlike Kir2.1, it has been reported that Kir2.3 channels can be inhibited directly by G<sub>q</sub> complexes (15). Moreover, within dendritic spines of cholinergic neurons, M1 receptor activation has been shown to lead to inhibition of Kir2.x channels through G<sub>q</sub> activation of phospholipase C, which depletes the second messenger phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) that is needed for Kir channel function (16). Kir7.1 likewise has a PIP<sub>2</sub> dependence for activity and was recently shown in RPE cells to be regulated by the oxytocin G<sub>q</sub>–coupled GPCR, through phospholipase C–mediated inhibition (17). However, the MC4R-mediated closure of Kir7.1 was found to be independent of G proteins (14). The mechanism by which this occurs is unknown, and examples exist of receptors interacting with Kir channels in the absence of G proteins and other second messengers. Specifically, Kir6.2 and the non-GPCR sulfonylurea receptor (SUR), a target of sulfonylurea drugs used to treat diabetes, form the K<sub>ATP</sub> channel (18). This heterooctamer composed of Kir6.2 subunits in 1:1 stoichiometry with SUR1/2 is expressed in the pancreas, and ATP binding to the SUR subunits increases channel open probability (19). SUR communicates with Kir6.2 through direct interaction between the C-terminal tail of Kir6.2 and an intra-cellular loop of SUR (20, 21).

In our initial investigations of potential molecular mechanisms of interaction for Kir7.1 with MC4R, we observed that co-expression of several GPCRs with Kir7.1 in HEK293T cells led to a dramatic reduction in complex glycosylated forms of Kir7.1 present in the cell. A review of the role of glycosylation within this family of inwardly rectifying potassium channels revealed that glycosylation of Kir1.1, a close family member, reduces open channel probability (22, 23). In patients with multiple sclerosis, autoantibodies against glycosylated forms of Kir4.1 have been reported, but the role for this in the pathophysiology of the disease is unclear (24, 25). A role for glycosylation of Kir7.1 has not been investigated. We report here on a novel functional role of GPCRs in the complex glycosylation and function of Kir7.1.

Results
Kir7.1 channel glycosylation is altered by the β2-adrenergic receptor (β2AR)

The α-MSH neuropeptide regulates food intake and energy expenditure through the MC4R, a G protein–coupled receptor. Studies of MC4R neurons suggested this receptor regulates neuronal depolarization and activation by ligand–mediated regulation of the conductance of Kir7.1 (14). Further, these studies supported a G protein–independent mechanism of regulation of Kir7.1. To investigate this unusual mode of Kir regulation, we first co-expressed a C-terminal FLAG-tagged Kir7.1 either with 3× HA–MC4R or as a control with the widely studied β2-adrenergic receptor (3× HA–β2AR) in HEK293T cells in the absence of ligand. We selected the β2AR as a control because the MC4R this receptor also couples to G<sub>q</sub>. Unexpectedly, we observed a significant reduction in the 50-kDa migrating doublet of Kir7.1 when co-expressed with β2AR, whereas MC4R caused a small increase in this form of the channel in some experiments (Fig. 1A). Treatment of cells with melancortin agonists, shown to reduce Kir7.1 currents in MC4R neurons of the paraventricular nucleus of the hypothalamus (14), had no effect on the typical amount or pattern of Kir7.1 bands observed (not shown). Using endoglycosidases, we confirmed the upper 50-kDa bands as the Endo H–resistant mature/complex glycosylated form of the channel, indicating that these bands are Kir7.1 that include glycans added in the Golgi complex (Fig. 1B). With Endo H treatment, the 37-kDa doublet migrated as a single band, indicating Endo H sensitivity. As Endo H–sensitive glycans are added in the endoplasmic reticulum, we identified this upper 37-kDa band as the core glycosylated form and classified this lower band as the unglycosylated form of Kir7.1. Additionally, treatment with the glycosidase PNGase F, which cleaves all N-linked glycans of proteins, resulted in a single migrating band at 37 kDa, confirming that Kir7.1 glycosylation is N-linked (Fig. 1B). Furthermore, we established a metric for the proportion of Kir7.1 complex glycosylation, by determining the ratio of mature/complex glycosylation, to immature core glycosylated plus unglycosylated forms of the protein. Although some variability in this ratio was seen, a value of ~1 was typically observed across multiple Kir7.1 expression trials in HEK293T cells in the absence of GPCR co-expression. Co-expression with β2AR reduced the ratio of glycosylation to less than 0.5, whereas MC4R exhibited a trend toward increasing this ratio, indicating opposing effects of these GPCRs (Fig. 1C).

Dose-responsive inhibition of Kir7.1 complex glycosylation with increased β2AR expression

With the striking observation of reduced Kir7.1 glycosylation following β2AR but not MC4R co-expression, we were curious whether differences in receptor expression levels could account for the proportion of Kir7.1 complex glycosylation observed. To examine this, we co-expressed Kir7.1 with increasing amounts of either MC4R or β2AR protein and assessed glycosylation by Western blotting analysis. We observed that increasing the amount of MC4R expressed had no effect on proportion of Kir7.1 complex glycosylation, whereas increasing amounts of β2AR potentiated the loss of complex glycosylation (Fig. 2, A and B). Moreover, co-expression with β2AR appeared to be increasing the amounts of core-glycosylated/immature Kir7.1 expressed. We determined the change in ratio of Kir7.1 glycosylation with changes in receptor expression, and β2AR mediated a progressive reduction in the ratio of complex to immature forms (unglycosylated and core glycosylated) of Kir7.1 (Fig. 2C). Increases in the amount of MC4R protein expressed did not have an effect on the ratio of mature/immature forms of Kir7.1. As a control, and to determine
whether the effect on glycosylation was specific to Kir7.1, we assessed whether overexpression of either GPCR would alter the glycosylation of the widely studied vesicular stomatitis virus glycoprotein (VSVG) tagged with GFP. VSVG–GFP is used to elucidate protein trafficking, where forms of the protein that have mature/complex glycosylation are Endo H–resistant. This can be determined by observing a band shift when comparing Endo H–treated versus untreated cell lysates of VSVG–GFP with either GPCR. We first treated cells transiently expressing VSVG–GFP only with a transfection of expression vector for the channel and the β2AR. 2.5 μg of expression vector for the MC4R was used to achieve receptor expression levels comparable with that seen with the β2AR (see Fig. 2). B, enzymatic digestion of whole-cell Kir7.1 lysates confirmed the 50-kDa Endo H–resistant doublet as the complex or mature glycosylated form, the 37-kDa band as the core glycan form, and the lowest band in lane 3 as the unglycosylated PNGase F–cleaved form. C, densitometric quantification of the blot shown in A, showing that MC4R had a modest increase in the ratio of mature to immature forms of Kir7.1 p < 0.05, whereas β2AR significantly reduced the ratio of mature forms of Kir7.1 p < 0.005, one-way ANOVA representative of three independent experiments. The error bars represent S.D. M.W; molecular mass.

**β2AR-reduced Kir7.1 mediated whole-cell currents**

Given the specific effect of β2AR on Kir7.1 glycosylation, we investigated whether this had an effect on the function of Kir7.1. To study the functional effect of the β2AR-mediated loss of Kir7.1 complex glycosylation, Kir7.1 whole-cell recordings were performed in the absence of any ligand in transfected HEK293T cells. Whole-cell patch clamp recordings were performed following Kir7.1 transfection and overexpression of either β2AR or MC4R. Because the Kir7.1 WT channel was found to have a small unitary conductance (2), we used a mutant form of the channel in which the methionine within the pore was replaced with an arginine typically found in the pore of other two-transmembrane domain Kir channels (Kir7.1-M125R). This mutant has been widely validated and used in multiple studies of Kir7.1 activity (2, 26, 27). Kir7.1-M125R expressed alone compared with co-expression with MC4R yielded no differences in whole-cell current, which aligned with previous observations (14) (Fig. 3, A and B). However, β2AR caused a >50% reduction in Kir7.1 whole-cell–mediated currents (Fig. 3, C and D). Because Kir7.1-M125R channels exhibit barium sensitivity, activity of expressed Kir7.1 was taken as the total amount of current inhibited by barium at the end of the experiment. In general,
reduced responses to changes in voltage were observed when Kir7.1 was co-expressed with β2AR (Fig. 3D). Because these experiments were performed in the absence of ligand, this suggested that glycosylation either reduces the amount of functional channel at the surface of the cell or directly alters channel function.

**β2AR-mediated loss of complex glycosylation does not alter total Kir7.1 surface expression**

We next investigated whether co-expression with β2AR altered the amount of Kir7.1 trafficked to the cell surface. Cells co-expressing β2AR or MC4R with Kir7.1 were biotin-labeled...
and assessed for cell surface expression by Western blotting analysis. This method allowed visualization of the different glycosylated forms of Kir7.1 reaching the cell surface. Surprisingly, Kir7.1 co-expression with β2AR and loss of complex glycosylation did not cause a reduction in the percentage of Kir7.1 protein on the cell surface (Fig. 4B). MC4R likewise had no effect on Kir7.1 surface expression. Additionally, we assessed whether the ratio of complex to immature forms of Kir7.1 at the surface changed in the presence of these GPCRs (Fig. 4C). This remained unchanged, with β2AR maintaining a <0.5 ratio of complex Kir7.1 glycosylation compared with the total glycosylated Kir7.1 expressed in the cell. A slight reduction in the ratio of surface mature/immature Kir7.1 following MC4R expression was observed, but this trend was not significant over multiple experiments. These results therefore indicate that complex glycosylation is not required for surface expression of Kir7.1.

**Lack of glycosylation of Kir7.1 alters channel gating**

Within the Kir family, the functional role of glycosylation has been studied for ROMK (Kir1.1) and GIRK1 (Kir3.1) (22). Although loss of glycosylation at Asn-119 in GIRK1 was reported not to be required for function, loss of glycosylation at Asn-117 in ROMK1 reduced open channel probability and whole-cell currents (23). Additionally, although Kir3.4 is reported to have an asparagine accessible for glycosylation, the site was found to be inactive (22). Thus, divergent roles for glycosylation exist within the family. Based on these studies, we aligned the Kir7.1 protein sequence with ROMK and GIRK1 and determined that Kir7.1 also shared a highly conserved glycosylation site (Fig. 5A). We mutated this site to a glutamine (Kir7.1-N95Q) and confirmed by Western blotting analysis the loss of both core and complex forms of glycosylation (Fig. 5B). Furthermore, we generated a model of Kir7.1 based on the Kir2.2 crystal structure using SWISS-MODEL, which revealed the likely glycosylation site to be in the outer loops gating the pore (Fig. 5C). This was suggestive of a functional role for glycosylation in Kir7.1. To assess this, we performed single-channel recordings using the M125R mutant of Kir7.1 to compare the WT fully glycosylated channel (Kir7.1-M125R) to the Unglycosylated mutant channel (Kir7.1-M125-N95Q).

WT Kir7.1 displayed clusters of openings with a main amplitude level of ∼1 pA (Fig. 6, A and D). Lack of glycosylation at the Asn-95 position of Kir7.1 channels caused openings to occur at an amplitude level of −0.6 pA (N95Q = 0.65 ± 0.03 pA, n = 6; WT = 1.11 ± 0.07 pA, n = 7; p = 0.0001) with a reduced open probability (N95Q = 0.23 ± 0.05, n = 6; WT = 0.41 ± 0.06, n = 7; p = 0.0484) (Fig. 6, B and E). Both channels opened to at least three open states (O1, O2, and O3), with no changes in the relative occurrence of opening events (N95Q = 9.09 ± 3.8 ms, n = 6; WT = 17.9 ± 3.5 ms, n = 7; p = 0.1188). We tested whether β2AR may be directly suppressing channel activity inde-
GPCRs differentially regulate Kir7.1 glycosylation

Figure 4. Co-expression of the β2AR does not reduce the total amount of Kir7.1 at the plasma membrane but alters the ratio of mature glycosylated forms of Kir7.1 at the surface. Transfection levels were as in Fig. 1. A, Western blotting analysis (WB) of surface biotinylation of cells transiently transfected with 3× FLAG–tagged Kir7.1 co-expressed with the 3× HA–β2AR or 3× HA–MC4R. The experiment was performed as described by Chandrasekhar et al. (39). The cells were labeled with sulfo-NHS-SS-biotin, and surface proteins were immunoprecipitated with streptavidin–agarose beads. At least 45 μg of prequantified total protein was immunoprecipitated and loaded per lane. 15 μg (one-fifth) of total input protein was loaded for comparison. B, GAPDH control indicated that cells remained intact throughout biotinylation. Surface quantification was determined by densitometry analysis of the mature/complex doublet versus immature. The percentage of surface biotinylation was determined by dividing the values in the surface lanes by the input lane values multiplied by five. Percentages of complex or immature Kir7.1 at the surface were compared. The error bars represent S.D. with no significance observed between conditions determined by one-way ANOVA. C, comparison of the ratio of mature to immature forms of Kir7.1 in whole-cell lysates versus cell-surface biotinylated proteins. The results are representative of three to five independent experiments (p < 0.05; p < 0.005). The error bars represent S.D. MW, molecular mass; NS, not significant.

Independent of altering glycosylation state, by studying the effects of β2AR expression on Kir7.1-M125R-N95Q whole-cell currents. However, current densities recorded at a test pulse of −180 mV were no different for Kir7.1-M125R-N95Q alone (110.8 ± 31.7 pA/pF) or Kir7.1-M125R-N95Q co-expressed with β2AR (107.4 ± 21.8 pA/pF, p = 0.8857, unpaired t test) (Fig. S3). These findings support our conclusion that β2AR modulates Kir7.1 channel function by inhibiting complex glycosylation. Thus, the lack of glycosylation leads to a clear reduction in function of individual Kir7.1 channels.

Other β-adrenergic receptors also alter Kir7.1 glycosylation

We next sought to determine whether suppression of complex glycosylation and function of Kir7.1 was unique to the β2AR. For this, we first tested whether the β1 and β3 adrenergic receptors also mediated alterations in Kir7.1 glycosylation. We saw a similar reduction in the proportion of complex glycosylation when co-expressing these other β-adrenergic receptors with Kir7.1 in HEK293T cells (Fig. 7A). Special care was taken to express the receptors at similar levels to allow adequate comparison for changes in glycosylation. β1AR and β3AR similarly reduced the ratio of Kir7.1 glycosylation as seen with β2AR, with β1AR having a slightly lower value (Fig. 7B). This, however, may be due to receptor expression of β1AR being slightly higher than β2AR (Fig. 7A). These results indicate that our original observation was not specific to the β2AR receptor but shared by others GPCRs tested within the family. We also attempted to screen a larger panel of GPCRs for effects on glycosylation (MC1R, MC3R, and OTXR) and did indeed see a reduction in Kir7.1 glycosylation ratio (Fig. S1). Although we were unable to attain consistent receptor expression levels for these receptors, the results suggest that many GPCRs suppress the complex glycosylation of Kir7.1.

Kir7.1 has a conserved Golgi export site, essential for complex glycosylation

As complex glycosylation occurs in the Golgi network, we sought to identify mechanisms by which GPCRs might suppress complex glycosylation of Kir7.1 described above. With recent reports of a Golgi export site required for surface expression of Kir channels (28, 29), we found that Kir7.1 shared a site similar to that in the N- and C-terminal regions of Kir2.1, 4.1, and 5.1 that has been identified as essential for Golgi export (Fig. 8, A and B) (28). Within the channels mentioned, deletion
of the serine and tyrosine (SY) residues within the C-terminal site was found to be sufficient to prevent Kir export from the cis-Golgi and trans-Golgi compartments of the cell, where complex glycosylation takes place (28, 29). Additionally, although WT Kir2.1 is not glycosylated, it was reported that deletion of SY in Kir2.1 also reduced complex glycosylation of an artificially placed asparagine residue (29). Given the high conservation of this site in Kir7.1, we tested whether deletion of these residues would alter WT Kir7.1 glycosylation. Deletion of the SY motif in Kir7.1 led to a significant loss of complex glycosylation with a ratio of <0.1 in Cos1 cells (Fig. 8, C and D). Cos1 cells were used because published studies of the export site in other Kir channels were performed in Cos cell lines (30). We further confirmed the loss of complex glycosylation by endoglycosidase digestion using Endo H and PNGase F (Fig. 8E). This indicates that the Golgi export residues SY in Kir7.1 are essential for complex glycosylation of Kir7.1. Although the SY mutant did have a significantly low glycosylation ratio, we also observed a glycosylation ratio of <0.8 in the WT channel in Cos1 cells (Fig. 8E), lower than the typical ratio of 1 observed in HEK293T cells. Because differential glycosylation of Kir channels have been reported across different tissue subtypes (31), we tested whether β2AR would likewise mediate a loss of glycosylation in the Cos1 cell line. The striking loss of Kir7.1 glycosylation observed within HEK293T cells when co-expressed with β2AR was repeated within Cos1 cells compared with the WT channel, and co-expression with MC4R again lacked the same effect (Fig. 8, F and G). This further indicated the strong specificity of the β2AR-Kir7.1 interaction.

**Kir7.1 forms heterotetramers of immature, unglycosylated, and fully glycosylated subunits**

Having established that complex glycosylation of Kir7.1 is required for function, we tested whether the channel exists as homo- or heterotetramers of subunits with no glycosylation, core glycosylation, and complex glycosylation. We co-expressed FLAG-tagged Kir7.1-N95Q or Kir7.1ΔSY with an HA-tagged WT Kir7.1 channel. HA-tagged WT Kir7.1 co-immunoprecipitated with both the unglycosylated (N95Q) and immature glycosylated (ΔSY) forms of the channel, in addition to the fully glycosylated WT control (Fig. 9). This suggests that nonfunctional Kir7.1 subunits may reduce channel function through subunit poisoning.

**Retinopathy-associated mutations of Kir7.1 also alter channel glycosylation**

Genetic studies have linked retinopathies of the eye to mutations in Kir7.1, demonstrating that Kir7.1 plays a key role in the function of the retinal pigment epithelium. Snowflake vitreoretinal degeneration and LCA are two Kir7.1-associated hereditary eye disorders, where known variants lead to loss of channel function (32). We wanted to determine whether glycosylation is also affected by any of these disease mutations, and a few known variants of Kir7.1, namely R162W, R162Q, Q117R, and L241P, were tested. These are all considered to be loss-of-function mutations except R162Q, which is linked to blindness but has no reported phenotype (32). Notably, none of these mutations are in known sites required for complex glycosylation. Interestingly, we observed a significant reduction in glycosylation in the L241P mutation (Fig. 10). The Q117R mutation appeared also to have a modest reduction in the proportion of complex glycosylation.

**Discussion**

GIRKs are Kir family members best understood to be regulated by GPCR signaling (1, 33). Our study reveals another mechanism by which GPCRs appear to regulate the function of another inward rectifier, Kir7.1, prior to cell surface expression and regulation by ligand binding. Our results demonstrate a highly specific effect of GPCRs on Kir7.1 glycosylation, which in
GPCRs differentially regulate Kir7.1 glycosylation

Figure 6. A and B, representative single-channel current traces from cell-attached patches of WT Kir7.1 (A) and Kir7.1<sup>N95Q</sup> (B) channels recorded from HEK293T cells. Patches were voltage-clamped at 130 mV. Openings are downward, and each representative trace was a continuous 5000-ms recording. C, average time histograms of openings (O1, O2, and O3) events are shown with the time constants (ms) and the relative contribution (%) of open events. D–F, bar graphs summarize the effects of Kir7.1<sup>N95Q</sup> on amplitude (D), mean open probability (NPO) (E), and mean open time of channels (F). The values are expressed as means ± S.E. (WT, n = 6; NQ, n = 7). Statistical differences were determined using unpaired t test relative to WT. C and O refer to the closed and open states, respectively.

turn directly regulates channel function. Additionally, although glycosylation of Kir7.1 has been mentioned in other publications (2, 32), no other study has characterized the glycosylation of the channel and its effect on function. Specifically, we have shown that a loss in the proportion of receptor subunits undergoing complex glycosylation is mediated by expression of a wide variety of GPCRs. Further, we observed that the reduction in Kir7.1 whole-cell currents when co-expressed with unliganded β2AR (Figs. 3 and 6) was indeed due to loss of complex glycans, an event occurring prior to trafficking of the channel to the cell surface. The MC4R has previously been reported to regulate Kir7.1, with agonists inhibiting and antagonists stimulating channel function; however, expression of the MC4R did not reduce complex glycosylation of the channel, with or without the presence of ligand. Nonetheless, it is intriguing to note that the MC4R is one of the few GPCRs we examined that leaves channel glycosylation intact.

Glycosylation has been previously reported for channels of the same family, Kir1.1 (ROMK) and Kir3.1 (22). Loss of glycosylation in Kir1.1 was shown to decrease whole-cell currents because of a significant reduction in open channel probability (23). We observed similar results in our N95Q glycosylation resistant mutant by single-channel recordings. Interestingly, Kir3.1, although having an active glycosylation site, is not reported to require glycosylation for function. Although Kir3.1 and Kir3.4 form complexes, Kir3.4 has a consensus glycosyla-
A functional role for glycosylation has not been investigated. Although higher amounts of complex glycosylation aids channel function. Additionally, the Kir4.1 channel has been shown to be differentially glycosylated in different tissues. As mentioned previously, Kir4.1 is the only inward rectifier with a clear reported role for glycosylation. In the case of CaR and Kir4.1, it was indeed reported that CaR Gq signaling and internalization by caveolin-1 (35, 36). This was not the case with β2AR’s effect on Kir7.1, wherein co-expression with β2AR did not reduce Kir7.1 surface expression. In the case of CaR and Kir4.1, it was indeed reported that mutant nonfunctional CaRs were unable to have an effect on Kir4.1 surface expression or activity (36). This has not been tested for GPCRs that we have shown to alter glycosylation of Kir7.1.

Although glycosylation is indeed not required for surface expression of ion channels such as Kir2.1, which is seemingly unglycosylated, we were surprised to see expression of immature forms of Kir7.1 at the cell surface. Particularly, the use of cell surface biotinylation allowed us to profile the different forms of the channel present at the surface and obtain a ratio of complex to immature plus unglycosylated forms. In co-expression with β2AR, the ratio of complex glycosylation remained unchanged at the surface, indicating that GPCR expression does not appear to alter trafficking of the channel. Furthermore, we were able to show that these channels can exist as heterotetramers of unglycosylated, partially glycosylated, and fully glycosylated subunits. Therefore, a change in the ratio of glycosylation most likely reflects a change in channel subunit composition. This would thereby contribute to subunit “poisoning” of Kir7.1 tetramers, further influencing the reduction in channel activity observed (Fig. 3, C and D). We also observed complexes of β2AR with the immature forms of Kir7.1 by co-immunoprecipitation in cells (Fig. S2). After cross-linking, only
GPCRs differentially regulate Kir7.1 glycosylation

A

RXXRXK

M1

M2

SYXXEIWW

C

SYLANNELW Kir2.1
SYPERSILW Kir4.1
SYPPRILW Kir5.1
SYPSEILW Kir7.1

C-TERMINAL

RSRFSWK Kir2.1
RRRFLT Kir4.1
RRRLH Kir5.1
YRRMVT Kir7.1

N-TERMINAL

B

Glycosylation sites
N terminal export patch
Golgi Export patch (c-terminal)

C

M.W (kDa)

WB: Anti FLAG

50

Kir7.1-3xFLAG

Kir7.1-3SYxFLAG

37

GAPDH

37

D

Ratio of Mature to Immature Glycosylated forms

K6

Kir7 3SYxFLAG

K6 Kir7 3xFLAG

G

Ratio of Mature/Complex to Immature forms of Kir7.1

K6 Kir7 3xFLAG

K6 Kir7 3SYxFLAG

K6 Kir7 3xFLAG + XRK

K6 Kir7 3SYxFLAG + XRK

F

Kir7.1 ΔSY3xFLAG in all lanes

WB: Anti FLAG

GAPDH

37

E

Endo H

PNGase F

Mature/Complex

Immature

Endo H Sensitive/Core
Unglycosylated

K6 Kir7 3xFLAG

K6 Kir7 3SYxFLAG

K6 Kir7 3xFLAG + XRK

K6 Kir7 3SYxFLAG + XRK

GAPDH

37
Figure 9. Kir7.1 forms heterotetramers of immature and unglycosylated subunits. HEK293T cells were transiently transfected with HA-Kir7.1 with either FLAG-tagged Kir7.1 N95Q, Kir7.1 ΔSY, or Kir7.1 WT as a control; transfection levels were as in Fig. 1. Cell lysates were immunoprecipitated with an HA antibody and immunoblotted with anti-FLAG. The blot is representative of three independent experiments. M.W, molecular mass; WB, Western blotting analysis.

Figure 10. A retinopathy-associated variant of Kir7.1 has altered complex glycosylation. Shown is Western blotting analysis (WB) of three known variants of Kir7.1, following transfection of Kir7.1 mutants indicated; transfection levels were as in Fig. 1. Whole-cell lysates of HEK293T cells expressing a known variant of Kir7.1 were analyzed for changes in glycosylation. Kir7.1 L241P in lane 5 not only had lower expression but also had an absence of complex glycosylation. *, 15 μg of protein were loaded versus 7.5 μg in all other lanes. The blot is representative of four independent experiments. M.W, molecular mass.

the immature forms of Kir7.1 co-immunoprecipitated with β2AR, although small amounts of complex Kir7.1 were present in the cell as observed in the input lane (Fig. S2). This suggests that immature channels may be the predominant form in the cells when co-expressed with certain GPCRs.

With β2AR forming complexes with the immature form of the channel, this suggests a possible mechanism for the GPCR-mediated loss of glycosylation. The protein titration experi-

Figure 8. A conserved Golgi transport signal is required for complex glycosylation of Kir7.1. A, schematic showing a two-pore domain Kir channel subunit with two transmembrane regions, M1 and M2. The consensus N- and C-terminal Golgi export patch residues are shown, with known essential residues shown in red. Clustal alignment of the N- and C-terminal regions of the Golgi export patch of other closely related Kir channels that have been previously identified (28). Kir7.1 shares a highly conserved patch, except for a C-terminal leucine residue where other channels have a tryptophan. B, SWISS-MODEL of Kir7.1 structure based on the crystal structure of Kir2.2 (PDB code 5KUK). Regions highlighted in blue show the glycosylation site in each monomer of the channel. The N- and C-terminal portions of the Golgi export patch of Kir7.1 are highlighted in green and red, respectively. C, deletion of the serine and tyrosine residues in the Golgi export patch of Kir7.1 led to a loss of complex glycosylation compared with the WT channel determined by Western blotting analysis (WB) in Cos1 cells. D, compared with the WT channel, essentially no complex glycans were observed by the significantly low ratio of 0.1 p < 0.005, (unpaired t test). E, enzymatic digestion of whole-cell lysates of the WT versus Kir7.1 SYΔ mutant from HEK293T cells confirmed that only the immature forms of Kir7.1 are expressed. F, β2AR also reduces the ratio of mature to immature glycosylated Kir7.1 in Cos1 cells. Western blotting analysis of whole-cell lysates from Cos1 cells co-expressing Kir7.1–3×FLAG with either 3× HA–β2AR or 3× HA–MC4R. 3× HA–β2AR also reduced Kir7.1 mature glycosylation in Cos1 cells as shown in the quantification by densitometric analysis in G (p < 0.05). Transfection levels in C, E, and F were as in Fig. 1. The data analyzed from four independent experiments were analyzed in GraphPad Prism with one-way ANOVA. The error bars for all data represent S.E. M.W, molecular mass.

GPCRs differentially regulate Kir7.1 glycosylation

ment showed a progressive increase in immature forms of the protein, with increased expression of β2AR. The amount of complex glycosylated Kir7.1, however, was generally reduced. A possible hypothesis is that β2AR forms complexes with Kir7.1 early in the endoplasmic reticulum and sterically blocks Kir complex glycosylation in the Golgi. Alternatively, the β2AR could also differentially stabilize immature forms of the protein. The potential role of G proteins in the formation of these proposed receptor–channel complexes should be examined in future experiments.

We have observed that deletion of the serine and tyrosine residues within the Golgi export site of Kir7.1 also inhibits complex glycosylation but leaves core glycosylation intact. The SY mutant of Kir7.1 described here may thus be a useful tool in future experiments to further validate the necessity of complex glycosylation for full channel function, suggested by the experiments in Fig. 2. Complex glycosylation is reported to occur at different stages during passage from the cis-medial-trans-Golgi before export to the surface (37). Within other Kir channels, the Golgi export site has been identified as a binding site of the AP-1 γ-adaptin protein, which is required for export to the cell surface (28, 29). In Kir2.1, deletion of these two residues reduced complex glycosylation of an artificial site placed within the channel by mutagenesis, with an intermediate glycosylation pattern observed (29). They argue that because complex glycosylation occurs in the trans-Golgi network, loss of the export site traps Kir2.1 in both the cis- and trans-Golgi networks, preventing further transport leading to incomplete complex glycosylation. However, in the work of Li et al. (28), the authors comment that compared with other Kir channels, Kir7.1 is the only channel that does not seem to share the full export site. Although we have confirmed the export site to be essential for complex glycosylation, these two residues do not appear to be required for Golgi export (data not shown); however, this was expected because we did see immature forms of the WT channel at the surface in biotinylation experiments (Fig. 4).

The AP-1 γ-adaptin protein is the only protein reported in literature to bind to the Golgi export site in Kir channels. The role of this protein in glycosylation has not been investigated, nor any other proteins that may bind to this site to mediate complex glycosylation. Our data suggest that GPCRs such as β2AR are indeed preventing this crucial step of glycosylation from occurring through the mechanism is unclear. It is plausible that GPCRs may indeed be blocking proteins from binding to this protein export site, which we have shown is required for glycosylation.
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Both the oxytocin receptor (17) and the MC4R (14) have been shown to regulate Kir7.1 function at the membrane in a physiologically relevant manner, with the former mediated via a PI3K intermediate. The mechanism(s) for regulation of Kir7.1 by MC4R remain to be determined, although the potential for Kir7.1 and MC4R to form complexes was shown by co-immunoprecipitation of tagged proteins from HEK293T cells (14). Although MC4R is a slightly smaller GPCR, its inability to block complex glycosylation relative to other GPCRs we tested is not understood. One hypothesis is that MC4R may only form complexes with Kir7.1 post-Golgi export, but this is unknown.

The data shown here provide evidence for molecular regulation of Kir7.1 structure and function by GPCRs. Within tissues where Kir7.1 is highly expressed, large variations in glycosylation have been recently observed using a knockin mouse expressing an HA-tagged Kir7.1 channel (38). Notably, the study showed predominantly immature forms of the channel in the lung, whereas full complex glycosylation was observed in the trachea (38). No explanation for variation in glycosylation patterns between these two tissues was given, but our data may suggest a possible mechanism by which such changes could occur.

Materials and methods

Cell lines and cell culture

HEK293T cells were grown in Dulbecco’s modified Eagle’s medium, with high glucose, t-glutamine, and phenol red supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% antibiotic-antimycotic (Thermo Fisher Scientific). The cultures were maintained in 5% CO2 environment at 37 °C. Cos1 cells were cultured similarly to HEK293T cell lines with medium containing sodium pyruvate. The protein transport inhibitor mixture from BD Biosciences was used at 0.5×, and the cells were incubated for 9 h prior to harvesting.

Transfection

Plasmid DNA constructs were transfected into HEK293T or Cos1 cells at 70–80% confluency using LipD293 reagent (SignaGen) according to the manufacturer’s instructions. The cells were allowed to grow for 24 h before harvesting. If cells were kept for 48 h, the medium was changed after 24 h.

Cell surface biotinylation

Cell surface biotinylation was performed as described in Chandrasekhar et al. (39). Briefly, HEK293T cells were transiently co-transfected at 70–80% confluency with Kir7.1–3×FLAG and either 3×HA–B2AR or 3×HA–MC4R. 24 h post-transfection, the cells were washed three times with PBS with calcium and magnesium (PBS2+). The cells were then incubated with 1 mg/ml of biotin-SS-sulfo (ApexBio) in PBS2+ twice for 15 min each. Excess biotin was quenched with two short washes followed by two 15-min incubations with 100 mM glycine in PBS2+. The cells were washed with PBS and then lysed with modified radioimmune precipitation assay buffer (see Western blotting protocol). The protein samples were quantified, and 25 µl of Pierce™ high-capacity streptavidin agarose beads (Thermo Scientific) were incubated overnight with 75 µg of protein lysate. The supernatant was removed, and the beads were washed three times with lysis buffer. The beads were first eluted with 2×LDS with 200 mM DTT then with 100 mM DTT in lysis buffer. Both elutions were combined and loaded on a 10% Bolt™ Bis-Tris Plus gel (Invitrogen) next to one-fifth of the input (15 µg of total cell lysate). Western blot analysis was continued as described.

Western blots and quantitative analysis

Post-transfection, the cells were lysed in a modified radioimmune precipitation assay buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, and protease inhibitor mixture (Sigma/Roche). The cells were scraped and incubated on ice with rocking for 30 min followed by a 10,000 rpm centrifugation for 10 min. The supernatant was collected and protein-quantified using a Pierce BCA protein assay kit. Lysates were prepared for denaturing gel electrophoresis by mixing with NuPAGE® LDS sample buffer (4×) (Invitrogen) with 400 mM DTT to a final concentration of 1×LDS, 100 mM DTT. Invitrogen Bolt™ 10% Bis-Tris Plus precast polyacrylamide gels were used to run 10–15 µg of protein at 200 V for 40 min using Bolt™ MOPS running buffer. The gels were run with Bolt™ running buffer with Bolt™ antioxidant added to the first chamber of the mini gel tank (Thermo Fisher Scientific). A Bio-Rad protein ladder, Kaleidoscope, Dual Color, or All Blue was also included on each gel. The gels were then transferred to polyvinylidene difluoride membrane (Millipore) using Bolt™ transfer buffer, with the Trans-Blot Turbo™ transfer system from Bio-Rad. The membranes were blotted with 5% nonfat dry milk in PBS with 1% Tween 20 for 30 min, prior to overnight incubation with antibodies. The blots were washed a minimum of three times for 10 min each before imaging with SuperSignal™ West Dura extended detection system. Images with samples of interest within the dynamic range were chosen for further quantitative analysis using the Bio-Rad Image Lab™ software. Ratios of mature to immature glycosylation were obtained by dividing the intensity of the upper 50-kDa doublet, by the lower 35-kDa doublet intensity. No normalization was performed on these values because the ratios are independent of protein loading. For comparison of receptor expression levels, intensities recorded were normalized to the loading control GAPDH. The data were analyzed using GraphPad Prism software. A one- or two-way ANOVA statistical test was used to determine significance (p < 0.05) with Tukey post-test for multiple comparisons.

Glycosidase treatments

Channel glycosylation was investigated using the endoglycosidases Endo H and PNGase F from New England Biolabs. Briefly, cells transiently transfected with Kir7.1–3×FLAG or Kir7.1Δ5Y–3×FLAG were harvested, and protein was quantified as previously described. 50 µg of lysate was denatured by addition of 10× glycoprotein denaturing buffer (New England Biolabs) followed by heating for 10 min at 55 °C. Samples being treated with Endo H were mixed with GlycoBuffer 3 and 2 µl of Endo H enzyme. The mixture was incubated for 1.5 h at 37 °C in a thermocycler. Samples treated with PNGase F were mixed with GlycoBuffer 2, plus 1 µl of the enzyme and also incubated
for 1.5 h at 37 °C. The treated samples were then mixed appropriately with 4× NuPAGE LDS buffer with 400 mM DTT and analyzed by Western blotting analysis as described. The samples were compared with untreated lysates and observed for band shifts in migration.

**Expression constructs**

Plasmids with full-length cDNA for the GPCRs β1AR, β2AR, β3AR, and MC4R were obtained from the cDNA Resource Center with 3× HA N-terminal tags in the pCDNA3.1+ expression vector. The full-length cDNA sequence for KCNJ13 (AJ066128.1) was cloned into the pCI-NEO expression vector (Promega) with a C-terminal 3× FLAG tag (Sigma). To create the HA-Kir7.1 construct, the full-length cDNA sequence for KCNJ13 was cloned into a pCDNA3.1+ vector with a single N-terminal HA tag. To create mutations in Kir7.1, primers were designed using the NEBaseChanger online tool and ordered from Sigma. Mutagenesis was performed using New England Biolabs Q5 site-directed mutagenesis kit (New England Biolabs). For electrophysiology, the cDNA sequence of Kir7.1 was cloned into pcDNA5/TO expression vector (Thermo Fisher Scientific) without tags. The VSVG-GFP construct was provided by the Kenworthy lab at Vanderbilt University.

**Antibodies**

Western blotting was performed using the following antibodies: monoclonal ANTI-FLAG® M2-peroxidase (HRP) antibody produced in mouse (Sigma–Aldrich) 1:10,000; HA tag (6E2) mouse mAb (HRP conjugate) (Cell Signaling Technology); GAPDH (D16H11)XP® rabbit (HRP conjugate) (Cell Signaling Technology); monoclonal ANTI-FLAG® M2-peroxidase (HRP) antibody produced in mouse (Sigma–Aldrich) 1:5000; and anti-GFP (HRP conjugate) (Abcam) 1:10,000.

**Structural models and protein alignments**

SWISS-MODEL (Swiss Institute of Bioinformatics) online software was used to generate a structural homology model of Kir7.1 based on the published crystal structure of Kir2.2 (PDB code 5KUK). Models were visualized using PyMOL (Schrodinger). Protein alignments were performed with either Clustal Omega (EMBL-EBI) or Lasergene MegAlign software (DNASTAR) using the UniProt entry human sequences for Kir7.1 (KCNJ13_O60928), Kir4.1 (KCNJ10_P78508), Kir5.1 (KCNJ15_Q99712), Kir3.1 (KCNJ3_P48549), Kir2.1 (KCNJ2_P63252), and Kir1.1 (KCNJ1_P48048).

**Whole-cell electrophysiology**

Whole-cell patch-clamp electrophysiology was performed as described by Raphemot et al. (18). Briefly, HEK-293T cells were transfected with Kir7.1-M125R—encoding plasmids (0.5 μg), MC4R plasmids (3.75 μg), or β2AR plasmids (3.75 μg) and EGFP plasmid (0.05 μg) as a marker for transfections. The following day, the cells were dissociated with trypsin and plated on poly-L-lysine–coated glass coverslips. The plated cells were allowed to recover for 1 h prior to experiments. The cover slips were placed in the recording chamber on an inverted microscope stage and perfused with a bath solution containing 135 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 10 mM HEPES, pH 7.4. A Flaming–Brown P-1000 micropipette puller was used to pull electrodes with resistances between 2 and 3 MΩ. The pipettes were filled with 135 mM KCl, 2 mM MgCl2, 1 mM EGTA, 10 mM HEPES, pH 7.3. Transfected cells were identified by EGFP fluorescence, and voltage-clamp conditions were used to record whole-cell currents. To obtain a current–voltage curve, the cells were voltage-clamped at a holding potential of −75 mV and then stepped to −150 mV for 500 ms before ramping to 150 mV at a rate of 2.4 mV/ms. The cells were superfused with 4 mM BaCl2 at the end of the experiment to fully block all Kir7.1 channels. This value was used to derive barium inhibitable current.

**Single-channel electrophysiology**

For electrophysiology experiments, the cells were plated onto 12-mm cover glass chips at 4 × 104 in 35-mm-diameter culture dishes and transfected after 24 h with 0.3 μg of cDNA of WT Kir 7.1 or Kir 7.1N95Q and 0.05 μg of EGFP (to identify transfected cells) using X-tremeGENE9 DNA transfection reagent (Roche Diagnostics). Channel activity was recorded 48–72 h after transfection in the cell-attached configuration. Pipettes had resistances of 6–10 MΩ when filled with a solution of the following composition: 135 mM NaCl, 5 mM KCl, 10 mM MgCl2, 2 mM CaCl2, 5 mM glucose, 10 mM HEPES, pH 7.4, with NaOH. Cells were bath-perfused with a high-K+ solution of the following composition: 135 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 5 mM glucose, 10 mM HEPES, pH 7.4, with KOH. The resting membrane voltage was assumed to be 0 mV. Currents were recorded using an Axopatch 200B amplifier, filtered at 2 kHz, sampled at 20 kHz using Digidata 1550, and saved using pCLAMP 10.4 (Axon Instruments). The data were analyzed offline using TAC 4.2 and TACFit 4.2 (Bruxton Corporation) software. Single-channel open and closed events were analyzed using the 50% threshold detection method and visually inspected before accepting the events. Single-channel openings occurred as bursts of one or more openings or cluster of bursts. Duration time and amplitude histograms were generated using TACFit 4.2 (Bruxton Corporation, Seattle, WA). Single-channel amplitudes (i) were calculated by fitting all-point histograms with single- or multi-Gaussian curves. The difference between the fitted “closed” and “open” peaks was taken as i. Duration histograms were fitted with exponential components in the form: Σ(ai/τi)exp(−t/τi), where a and τ represent the relative area and time constant of the i component, respectively, and t is the time. Numerical data were expressed as means ± S.E. Statistical analysis was performed using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla, CA). Statistical significance was taken as p < 0.05 using unpaired two-tailed Student’s t test.

**GPCRs differentially regulate Kir7.1 glycosylation**

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