Characterizing the roles of multiple Gβγ binding sites on Kir3 channels

by

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ABSTRACT (english)

Kir3 channels contain multiple Gβγ binding sites within their N- and C-terminal domains. However, the channel opens when only a few binding sites are occupied by Gβγ, suggesting that some sites subserve roles other than agonist-dependent interactions. We delineated these roles on Kir3.2 channels with mutations in their N- or C-terminal Gβγ binding sites.

The N-terminal mutant displayed a high affinity site for Gβγ but this affinity was reduced with the C-terminal mutant. Also, the N-, but not the C-terminal mutant could traffic to the cell surface. Carbachol stimulation of the muscarinic M_2 receptor, resulted in conformational changes between Gβγ and the wildtype channel, which were not engendered by either of the mutants. Lastly, neither of the mutant channels could be opened following receptor activation.

We conclude that the C-terminal Gβγ site may be required for 'precocious' interactions, while the N-terminal site may be needed for signalling following receptor activation.

ABSTRACT (french)

Les canaux Kir3 possèdent de multiples sites de liaison pour les protéines Gβγ, localisés dans leurs domaines N- et C-terminaux. Cependant, la liaison des protéines Gβγ à seulement quelques-uns de ces sites suffit à produire l'ouverture du canal, ce qui suggère que certains sites remplissent des rôles autres que ceux résultant de l'interaction avec les protéines Gβγ lors d'un l'activation du récepteur. Nous avons investigué ces rôles en utilisant des canaux Kir3.2 ayant des mutations au niveau des sites de liaisons des protéines Gβγ dans les domaines N- et C-terminaux.

Nous démontrons que le mutant de la partie N-terminale possède un site de haute affinité pour les Gβγ, mais que cette affinité est réduite par la présence du mutant de la partie C-terminale. De plus, seul le mutant de la partie N-terminale a la capacité d'être acheminé à la surface cellulaire. Une stimulation du récepteur muscarinique M_2 par le carbachol résulte en un changement de conformation entre les protéines Gβγ et le canal de type sauvage, ce qui n'est pas engendré par les mutants. Finalement, aucun des canaux mutants n'a pu être ouvert suivant l'activation du récepteur.

Nous concluons que le site de liaison situé dans la région C-terminale puisse être requis pour des interactions précoces alors que celui en N-terminal puisse être nécessaire à la signalisation suivant l'activation du récepteur.

LIST OF ABBREVIATIONS

ATP – adenosine triphosphate

BGH – bovine growth hormone

Bmax – maximal BRET

BRET – bioluminescence resonance energy transfer

BRET2 – bioluminesnece resonance energy transfer 2

$BRET_{50}$ - half maximal BRET

BSA – bovine serum albumin

CMV – cytomegalovirus

DABCO – 1,4-diazabicyclo[2,2,2]octane

DDM – n-dodecyl-D-maltoside

DMEM – Dulbecco's modified eagle medium

DN – dominant negative

EDTA – ethylenediaminetetraacetic acid

ER – endoplasmic reticulum

FRET – fluorescence resonance energy transfer

G proteins - guanine nucleotide binding proteins

GDP – guanosine diphosphate

GFP – green fluorescent protein

GPCR – G protein-coupled receptor

GTP – guanosine triphosphate

HA – hemagglutinin

HEK293 – human embryonic kidney

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IRK channels – inwardly rectifying potassium channels

Kir3 channels – inwardly rectifying potassium channels

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PEI – polyethylenimine

PIP_2 – phosphatidylinositol 4,5 bisphosphate

PMSF – phenylmethanesulfonylfluoride

PVDF – polyvinylidene fluoride

RET – resonance energy transfer

RluA – luciferase activity

Rluc – renilla luciferase

SDS – sodium dodecyl sulfate

SEM – standard error of the mean

TAP – tandem affinity purification

TBS-T – tris buffered saline tween

TEV – tobacco etch virus

WT – wildtype

β_2AR – β_2 adrenergic receptor

βARK-CT – carboxy terminus of beta adrenergic receptor kinase

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INTRODUCTION

Historical perspectives of G protein signalling

During the first few decades of the twentieth century, scientists focused on understanding how cells transformed an extracellular stimulus into a cellular response [1-2]. Although the concept of a receptor had been acknowledged as a means to recognize extracellular stimuli, there remained many gaps in our knowledge about how information flowed from the receptor to produce a cellular response [2]. With the initial identification of receptors, as well as transducers and downstream effector molecules, arose questions about the interactions between them [3].

In 1972, Singer and Nicholson proposed the fluid mosaic model in an attempt to characterize the structure and organization of cell membranes [2]. According to this model, all proteins were free to move independently within the lipid bilayer and transiently interact as a result of random collisions [2-4]. Thus, protein-protein interactions in G protein signalling cascades were thought of as transient events [2]. Although this model was sufficient to explain signal transduction in tissues such as the rod outer segment disk of the mammalian visual system, where there is only one receptor, one transducer and one effector, it soon failed to account for the specificity of cellular signalling in other complex systems [2].

Most cells express a large number of receptors, transducers (also known as guanine nucleotide binding proteins or G proteins) and effector molecules

(shortened now as *effectors*). For example, in the rat portal vein myocyte, there are reportedly 11 different receptors, 7 Gα subunits, 5 Gβ subunits, 8 Gγ subunits and 6 different effectors [2]. In humans, there are hundreds of receptors, at least 20 different Gα subunits, 5 Gβ subunits, 13 Gγ subunits and dozens of effectors [5-6]. Antisense approaches have shown that receptors require specific G protein heterotrimer combinations in order to elicit a specific response [5]. For example, vasoactive intestinal peptide receptors mediate the stimulation of adenylyl cyclase by activating $G\alpha_s\beta_2\gamma_2$, while somatostatin receptors mediate the inhibition of adenylyl cylase by coupling to $G\alpha_{i2}\beta_1\gamma_3$ [2]. As such, many specific mechanisms need to be in place to propagate a given signal into a particular effector response [7].

Other evidence for the existence of signalling specificity stems from studies with the inwardly rectifying potassium (Kir) channels. β_2-adrenergic receptors (β_2AR) have been shown to activate $G\alpha_s$ proteins which can in turn, stimulate adenylyl cyclase [8], while the $G\alpha_i$-coupled muscarinic M_2 receptors activate the Kir channels through the Gβγ subunits. If random interactions occurred, one would expect the Gβγ dimers potentially liberated from heterotrimeric G proteins upon β_2AR activation to open the Kir channels [2]. However, the β_2AR does not normally open the Kir channels, suggesting again that there are mechanisms that confer signalling specificity [2, 9].

The random collision model, thus not only failed to account for signalling specificity, but also appeared to have a conceptual problem with regards to the

speed of G protein-mediated signalling events. Since effector responses can be elicited within milliseconds following agonist stimulation, this model also encountered difficulties in accounting for the speed that directs specific signalling interactions if signalling partners had to associate and dissociate with each receptor activation cycle [2].

A novel view of G protein signalling

How then is fidelity in receptor signalling achieved, given that many systems use the same components of the G protein signalling machinery? Although G protein signalling had always been perceived as a series of transient interactions between receptors, G protein subunits and effectors at the cell surface, there is now growing evidence from yeast, *Drosophila* and mammalian systems for an alternative organizational paradigm in which proteins interact to form macromolecular signalling complexes [9-10]. The assembly of signalling complexes undoubtedly serves as a robust model to account for the specificity observed in signalling events [4]. Macromolecular protein complexes also have several advantages in that they can allow for a signal to propagate quickly, reduce the noise or "cross-talk" associated with non-specific collisions, and also reduce the energy costs associated with the maintenance of localized protein complexes [4].

In the last decade or so, many G protein-coupled receptor (GPCR) investigators have focused on the composition of these protein complexes and where they might assemble and reside in the cell [9, 11-13]. Recent studies using

molecular imaging techniques in living cells such as fluorescence or bioluminescence resonance energy transfer (FRET or BRET) have demonstrated that various signalling proteins remain associated with one another during signal transduction [4, 13-14]. In fact, biochemical studies have identified receptor-G protein [15], G protein- effector [3, 9] and receptor-effector interactions [13], all of which occur in the absence of receptor activation [2]. This further suggests that protein interactions within these complexes occur prior to their arrival at the

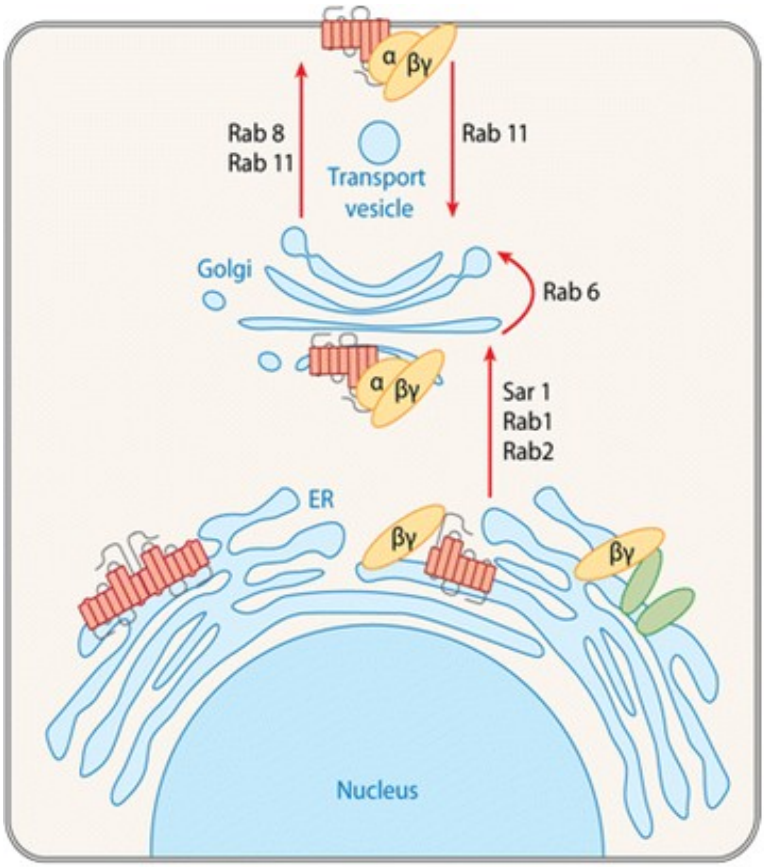


Figure 1. Assembly and trafficking of signalling complexes. Signalling proteins are thought to interact in the ER soon after their biosynthesis. These interactions include receptor-receptor, receptor-G protein and G protein-effector interactions. Once these proteins are assembled into a complex, they are trafficked to the cell surface by a number of Sar and Rab GTPases, which mediate the trafficking of proteins within distinct compartments of the exocytic and endocytic pathway. Upon reaching the cell surface, the signalling complex responds to extracellular stimuli. Figure from [16].

plasma membrane, leading to the question of where they might initially interact [3, 11]. A number of studies have demonstrated that these proteins initially interact at the endoplasmic reticulum (ER) soon after their biosynthesis [2-3, 9, 11]. These include the formation of receptor dimers [11], receptor-Gβγ interactions [11] and Gβγ-Kir3 channel interactions [3, 9]. As seen in Figure 1, once these complexes are formed inside the cell during protein biosynthesis, they are trafficked to the cell surface via trafficking pathways dependent on Sar or Rab GTPases, which govern distinct steps of both the exocytic and endocytic trafficking pathways [11, 17]. For example, Rab1 regulates ER-Golgi transport, whereas Rab6 regulates intra-Golgi transport [6, 17]. Once at the cell surface, these complexes can be activated by an external ligand in order to propagate a given cellular response [11]. Therefore, decisions regarding signalling specificity and complex formation can be made early during biosynthesis [3, 9, 11].

GPCRs and G protein signalling

GPCRs are the largest family of transmembrane proteins and mediate most cellular responses to a variety of ligands such as hormones, neurotransmitters, lipids, peptides and even photons [5, 18-19]. Our initial observations of GPCR structure and function arose from the experimental framework of the rhodopsin receptors in the visual system [20-23]. We now know that all GPCRs are characterized by the presence of 7 membrane-spanning α-helices, which are separated by an alternating pattern of extracellular and intracellular loops [1, 24]. In addition, all GPCRs also share an extracellular amino-terminal domain and an

intracellular carboxy-terminal domain [24]. Information is transmitted to the receptor through ligands which bind from the extracellular surfaces of the receptor [18]. However, in addition to being activated by ligands, GPCRs can also demonstrate spontaneous, ligand-independent activity [21, 25].

From the receptor, signals are transmitted to the heterotrimeric G protein, which is composed of a Gα subunit and the dimeric Gβγ subunit [6, 26-27]. As mentioned previously, there are multiple Gα, Gβ and Gγ proteins, in addition to a number of splice variants [27]. Gα subunits can be divided into four main classes based on their sequence and functional similarities: Gαs, Gαi, Gαq/11 and Gα12 [18, 26]. Given the vast number of Gβ and Gγ subunits, one would expect a large number of possible Gβγ dimer combinations [5]. However, all dimeric pairs have not been shown to exist *in vitro* [16].

The standard model of G protein signalling proposes that the binding of an agonist to a GPCR promotes a conformational change in the intracellular domains of the receptor, allowing it to act as a guanine nucleotide exchange factor on the Gα subunit and thus, facilitating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα subunit [5, 19, 28]. This results in the activation of the G protein and allows the Gα subunits and the Gβγ subunits, to either independently or in concert regulate the function of many downstream effectors including adenylyl cyclase, phospholipase, protein tyrosine kinase, MAP kinase, and even a number of ion channels [1, 5, 28-29]. These effectors can be regulated indirectly (through the production of second messengers and their

activated protein kinases) or directly through the physical association of the proteins [7, 29-31].

Although classic models of G protein signalling have depicted subunit dissociation accompanying G protein activation, recent chemical and molecular cross-linking studies have begun to challenge this notion by showing that physically tethered heterotrimers may still be functional in terms of signalling [10, 32]. Furthermore, FRET-based assays in human embryonic kidney (HEK293) cells have indicated that the G protein heterotrimer undergoes molecular rearrangement during activation rather than complete dissociation [16]. These data suggest that conformational changes in the G protein, rather than subunit dissociation *per se*, are sufficient to reveal distinct effector interacting surfaces [10, 16]. However, there is also accumulating evidence for the support of the original hypothesis [16]. Thus, the consensus today is one of where both scenarios are possible, with a predisposition towards the notion of partial, rather than complete subunit dissociation [16, 32]. However, the degree of subunit dissociation appears to depend on the particular heterotrimer in question. For example, Gαi-containing heterotrimers are able to dissociate from Gβγ more readily than Gαs-containing heterotrimers [33]. This may in turn, affect the function of downstream effectors which are regulated by these G proteins [33].

Effectors: Kir3 channels

A) An introduction

There are seven known families of inwardly rectifying potassium (Kir) channels, which by virtue of their properties, play a role in establishing the resting membrane potential and maintaining potassium homeostasis [34-37]. In cardiac myocytes, activation of Kir channels underlies the acetylcholine-induced decrease in excitability, which serves to reduce the heart rate following vagal activity [38-39]. In the central nervous system, Kir channels mediate the postsynaptic inhibitory effects of eight different neurotransmitters [38, 40-41].

Of particular interest here is the mammalian family of Kir3 channels (also known as GIRK or G protein-gated Inwardly Rectifying Potassium channels), comprised of four members: Kir3.1, Kir3.2, Kir3.3 and Kir3.4 [39, 42]. These subunits form functional channels as either homo- or hetero-tetramers (depending on their composition) at the cell surface [43-45]. Kir3 channels are predominantly expressed in the brain, heart and pancreas, although their tissue expression patterns are also dependent on their subunit combinations [38, 42-43]. For example, the Kir3.1- and Kir3.2-containing tetramers are mainly expressed in the brain, notably in the cerebellum, cerebral cortex and hippocampus, while the Kir3.1- and Kir3.4-containing channels are mainly expressed in the heart [38, 41, 44].

B) Clinical relevance of studying Kir3 channels

Although much is known about diseases with other members of the Kir channel superfamily, less is known about the Kir3 channels. Nevertheless, several animal models indicate that alterations in Kir3 channel function might be detrimental in humans.

The weaver mouse model, is probably one of the best studied models of Kir3 channel pathologies [46]. The weaver phenotype arises from a loss-of-function mutation (G156S) in the highly conserved H5 pore region of the Kir3.2 subunit [30, 37]. This mutation leads to a loss of potassium selectively and manifests itself as severe ataxia and sporadic seizures, which stem from a lack of neuronal differentiation in the cerebellum [30, 43].

The Kir3 channels are also believed to be the targets of many antidepressant and antipsychotic drugs used to treat various neurological disorders [47]. Classical antipsychotics such as clozapine and haloperidol, in addition to antidepressants, appear to inhibit Kir3 channel currents, thereby increasing the likelihood of neuronal firing [46]. Moreover, since addictive drugs such as cocaine, opioids and cannabinoids have been shown to activate Kir3 channels, inhibitors of the channel could potentially be used in the treatment of certain types of drug abuse [48-49].

Recent studies with knockout mice also suggest that the Kir3 channels may be implicated in learning [49], pain [49-52] and epilepsy [53]. The Kir3.2 subunit in particular, has been implicated in a mouse model of Down syndrome [54].

Preliminary work also suggests that the Kir3 channels are implicated in breast [55] and lung cancer [56] in humans. Given their predominant expression in the heart, the Kir3 channels may also be involved in certain cardiac pathologies, especially since knockout mice lacking the Kir3.4 subunits, are unable to regulate their heart rate [37].

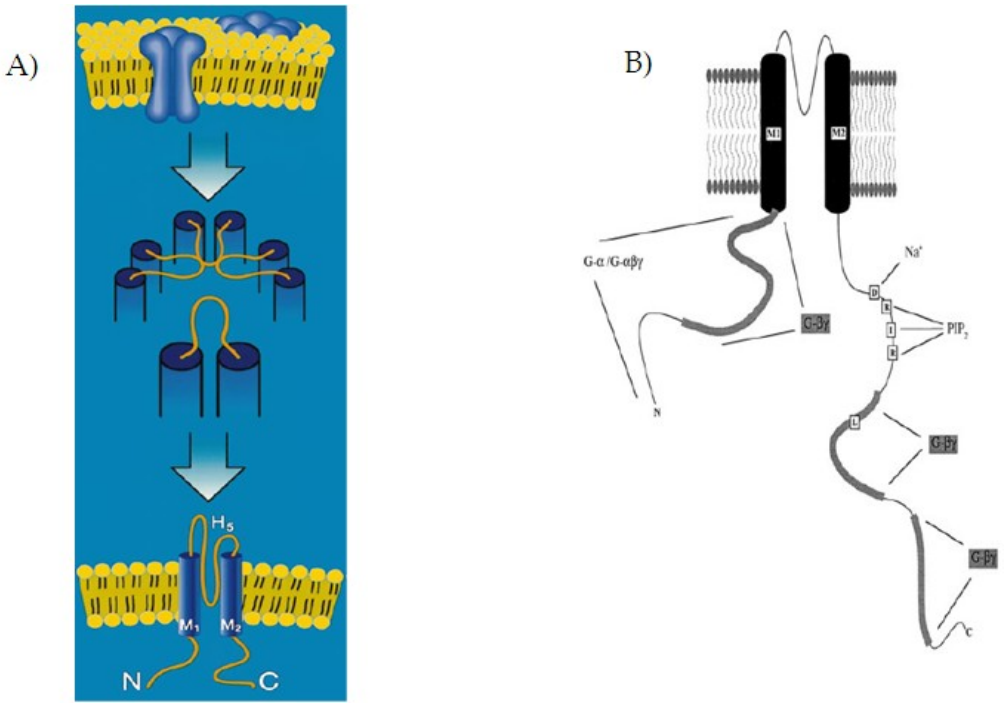


Figure 2. Structure of the Kir3 channel. (A) The channel is embedded in the plasma membrane (top panel) and is a tetramer of four subunits (middle panel). As shown in the lower panel, each subunit contains two membrane-spanning domains (M_1 and M_2) as well as a pore region (H_5). In addition, the channel consists of an amino (N) and carboxy (C) terminus which extend into the intracellular face of the plasma membrane. Figure from [37]. (B) Each of the N- and C- terminal domains of the Kir3 channel contain regions responsible for G protein, PIP_2 and sodium (Na^+) binding. G protein subunits can bind to both the N- and C-termini of the channel and each subunit may contain 2-3 binding sites for Gβγ. PIP_2 binds to the C-terminus of the channel through its interaction with positively charged amino acids. Na^+ is suggested to neutralize the negatively charged aspartate in the C-terminus so as to favor PIP_2 binding. Figure from [30].

C) Channel structure

Crystal structures of two bacterial homologues of potassium channels, KcsA and KirBac1.1, demonstrated for the first time, the architectural features of the Kir channel family [35, 57]. As seen in Figure 2A, each subunit of the tetrameric Kir channel is made up of two transmembrane spanning helices (~90 amino acids) which surround a highly-conserved pore region [38-39, 45, 57].

Two-thirds of the amino acid sequence of the different Kir3 subunits are characterized by large hydrophilic amino (N) and carboxy (C) terminal tails which extend from 90 to over 200 amino acids into the cytoplasm, respectively [39]. These large cytosolic domains are the targets and the transducers of the modulatory effects of intracellular signals [57-59]. As such, a diverse set of intracellular molecules such as G proteins, membrane phospholipids, sodium and magnesium, among many others, affect channel gating through these domains (Figure 2B) [58, 60].

The pore of the channel, which spans a length of ~100 amino acids, is located between the two transmembrane spanning helices and measures ~30Å in length and 7-15Å in diameter [59, 61]. The outer wall of the pore is composed of β-sheets that are surrounded by a number of polar residues [61]. The pore itself, is characterized by a descending pore helix followed by an ascending and well-conserved potassium channel signature sequence, TXGYG [40, 61]. Point mutations within this sequence have been shown to abolish potassium selectivity

and thus, the pore loop is thought to harbor the selectivity filter of the channel [35, 60, 62].

The four cloned human Kir3 subunits encode proteins of 393 to 501 amino acids, which share approximately 36% sequence identity [63]. In the transmembrane domains and the pore regions of the channels however, the level of sequence identity increases to ~80-90% [63]. The Kir3.1 subunit is the largest subunit of the Kir3 channels, and also the most divergent of the four since it shares only 44% sequence identity with the other three subunits [44]. The Kir3.2, Kir3.3 and Kir3.4 subunits are more closely related, sharing up to 62% sequence identity [43]. All four human Kir3 subunits share high sequence similarities with most mammalian species [57].

D) Channel trafficking

The post-translational trafficking itineraries of the Kir3 channels after their exit from the ER vary greatly depending on the channel subunit [64-65]. Studies have shown that the Kir3.1 subunit is unable to localize to the plasma membrane when expressed alone in heterologous cells, due to the absence of an ER export signal [64, 66-67]. Only when expressed with either the Kir3.2 or Kir3.4 targeting subunits, can the Kir3.1 subunit be trafficked to the plasma membrane as part of a functional channel [65-66]. In contrast to the Kir3.1 subunit, both the Kir3.2 and Kir3.4 subunits have been reported to form functional homomeric channels at the cell surface when expressed alone [65]. Interestingly, the Kir3.3 channel subunit contains a lysosomal targeting signal (YWSI motif), targeting the channel to the

late endosomal/lysosomal pathway where degradation occurs [43, 49, 63, 68]. For this reason, the Kir3.3 subunit is thought to help regulate the overall number of Kir3 channels at the cell surface probably through heteromultimerization with other channel subtypes [65]. Although much is known regarding these early Kir3 channel trafficking events, the fate of these channels after internalization from the cell surface remains unknown [66].

E) Channel gating

The biophysical fingerprint of Kir3 channels is their characteristic inward rectification, which depends critically on positively charged polyamines that reside in the cytoplasmic side of the cell [59, 62, 69]. Under experimental conditions, this inward rectification can give rise to large inward currents at hyperpolarized potentials and small outward currents under depolarized potentials [36, 38]. The limited potassium flux at depolarized potentials is due to the blockade by intracellular magnesium and polyamines such as spermine and spermidine [35, 69-70]. Thus, unlike the family of voltage-gated potassium channels, the Kir3 channels structurally lack an intrinsic voltage sensor segment, despite the fact that their activity is voltage-dependent [71].

Kir3 channel gating is also dependent on the presence of membrane phosphatidylinositol 4,5 bisphosphate (PIP_2) levels (Figure 2B) [34, 57, 70, 72]. As such, Kir3 channel currents are enhanced in the presence of PIP_2 and its depletion usually leads to channel rundown [34, 58]. The PIP_2-binding domains have been localized to the N- and C-termini of the Kir3 channel [30, 72].

F) Gβγ subunits and Kir3

F1. Gβγ – central activators of Kir3

In addition to polyamines and PIP_2, the heterotrimeric Gβγ subunits also play a crucial role in modulating Kir3 channel activity [30, 72]. Although it was clear for many years that the Kir3 channels were coupled to and activated by pertussis toxin-sensitive GPCRs such as the muscarinic M_2, δ-opioid, dopamine D_4 or somatostatin receptors, the G protein subunit directly responsible for channel activation was actively debated [30, 40, 73]. Logothethis et al. were the first to show that it was Gβγ rather than Gα that modulated channel activation in a membrane-delimited manner [40, 74-75]. The Gα subunits were instead shown to determine the specificity of G protein action [38, 73, 76-77]. For example, chimeric studies have revealed the Gαi subunit, but not the Gαs or Gαq subunits, as critical determinants of Gβγ specificity for Kir3 channel modulation [38]. Thus, Kir3 channel activity is dependent on the specific Gα subunit to which the Gβγ subunits are associated [38, 73].

The ability of the Gβγ dimer to mediate effector signalling sparked significant interest in the field of G protein signalling since historically it was the Gα subunits which were thought to be important for signal propagation [7, 76, 78]. Gβγ dimers were only thought to be required for the inactivation of the Gα subunits, allowing them to re-associate with the receptor [7, 77]. Interestingly, the Kir3 channels were one of the first direct effectors identified for Gβγ, providing an excellent model for studying Gβγ-effector interactions [75, 79]. Today we know

that Gβγ subunits modulate many other effectors such as phospholipase C, phosducin, adenylyl cyclase and even some voltage-gated calcium channels, just to name a few [7, 80-82].

The contribution of the Gβγ subunits to Kir3 channel activation was initially controversial, but there are now numerous lines of evidence that support the notion of Gβγ dimers as central activators of these channels. For instance, purified Gβγ subunits from the bovine brain have been shown to activate Kir3 channels [71]. This finding was later confirmed in heterologous expression systems [30] as well as in excised patches from atrial myocytes [31]. Overexpression of Gβγ subunits in *Xenopus* oocytes was also shown to increase basal Kir3 channel currents [31, 83]. Additionally, Gβγ sequestration through phosducin or Gβγ-binding peptides has been shown to render the channel inactive, even when stimulated by neurotransmitters, thereby reinforcing the role of the Gβγ subunits in Kir3 channel activation [30].

Recent mutagenesis work has also confirmed the contribution of the Gβγ subunits in Kir3 channel regulation. Specific point mutations in the $G\beta_1$ subunit have been shown to block the ability of the G protein to regulate Kir3 channel activity [84]. Likewise, mutations in the C-terminal extremity of the $G\gamma_2$ subunit, demonstrated that this subunit was required for the activation of the channel [83].

F2. Binding of Gβγ to Kir3

Using channel fusion proteins, a number of studies have demonstrated the direct binding of Gβγ subunits to the full-length Kir3 channel or to segments of the intracellular N- and C-terminal domains of the channel [30-31].

Electrophysiological studies have shown that deletion of the Gβγ binding site on the N-terminus of Kir3.1 is associated with the loss of the fast activation and deactivation kinetics which usually accompanies Kir3 channel activation [30]. Similarly, peptides derived from the N- and C- termini of the Kir3 channel were shown to block G protein modulation of the channel in excised patches [40]. Co-immunoprecipitation of Kir3.1 and Kir3.4-containing subunits and Gβγ from atrial membranes with antibodies against Kir3.1 and Kir3.4, have also supported the idea of an interaction of the G protein with the channel [71, 78]. Thus, direct interactions between the Gβγ subunits and the channel are required for channel activation [74].

Much recent work has also focused on the interaction sites of Gβγ with Kir3 channels. For example, point mutation studies have helped identify critical regions of the channel important for Gβγ binding. Mutation of a histidine-64 residue in the N-terminal of Kir3.4 and a leucine-268 residue in the C-terminal of Kir3.4 have been proven to be critical for Gβγ-mediated channel activity [39]. Similar mutations in the Kir3.1 channel have also helped identify residues which regulate the Gβγ-Kir3.1 channel interaction [39]. The reverse approach of using Gβ mutations has also aided in the discovery of functionally important channel

interaction sites with Gβγ. For example, mutations at threonine-86, threonine-87 and glycine-131, all located on the outer loops of the $G\beta_1$ subunit, were shown to substantially reduce Kir3 channel activation, suggesting that these residues lie in the $G\beta_1$-Kir3 channel interface [84].

X-ray crystallography has revealed that the Gβ protein has the structure of a seven-bladed propeller with four anti-parallel β strands per blade [85]. The Gα subunit has been shown to interact with the Gβ subunit through the top of the propeller, while the Gγ subunit appears to interact with the Gβ subunit through the bottom of the propeller [7, 79, 86]. Substitution of alanine for tryptophan-332 on the Gα/Gβ-interacting surface impaired the interaction between $G\beta_1$ and the Kir3 channel [84]. Therefore, the interaction sites between the Gβ and Gα subunits may help create the interaction sites of the channel [87]. In fact, recent studies have established that the Gβ subunit can interact with the Kir3 channel only when the Gα subunit is detached from the Gβ subunit [79]. However, Gβ surfaces which lie outside the Gα/Gβ–interface may also contain Gβ-channel interaction sites [79, 84]. Additionally, distinct effector binding domains within Gβγ may differentially regulate effector functions since mutations of Gβγ have been shown to alter the regulation of specific effectors without affecting other Gβγ-dependent functions [28, 79, 81, 87].

F3. Multiple Gβγ binding sites on Kir3 channels

GST pull-down assays, mutational mapping studies and structural analyses have all identified multiple binding sites for the Gβγ subunits on the Kir3 channel

[39, 79, 81]. A multiplicity of Gβγ-binding sites has also been observed with other effectors such as voltage-gated calcium channels and adenylyl cyclase [81]. In fact, a recent crystal structure of the Gβγ-phosducin complex also confirms the multiple sites of interaction between these two proteins [88]. Interestingly, a number of direct interaction sites between the Gβγ subunits and many GPCRs have also been reported [8, 16].

Studies with the Kir3 channel have established that there are in fact two or three separate Gβγ-binding segments on each of the N- and C- terminal domains of each channel subunit (depending on the subunit), resulting in a total of eight to twelve putative Gβγ-binding segments per channel tetramer (Figure 2B) [31, 40, 74, 79, 81]. In three-dimensional space, whether this many binding sites can be accommodated on the channel is still unknown [31]. Cross-linking experiments have reported the binding of four Gβγ subunits per tetramer, attributing one Gβγ binding site per channel subunit [69, 89-90]. These putative Gβγ binding sites have been localized to residues 34-86 on the N terminus and residues 318-374 and residues 390-462 on the C terminus of the Kir3.1 subunit [40, 43]. Although interactions with the C terminus of the Kir3.1 subunit appear to be stronger than those with the N-terminus [39], it is in fact the N-terminus that seems to interact exclusively with the GDP-bound Gαβγ heterotrimer [78-79].

Objectives, hypothesis and rationale of the study

Studies using chimeras between the Kir3.4 subunit and the Gβγ-insensitive inwardly rectifying potassium (IRK) channel have helped identify a C-terminal

leucine residue, mutation of which abolished the Gβγ–mediated agonist-induced channel activation, while preserving the agonist-independent (basal) channel activity [90]. These results suggest that functionally important interactions of Gβγ with the C-terminus of the Kir3 channel are required for the activation of the channel [90].

Furthermore, studies in excised patches have indicated that the binding of Gβγ to only a subset of the many sites on the channel, is sufficient to fully open the channel, suggesting that only some sites are involved in mediating channel activation [91]. What then, are the roles of the other Gβγ binding sites on the Kir3 channel?

Some studies suggest that there may be functionally distinct roles for the different Gβγ binding sites on the Kir3 channel [79]. For example, He et al. discovered that a leucine-339 residue on the Kir3.4 channel interacted with Gβγ only after its release by receptor stimulation [92]. The histidine-64 and leucine-268 residues were instead found to interact with Gβγ to produce basal activity and enable overall Gβγ-mediated stimulation. Thus, each of these residues in the Gβγ binding region appeared to have different roles with regards to Kir3 channel modulation. However, no one has systematically studied these sites of interaction, especially on the Kir3.2 channel, and characterized what their roles may be.

Since BRET experiments in our laboratory have previously established that Kir3 channels can interact with Gβγ in the ER, we hypothesized that some channel-Gβγ interactions are necessary for the assembly of Kir3 signalling

complexes before they can be targeted to the cell surface [3]. However, as mentioned above, there is also evidence in the literature to suggest that some channel-Gβγ interactions are required for the activation of the channel once the signalling complex has reached the cell surface [91].

Based on these data, one might hypothesize that there are different sites on the channel which mediate these two distinct signalling events. As such, some Gβγ binding sites on the channel may be required for the early or 'precocious' interactions between the channel and the G protein inside the cell, during the assembly and trafficking of signalling complexes to the cell surface. Other Gβγ binding sites may be required to mediate channel activation at the cell surface, following complex localization at the cell surface.

In the context of a large signalling complex with many potential sites of interaction with the G protein, it is critical that we decipher the roles of the individual Gβγ binding sites on the Kir3 channel and understand which sites play a role in the 'precocious' interactions versus the signalling interactions.

To this end, we undertook a series of experiments to differentiate between the roles of the Gβγ binding sites on the N- and the C-termini of the Kir3.2 channel by studying channels in which either the N- or the C-terminal Gβγ binding sites were removed (Figure 3). More specifically, we wanted to test whether the Gβγ binding sites on one domain of the Kir3.2 channel, could be important for assembly and trafficking events, while Gβγ binding sites on the other domain, could be critical for the functional regulation of the channel.

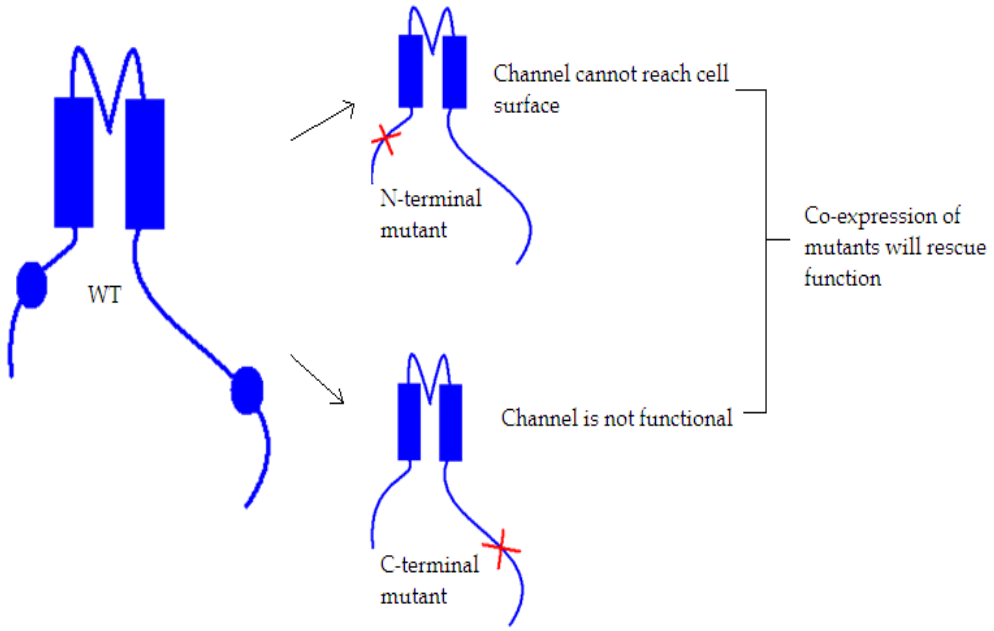


Figure 3. Hypothesis of the study. In order to study the roles of the N- and C-terminal Gβγ binding sites on the Kir3.2 channel, we engineered two constructs – one with a deletion or mutation in the N-terminal Gβγ binding site and the other, with a deletion or mutation in the C-terminal Gβγ binding site. We hypothesized that one construct (either the N- or the C-terminal mutant) would either fail to traffic to the cell surface, or be able to reach the cell surface, but not be functional. Thus, when only both mutants are co-expressed, would a functional channel be re-constituted, where it is able to both traffic and signal at the cell surface.

The Kir3.2 channel served as a good model to use since, in principle, it could be studied on its own, without having to co-express it with another Kir3 channel subunit to target it to the cell surface. This is in contrary to studies with the Kir3.1 channel subunit, which alone, cannot be targeted to the cell surface.

We predicted that a channel lacking either the N- or C-terminal Gβγ binding site would not correctly be assembled and targeted to the cell surface, or instead, be able to reach the cell surface, but become incapable of signalling in response to a ligand (Figure 3). Only when both channel deletions are co-expressed (each of which lacks one of the putative Gβγ binding sites), would we expect to re-

constitute a functional Kir3 channel, which is able to both traffic to the cell surface, and signal in response to GPCR activation.

MATERIALS & METHODS

Constructs

Constructs encoding Kir3.2-Rluc (*Renilla* luciferase), Kir3.2-Myc, Kir3.1-HA, eGFP-tagged $G\beta_{1\text{-}5}$, GFP_{10}-$G\gamma_{2,}$ wildtype (WT) and dominant (DN) Sar1 and Rab1 constructs were used as previously described and all constructs used for BRET experiments were shown to be functional [9, 11]. Flag-$G\beta_{1\text{-}5}$, HA-$G\gamma_2$ and the HA-tagged muscarinic M_2 receptor were obtained from the UMR cDNA Resource Center (www.cdna.org). The extracellulary tagged Flag-Kir3.1 construct was a generous gift from Dr. Deborah J. Nelson (University of Chicago, Illinois). Constructs encoding GFP_{10}-$G\beta_1$ and CD8-Rluc were obtained from Dr. Michel Bouvier (Université de Montreal, Quebec). The Flag-tagged dopamine D_4 receptor was provided by Dr. Hubert Van Tol (University of Toronto, Ontario).

Gβγ deletion constructs

The exact location of the Gβγ binding regions on the Kir3.2 channel has previously been identified [40]. We selected these regions and prepared Kir3.2 channel constructs in which the N- and C-terminal Gβγ binding sites were removed. The Kir3.2-RlucΔN52-96, Kir3.2-MycΔN52-96, Kir3.2-RlucΔC318-368 and Kir3.2-MycΔC318-368 constructs were created through a series of PCR reactions using CMV forward and BGH reverse primers, as well as Gβγ deletion specific primers.

For the N-terminal deletions constructs, the primers designed were:

Fwd: 5’ GAT CGG ACC AAA AGG AAA GTC ATG GTT TAC ACA GTG 3’

Rev: 5’ CAC TGT GTA AAC CAT GAC TTT CCT TTT GGT CCG ATC 3’

For the C-terminal deletion constructs, the primers designed were:

Fwd: 5’ GGA AGC CAC AGG GAT GAC AGA GCT GGC CGA GTT AGC 3’

Rev: 5’ GCT AAC TCG GCC AGC TCT GTC ATC CCT GTG GCT TCC 3’

The Kir3.2-Rluc deletion constructs were cloned into pcDNA3.1, whereas the Kir3.2-Myc deletion constructs were cloned into pcDNA3. All constructs were initially prepared using two separate PCRs in order to remove the Gβγ binding site of interest. A third PCR was then performed to combine the two separate channel fragments so as to produce a channel with the desired deletion. Next, a digest of the wildtype channel and the deletion channel with the appropriate restriction enzymes (see below) served to separate the plasmid into two parts: the channel sequence containing the deletion and the vector. Following digestion, samples were run on an agarose gel and two bands, one containing the channel with the deletion, and the other containing the vector, were isolated, purified and ligated. Transformation of this new construct, and subsequent digestion of this construct with the appropriate enzymes, was used to confirm that the clone of choice had been made. All constructs were additionally confirmed by bidirectional sequencing.

The Kir3.2-RlucΔN52-96 construct was cloned into the *Xba*I and *Bst*XI sites of the vector. The Kir3.2-MycΔN52-96 construct was cloned into the *Bam*HI

and *Not*I sites. Kir3.2-RlucΔC318-368 was cloned into the *Pvu*II site. Kir3.2-MycΔC318-368 was cloned into the *Eco*RI and *Not*I sites.

Gβγ point mutation constructs

All point mutation constructs were generated using the Quickchange site-directed mutagenesis kit (Stratagene). The Kir3.2-Rluc (H67F), Kir3.2-Myc (H67F), Kir3.2-Rluc (L271I) and Kir3.2-Myc (L271I) constructs were created through PCR reactions using specifically designed forward and reverse primers which included the point mutation.

For the N-terminal point mutation constructs, the primers designed were:
Fwd: 5' GTG CAA TGT TCA TTT CGG CAA CGT GAG GGA G 3'
Rev: 5' CTC CCT CAC GTT GCC GAA ATG AAC ATT GCA C 3'

For the C-terminal point mutation constructs, the primers designed were:
Fwd: 5' GGG GAT GAC CGT ATA TTT CTG GTG TCA CCG 3'
Rev: 5' CGG TGA CAC CAG AAA TAT ACG GTC ATC CCC 3'

Following the PCR, all point mutation constructs were treated with *Dpn*I to remove methylated sites. The constructs were then grown and their sequences were confirmed prior to use.

Cell culture and transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose (Invitrogen) containing 5% fetal bovine serum (Hyclone).

Cells stably transfected (not clonally) with Kir3.2 or Kir3.2-Rluc were grown in the presence of G418 sodium sulphate (500 μg/ml; Wisent) in order to maintain selection of the Kir3.2- or Kir3.2-Rluc-containing vector. Cells were grown in 6 well plates at an initial density of 3 x 10^5 cells/well and transfected twenty-four hours later. Cells were transfected with Lipofectamine 2000 (Invitrogen) as per manufacturer's recommendations or PEI (polyethylenimine; Polyscience). PEI (1mg/ml stock) was used at 1:3 ratio with DNA. Experiments were carried out forty-eight hours after transfection.

BRET

HEK293 cells were transfected with Rluc- (donor) or GFP-tagged (acceptor) fusion proteins. Forty-eight hours after transfection, cells were washed twice with phosphate buffered saline (PBS) and resuspended in PBS + 0.1% glucose. The cells were then harvested and dispensed into 96-well microplates (white Optiplate; Perkin-Elmer Life and Analytical Sciences).

There are several generations of BRET pairs - although the most common are BRET1 and BRET2, which rely on different luciferase substrates and specific GFP variants, thereby conferring distinct spectral properties. In this study, all experiments used the BRET2 system, in which coelenterazine 400a (Cedarlane) was used as a substrate, at a final concentration of 5μM. Signals were obtained on a Packard Fusion instrument (Perkin-Elmer Life and Analytical Sciences) using either a 410/80-nm (luciferase) or a 515/30-nm (GFP_{10}) band pass filter.

The presence of a protein-protein interaction was determined by the amount of light which passes through the 515/30-nm filter relative to that which passes through the 410/80-nm filter. This ratio is known as the BRET ratio. Transfection conditions were optimized to maintain constant Rluc and GFP expression in order to avoid possible variations in the BRET signal due to differences in the expression levels of the donor and acceptor proteins. To verify that this was the case, luciferase values and GFP fluorescence levels were measured during each experiment. In saturation experiments, these values were used to calculate GFP/Rluc ratios which are plotted along the x-axis. The BRET background was determined under conditions where resonance energy transfer between Rluc and GFP could not occur. This was accomplished by expressing CD8-Rluc and GFP-tagged proteins which do not interact physiologically, thereby serving as negative controls.

Confocal Microscopy

Twenty-four hours after transfection, all cells were seeded onto glass coverslips pre-treated with 10 μg/ml laminin (Sigma) and allowed to attach. Twenty-hour hours after seeding, cells were incubated with an anti-Flag polyclonal antibody (Sigma) diluted 1:200 in DMEM and incubated for 60 minutes at 37°C and 5% CO_2 for non-permeabilized cells. Non-permeabilized conditions were only used when extracellularly labeled Flag-tagged constructs were being studied, in order to visualize cell surface proteins levels.

Cells that did not require permeabilization were instead directly fixed with 2% paraformaldehyde for 20 minutes, followed by three washes with PBS 1X at a pH of 7.4. The cells were then treated with 2% normal goat serum (Jackson Immuno Research Laboratories INC) and 0.2% Triton X-100 (Sigma) for 60 minutes in order to permeablize the cells and block non-specific binding. Cells were incubated overnight at 4°C with primary antibodies against Rluc (Chemicon International), c-myc (Covance) or Flag (Sigma), all of which were diluted 1:200 in PBS 1X containing 1% normal goat serum and 0.04% Triton X-100. Following the overnight incubation, cells were washed three times with PBS 1X and incubated for 45 minutes at room temperature with goat anti-mouse or rabbit secondary antibodies conjugated with Alexa Fluor 488 or 647 (Molecular Probes). These secondary antibodies were diluted to a ratio of 1:600 in 1% normal goat serum and 0.04% Triton in PBS 1X. Cells were washed three times, mounted onto microscope slides with a drop of mounting media containing 0.1% DABCO and glycerol (Sigma). Cells were examined on an inverted laser-scanning microscope (LCM 510; Zeiss) using a 63/1.4 oil Plan-Apochromat objective.

Immunoprecipitation

Approximately forty-eight hours after transfection, cells were washed three times with PBS 1X and harvested in lysis buffer containing 150mM NaCl, 5mM EDTA, 50mM Tris-HCl pH 7.4, 1% Triton, 2mM PMSF and 5µl/ml protease inhibitor (which contains 10mg/ml benzamidine, 5mg/ml leupeptin and 5mg/ml trypsin inhibitor). Cells were incubated on ice for 3 hours and vortexed occasionally at low speed. Non-solubilized material was removed by centrifugation

at 10,000 x g for 15 minutes at 4°C. 0.01g/sample of Protein A-Sepharose beads (Sigma) and 1% BSA were resuspended in lysis buffer for 30 minutes at 4°C with gentle shaking before use. To pre-clear the samples, 500µg of cell lysate and the Protein A-Sepharose beads were incubated together for 30 minutes at 4°C. Beads were spun down and the supernatant was incubated for 1 hour at 4°C with 3µl of anti-Myc (Covance) or anti-HA monoclonal antibodies (Covance). 0.01g/sample of Protein A-Sepharose beads resuspended in lysis buffer were added to the mixture and incubated with shaking at 4°C overnight. Lysates were washed 5 times by centrifugation and the beads were resuspended in protein loading buffer prior to performing a Western blot.

Western Blotting

Twenty-four hours after transfection and following subsequent cell lysis, the concentration of solubilized proteins was determined using the Bio-Rad protein assay, which employs the Bradford method to estimate protein concentration [93]. Protein samples were then diluted in loading buffer (Tris-HCl pH 6.8, 8% SDS, 40% glycerol, bromophenol blue and β-mercaptoethanol) and heated for 15 minutes at 65˚C. Samples were then loaded onto 7.5% acrylamide gels (25µg protein/lane). Following electrophoresis, proteins were transferred onto nitrocellulose membranes (BioRad). The membranes were subsequently blocked with 5% milk dissolved in PBS 1X and 0.1% Tween for 1 hour at room temperature in order to minimize non-specific staining. Membranes were incubated overnight at 4˚C with antibodies against c-Myc (Covance), HA (Covance) or Rluc

(Chemicon International), all of which were diluted 1:1000. Following the incubation, membranes were washed with PBS 1X and 0.1% Tween, 3 times for 10 minutes each at room temperature. HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit, 1:20,000; Sigma) were applied to the membranes for 1 hour at room temperature while shaking. After another series of washes, the chemiluminescence method was used to detect antibody staining.

Electrophysiology

Kir3-mediated currents were recorded using the whole-cell configuration of the patch clamp technique using an Axopatch 200B amplifier (Axon Instruments). Patch pipettes were pulled from filamented borosilicate glass (Clark Electromedical) and had a resistance of 2-3.5 MΩ when filled with pipette solution. Low potassium external bath solution contained 140mM NaCl, 20mM KCl, 0.5mM $CaCl_2$, 2mM $MgCl_2$ and 10mM HEPES at a pH of 7.4. High potassium external solution contained 140mM KCl, 0.5mM $CaCl_2$, 2mM $MgCl_2$ and 10mM HEPES at a pH of 7.4. Internal solution contained 110mM KCl, 1.2mM $MgCl_2$, 5mM EGTA, 10mM HEPES, 2.56mM ATP-K_2 and 0.3mM GTP-Na_2 at a pH of 7.2.

All currents were recorded at room temperature, forty-eight hours after transfection. HEK293 cells were voltage-clamped and perfused continuously with a low potassium-containing recording solution. After stable current recordings were achieved, cells were perfused with a high potassium-containing external solution, and once the cells recovered, currents were recorded again.

For the current-voltage protocols, currents were recorded by 20 msec voltage commands from -100mV to +50mV, delivered in 10mV increments. Membrane currents were filtered at 2kHz, digitized at 5kHz, and data were collected and analyzed using a Digidata 1200B interface (Axon instruments) and the pClamp suite of software (version 10.0; Axon instruments). Cell capacitance was approximately 15 pF and the series resistance was less than 10 MΩ.

All current recordings made in the presence of a drug, were performed on cells co-expressing both the Kir3 channel as well as a GPCR such as the muscarinic M_2 receptor or the dopamine D_4 receptor. The addition of a drug was expected to activate the GPCR, which could then in turn, activate the Kir3 channel. In this way, we were able to measure Kir3 channel activation by simply applying a drug to the cell.

During these recordings, the cells were held at -60mV and the electrical perfusion system was continuously running, so as to supply the cells in the bath with fresh external solution. The drug was manually applied to the cell through a valve which could control when the drug was directly released onto the cell. Once the response to the drug had peaked (ie. reached a maximal peak amplitude), perfusion of the drug was manually closed by a valve in the perfusion set-up. Thereafter, the cell was allowed to recover back to basal levels as the drug was washed out from the bath.

Carbachol (Sigma) and dopamine (Sigma) were both used at 1μM for BRET and electrophysiology experiments. Both drugs were dissolved in PBS solution prior to use.

Tandem Affinity Purification

In experiments designed to examine the maturation status of the Kir3.1 channel in response to its co-expression with wildtype or mutant Kir3.2 channels, we used a tandem affinity purification (TAP)-tagged Kir3.1 construct. The TAP tag incorporates a streptavidin binding domain and an HA tag, in addition to a calmodulin binding domain and a tobacco etch virus (TEV) cleavage site.

The TAP-Kir3.1 construct was generated from the HA-Kir3.1 construct described above using PCR. PCR products were run on an agarose gel and the relevant bands were cut out. Both the Kir3.1 DNA fragment and the pIRES-Glue-N1 vector fragment (a generous gift from Stéphane Angers at the University of Toronto, Ontario) were then ligated. Finally, this construct was verified by bidirectional sequencing.

For purifications, we used lipofectamine 2000 to transfect 10 μg of TAP-Kir3.1 and 10 μg of either Kir3.2-myc WT, ΔN52-96, ΔC318-368, H67F or L271I into a T175 flask containing HEK293 cells. Cells were harvested forty-eight hours after transfection in 15ml of PBS 1X. Cells were spun down for 8 min at 2000 rpm, and each T175 flask was resuspended in 12ml of lysis buffer (Tris 5mM pH 7.4, EDTA 2mM, protease inhibitor cocktail (trypsin inhibitor 5 μg/ml, benzamidine 10

μg/ml, leupeptin 5 μg/ml, diluted in water). The cells were then lysed by Polytron (2 bursts of 10 seconds each). Cells were spun again for 5 min at 1000 rpm to remove unbroken cells/debris. The supernatant was then discarded since only the pellet contained cell membranes. We then solubilized the membrane pellet in approximately 800-900μl of solubilization buffer (Tris 75mM pH 8, EDTA 2mM, $MgCl_2$ 5mM, 0.5% dodecyl-maltoside (DDM) with the protease inhibitor cocktail as before), all diluted in water. Samples were solubilized while shaking overnight at 4°C. The next day, the soluble and insoluble fractions were separated by centrifugation for 20 min at 18,000 rpm and the pellet was discarded. Protein concentrations were determined and 30 μg of protein was used to run the total lysate. In the mean time, 50μl of streptavidin beads/sample in 1ml of solubilization buffer (same as above) were washed 3 times for 2 min at 1600 rpm. Once the beads were washed, the solubilized membranes were added to the beads and rocked for 2-3 hours at 4°C. The beads were spun down for 2 min at 1600 rpm and washed 4 times in 250μl of solubilizaton buffer (same as above). After the fourth wash, beads were resuspended in 1X Laemmli buffer and samples were heated for 15 min at 65°C. For western blots 25 μL of eluate/sample was loaded onto 10% SDS gels which were eventually transferred to PVDF membranes. Membranes were blocked for 1 hour in 5% milk in Tris buffered saline in tween (TBS-T), and incubated with a primary antibody (mouse anti HA (1:5000)) overnight with shaking at 4°C. The next day membranes were washed 3 times in TBS-T, followed by incubations with secondary antibodies.

Data analysis and statistics

All BRET data were plotted and analyzed using GraphPad Prism. Results are shown as means +/- SEM. Two group comparisons were done using an unpaired Student's two-tailed *t*-test.

All confocal images were viewed using the Zeiss Meta 510 system software. The intensity of the fluorescence signals were quantified using the Image J software. Three fluorescence readings were taken from each image and the average was plotted as an arbitrary value.

All electrophysiology traces were analyzed with the Clampfit software (version 10.0; Axon Istruments) and then plotted using GraphPad Prism, Adobe Illustrator and Microsoft Paint.

Western blots and co-immunoprecipitation images were assembled with Adobe Illustrator.

RESULTS

Assembly and trafficking of Kir3.2 channel-G protein complexes

In a previous study by Robitaille et al., we investigated the Kir3.2 channel-G protein interaction using BRET [3]. To do this, HEK293 cells were transfected with wildtype Kir3.2-Rluc, GFP_{10}-$G\gamma_2$ and various Flag-Gβ subunits, after which BRET experiments could be performed. A BRET signal is generated when the two proteins of interest (the channel and the G protein) are within 10 nm of one another. Thus, based on their proximity to one another, we can infer that the two proteins are interacting.

As seen in Figure 4, we observed a BRET signal (measured as the BRET2 ratio on the y-axis) in cells expressing Kir3.2-Rluc and $G\beta_1$ or $G\beta_2$, and to a lesser extent $G\beta_3$. $G\beta_4$ and $G\beta_5$ did not interact with the channel. This suggests that the Gβ subunits showed specificity in their interactions with the Kir3.2 channel when the complex is assembled inside the cell. Since the BRET signal was at a maximum when the cells co-expressed either $G\beta_1$ or $G\beta_2$ with $G\gamma_2$, we performed all future experiments with the $G\beta_1\gamma_2$ combination. Incidentally, other groups have also used this particular Gβγ combination for BRET experiments [11, 13]. As a negative control, we used CD8-Rluc, a construct known to not interact with the G protein. To confirm that the fluctuations in BRET signals were not due to varying expression levels of the Gβ proteins, we also performed a Western blot to measure Flag-Gβ protein levels. $G\beta_1$, $G\beta_2$ and $G\beta_5$ were all expressed at similar levels compared to $G\beta_3$ and $G\beta_4$ (inset in Figure 4A).

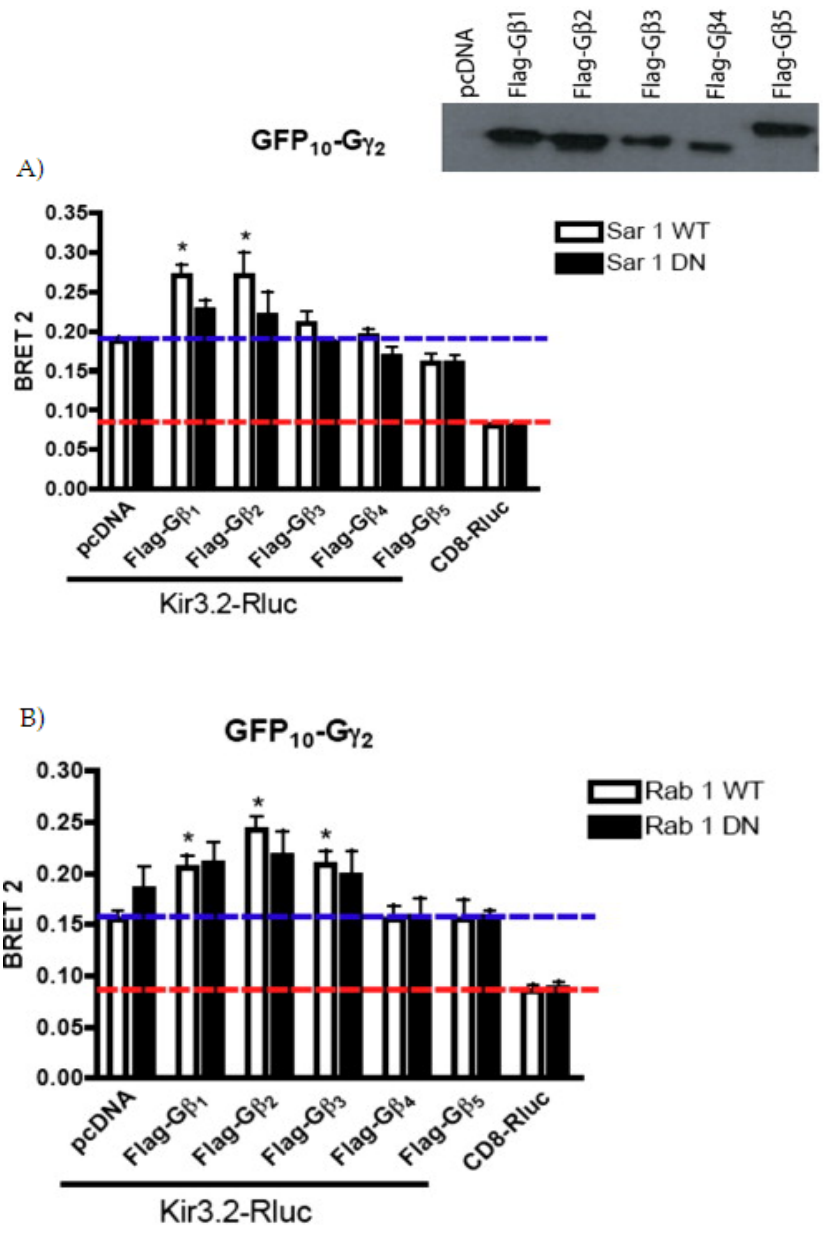


Figure 4. Gβ subunits show specificity in interactions with the Kir3.2 channel. BRET experiments were used to study the Gβγ-Kir3.2 channel interaction. (A) HEK293 cells were transfected with Kir3.2-Rluc, GFP_{10}-$G\gamma_2$ and either pcDNA3 or $G\beta_1$, $G\beta_2$, $G\beta_3$, $G\beta_4$ or $G\beta_5$. These conditions were studied in the presence of WT or DN Sar1. Inset: Similar expression levels of the individual Flag-Gβ subunits was verified by western blot with anti-Flag antibodies. B) Similar experiments were performed in the presence of WT or DN Rab1. No differences were identified when using the WT or DN Sar1 or Rab1 proteins. The dotted blue line indicates BRET fostered by endogenous Gβ subunits. The dotted red line indicates a non-specific BRET interaction when using the negative control, CD8-Rluc. * indicates $p < 0.05$ compared to pcDNA3 for the different Gβ subunits (unpaired t-test). Data are expressed as BRET mean ± SEM (n=3). Figure from [3].

As we showed for Kir3.1 [3], we were interested in how the Kir3.2 channel-G protein interaction might be affected by modulating the exocytic trafficking pathway. Thus, we repeated the BRET experiments in the presence of wildtype or DN Sar1 or Rab1 proteins. As mentioned above, Sar1 and Rab1 proteins are GTPases which are required for the trafficking of signalling complexes from the ER to the golgi. Thus, DN GTPases can essentially perturb the trafficking of the signaling complex to the cell surface.

Neither DN Sar1 (Figure 4A) nor DN Rab1 (Figure 4B) proteins were found to affect the BRET interaction between the Kir3.2 channel and the G protein relative to the wildtype GTPases, suggesting that the "precocious" channel-G protein interactions occurred before the complex was trafficked to the plasma membrane.

From our preliminary work in this earlier paper, it was clear that the Kir3.2 channel could interact with the Gβγ dimer. Furthermore, since the Kir3.2 channel could traffic on its own to the cell surface, we proposed that the channel could interact with the G protein both at the cell surface, but also inside the cell since a significant proportion of the channel has been shown to reside inside the cell [3, 49]. However, given that there are many Gβγ binding sites on the channel, the roles of which are unknown, we then turned to study the different binding sites in order to characterize their roles in Kir3.2 channel modulation.

Expression of the Gβγ deletion and point mutation channel constructs

In order to study the roles of the various Gβγ binding sites on the Kir3.2 channel, we referred to a study by Ivanina et al. which, through GST-pulldown experiments, had previously identified regions on the Kir3.2 channel that interacted with the Gβγ subunit [40]. Using these regions as a starting point, we constructed Myc-tagged and Rluc-tagged Kir3.2 channels in which the Gβγ

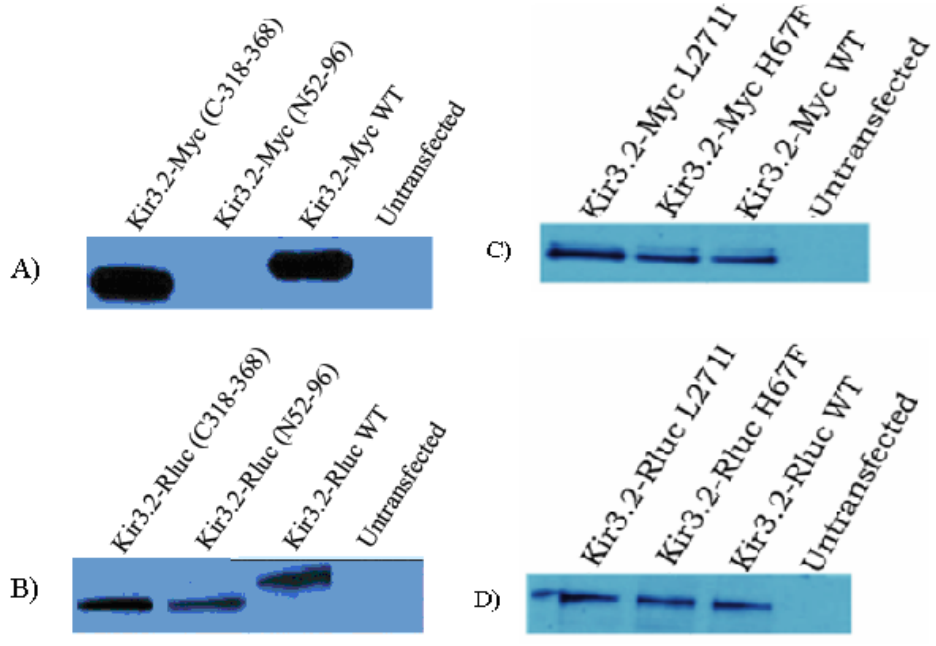


Figure 5. Expression of the Kir3.2 channels in HEK293 cells. A Western blot confirms that (A) the Kir3.2-Myc C-terminal deletion (Δ318-368) construct, (B) the Kir3.2-Rluc N-terminal deletion construct (Δ52-96) and the Kir3.2-Rluc C-terminal deletion (Δ318-368) construct could be expressed in HEK293 cells and at similar levels as the wildtype channels. The Kir3.2-Myc N-terminal deletion (Δ52-96) construct, however, could not be expressed. (C and D) All point mutation constructs (H67F for the N-terminal and L271I for the C-terminal) could be expressed. Western blots were labeled with anti-Myc or anti-Rluc antibodies.
Expected band size for the Rluc-tagged wildtype channel ≈ 84kDa, Rluc-tagged NΔ52-96 channel ≈ 78kDa, Rluc-tagged CΔ318-368 channel ≈ 78kDa, Myc-tagged wildtype channel ≈ 48kDa, Myc-tagged NΔ52-96 channel ≈ 43kDa, Myc-tagged CΔ318-369 channel ≈ 43kDa, Rluc-tagged wildtype and mutant channel ≈ 84kDa, Myc-tagged wildtype and mutant channel ≈ 48kDa (n=3).

binding sites from the intracellular N-(Δ52-96) or C-(Δ318-368) terminal domains was removed.

Prior to using these constructs in any experiments, we wanted to ensure that they could indeed, be expressed in our heterologous expression system. Probing Western blots with anti-myc and anti-Rluc antibodies revealed the presence of the Kir3.2 channels in HEK293 cells. However, although we were able to detect the expression of both the Kir3.2-RlucΔ52-96 and Kir3.2-RlucΔ318-368 construct, as well as the Kir3.2-MycΔ318-368 construct, we were unable to detect expression of the Kir3.2-MycΔ52-96 construct on a western blot (Figure 5A and 5B).

Due to difficulties associated with the deletion constructs later during the study (see below), we then turned to an approach less disruptive to overall channel structure in order to understand the roles of the different Gβγ binding sites on the channel. Through biochemical and electrophysiological approaches, He et al. identified a critical N-terminal (histidine-64) and C-terminal (leucine-268) residue in the Kir3.4 subunit, which when mutated, significantly reduced Gβγ binding [39]. Mutations at similar regions of the Kir3.1 subunit also proved to confer reduced Gβγ sensitivity [39]. As a result, we mapped out the equivalent residues in the Kir3.2 subunit and made point mutations at residues which were expected to reduce Gβγ binding. These mutations corresponded to the H67F residue in the N-terminal domain and L271I residue in the C-terminal domain of the Kir3.2 channel. In contrast to the deletion constructs, all four point mutation constructs

(Kir3.2-Rluc H67F, Kir3.2-Rluc L271I, Kir3.2-Myc H67F and Kir3.2-Myc L271I) could be detected on a Western blot (Figure 5C and 5D).

Relative affinities of the Gβγ-channel interaction

Since the Rluc-tagged Kir3.2 deletion constructs could be well expressed in HEK293 cells, we decided to use them in BRET experiments to determine as to whether they, like the wildtype Kir3.2 channels, could interact with G proteins. To this end, we performed a BRET saturation experiment in which we could measure the specificity and relative affinity between Kir3.2-Rluc and GFP_{10}-$G\gamma_2$. In such an experiment, the donor (Rluc-tagged) protein is maintained at a constant level, while the expression of the acceptor (GFP-tagged) protein is increased.

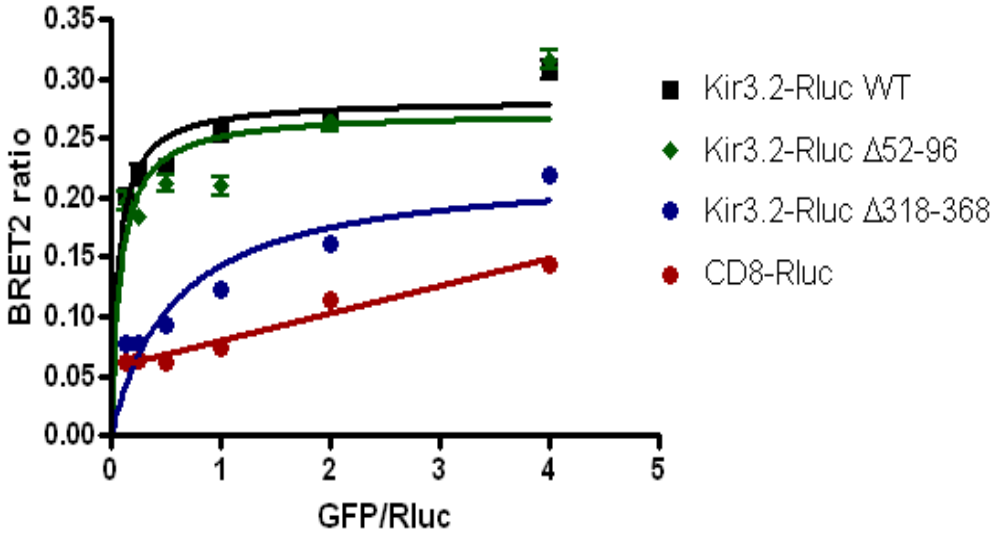
One attribute of using resonance energy transfer (RET) to study protein-protein interactions is that changes in the efficiency of resonance energy transfer can reveal changes in the interaction between proteins [14]. For G protein-mediated signaling pathways, these changes often occur when a ligand binds to its cell surface receptor [75, 94]. However, a change in RET carries with it some ambiguity because it indicates one or a combination of two different things: 1) a change in the affinity of proteins for each other or 2) a change in intermolecular distance or orientation (*ie.* a conformational change) within a protein complex [9]. One approach to distinguishing between these possibilities is to express varying amounts of the acceptor-tagged protein with fixed amounts of the donor-tagged protein [9, 95]. RET signals will be sensitive to the ratio of tagged proteins if they interact specifically with each other [95]. By fixing the expression of the donor-

tagged protein and increasing the expression of the acceptor-tagged protein, the RET between these proteins will increase, approaching a maximum value asymptotically, thus producing, in effect, a saturation curve [9]. An agent, such as a receptor ligand, that causes either an increase or a decrease in the amount of acceptor-tagged protein needed to attain half-maximal BRET (the $BRET_{50}$ in this case) is responsible for, respectively, decreasing or increasing the affinity of the tagged proteins for each other [96]. Conformational changes which do not result from changes in affinity will alter the maximum BRET signal (Bmax) without changing the $BRET_{50}$ [9]. One caveat that must be stated is that since the $BRET_{50}$ or Bmax values are unique to each pair of interactors, we cannot compare these values directly between Gβγ and the WT or mutant channels.

As seen in Figure 6, HEK293 cells expressing the tagged channel and G protein exhibited hyperbolic increases in the BRET signal while approaching a maximum asymptotic value. The fact that the BRET between the channel and the G protein reached a plateau value as the amounts of the GFP-tagged protein increased, indicated a specific protein-protein interaction.

We found that the affinity of the G protein for the Kir3.2-RlucΔ52-96 channel was similar to that of the G protein for the wildtype channel, given the similar $BRET_{50}$ values for both the N-terminal deletion construct (0.08) and the wildtype construct (0.06) (Figure 6). The Bmax values for the two channels was also similar (0.27 for the Kir3.2-RlucΔ52-96 channel and 0.28 for the wildtype channel). However, in the presence of the Kir3.2-RlucΔ318-368 channel, the

A)



B)

	$BRET_{50}$	std error	**Bmax**	std error
Kir3.2-Rluc WT	0.06	0.01	0.28	0.01
Kir3.2-Rluc Δ52-96	0.08	0.02	0.27	0.01
Kir3.2-Rluc Δ318-368	0.59	0.08	0.23	0.01

Figure 6. **Affinity of the Gβγ-deletion channel interaction.** BRET saturation assays were used to determine the relative affinities of the G protein for the different channel constructs. (A) HEK293 cells were transfected with Kir3.2-Rluc (wild-type or deletion channels) as well as $G\gamma_2$-GFP_{10} and Flag-$G\beta_1$. The affinity of the Gβγ-channel interaction was similar to the wildtype channel when the Gβγ site on the N-terminal (Δ52-96) was removed. However, the affinity of the Gβγ-channel interaction reduced by almost 10-fold when the Gβγ site on the C-terminal (Δ320-370) was removed. In both cases, CD8-Rluc was used as a negative control. Data represent mean +/- SEM of three separate experiments. (B) A table of $BRET_{50}$ and BRETmax values as well as their standard errors is listed for the wildtype, N-terminal (Δ52-96) and C-terminal (Δ318-368) deletion channels from the experiment in (A).

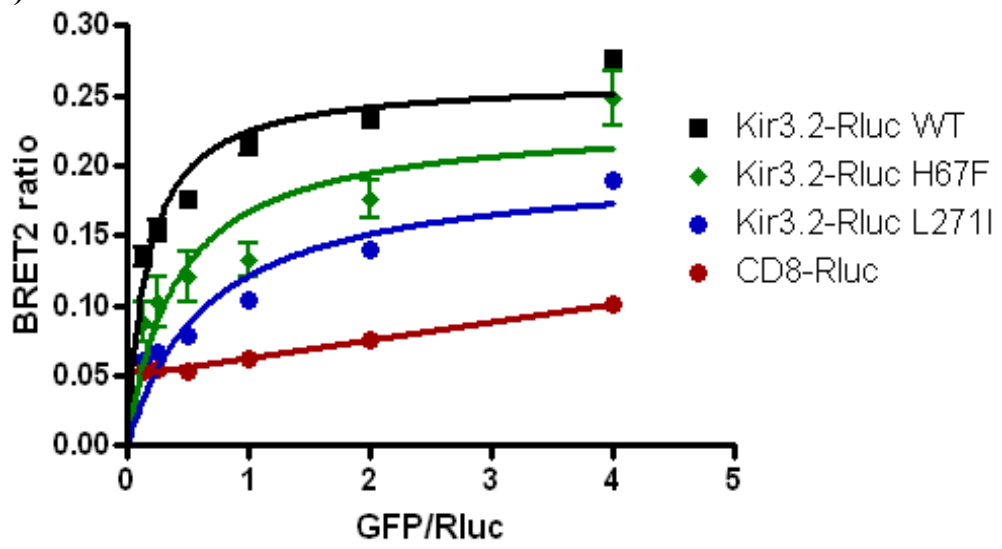
$BRET_{50}$ value increased to 0.59, while the Bmax value decreased. We found that the affinity of the G protein for the Kir3.2-RlucΔ52-96 channel was similar to that of the G protein for the wildtype channel, given the similar $BRET_{50}$ values for both the N-terminal deletion construct (0.08) and the wildtype construct (0.06) (Figure 6). The Bmax for the two channels was also similar (0.27 for the Kir3.2-RlucΔ52-

96 channel and 0.28 for the wildtype channel). However, in the presence of the Kir3.2-RlucΔ318-368 channel, the $BRET_{50}$ value increased to 0.59, while the Bmax value decreased to 0.23, thereby reducing the apparent affinity of the G protein for the C-terminal deletion channel by nearly 10-fold compared to that of the wildtype Kir3.2 channel.

To confirm and extend these data, we then repeated this BRET saturation experiment with our point mutation constructs. As seen in Figure 7, the affinity of the G protein for the Kir3.2-Rluc H67F mutant ($BRET_{50}$=0.41) was lower than that of the G protein for the wildtype channel ($BRET_{50}$=0.06). The Bmax values for both these channels were similar, however (0.23 for the N-terminal mutant and 0.28 for the wildtype channel). In contrast to the wildtype channel, the Kir3.2-Rluc L271I mutant exhibited a $BRET_{50}$ value of 0.67, indicating a reduced affinity of the C-terminal mutant for the G protein, compared to the wildtype channel. Thus, even though the individual $BRET_{50}$ and BRETmax values were different for both the deletion and point mutation constructs, both constructs still displayed similar trends with regards to their relative affinities for the G protein.

In both sets of experiments, CD8-Rluc was used as a negative control for non-specific BRET. CD8-Rluc is an integral membrane protein and as such is used because it localizes to the same place in the cell as Kir3, but does not physiologically associate with it [9]. CD8-Rluc proved to be a suitable negative control since comparable amounts of this protein in place of Kir3.2-Rluc produced small increases in the BRET signal, indicating a weak, non-saturable and thus non-specific interaction with the tagged acceptor proteins.

A)



B)

	$BRET_{50}$	std error	**Bmax**	std error
Kir3.2-Rluc WT	0.06	0.01	0.28	0.01
Kir3.2-Rluc H67F	0.41	0.12	0.23	0.02
Kir3.2-Rluc L271I	0.67	0.09	0.20	0.01

Figure 7. **Affinity of the Gβγ-mutant channel interaction.** BRET saturation assays were used to determine the relative affinities of the G protein for the different channel constructs. (A) HEK293 cells were transfected with Kir3.2-Rluc (wild-type or point mutation channels) as well as $G\gamma_2$-GFP_{10} and Flag-$G\beta_1$. The affinity of the Gβγ-channel interaction was marginally reduced (compared to the wildtype channel), in the presence of the N-terminal point mutation (H67F). The affinity of the Gβγ-channel interaction reduced by ~4-fold in the presence of the C-terminal point mutation (L271I). In both cases, CD8-Rluc was used as a negative control. Data represent mean +/- SEM of three separate experiments. (B) A table of $BRET_{50}$ and BRETmax values as well as their standard errors is listed for the wildtype, N-terminal (H67F) and C-terminal (L271I) mutant channels from the experiment in (A).

Additional controls were also used to minimize false positives. As seen in Figure 8A, we observed similar levels of luciferase (RluA) expression across the various transfected conditions, confirming that the BRET signal was not fluctuating due to changes in luciferase activity. Total GFP fluorescence readings

were also taken and as expected, were seen to increase with increasing concentrations of the transfected acceptor protein (Figure 8B).

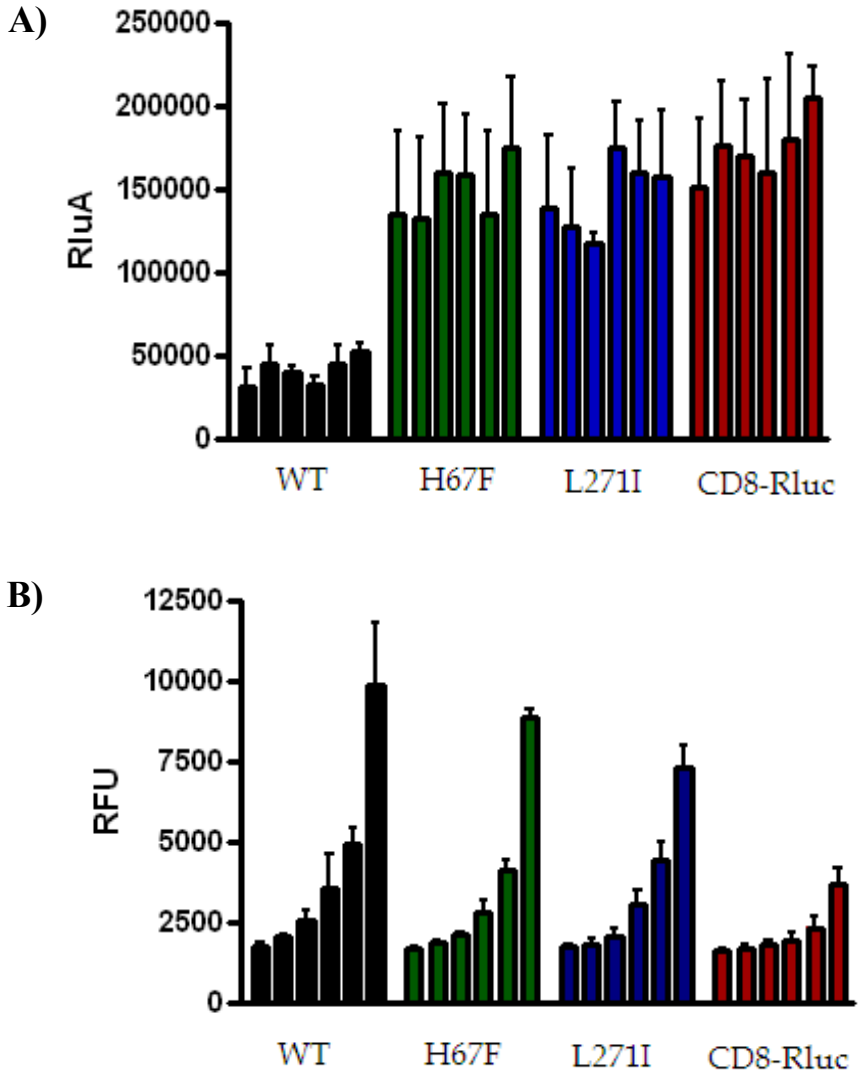


Figure 8. Expression levels of BRET acceptors and donors. (A) Luciferase values were measured (RluA) for each transfected condition used to perform the BRET saturation experiments in Figures 6 and 7. The RluA values for each of the six concentrations of acceptor protein (0.06, 0.12, 0.25, 0.50, 1 and 2 µg of transfected DNA) are relatively constant within each condition. They are also fairly consistent between conditions, except for the condition containing the wildtype channel which constantly exhibited lower luciferase signals (n=3). (B) Total GFP fluorescence values were also measured for each transfected condition. As expected, these values increased with increasing concentrations of the acceptor protein. Data represent mean +/- SEM of three separate experiments.

Trafficking of the Kir3.2 channels

We hypothesized that the N- and C-terminal Gβγ binding sites on the Kir3.2 channel would serve different roles in signaling. As such, we expected one domain to be responsible for the early or "precocious" interactions between the channel and G protein which would be required for its assembly and trafficking to the cell surface, while the other domain would be required for signaling at the cell surface upon GPCR activation.

In order to test these different possibilities, we first studied the trafficking abilities of the N- and C-terminal deletion constructs by performing confocal imaging experiments. As mentioned earlier, the Kir3.2 channel subunit can both reside inside the cell and also traffic to the cell surface as a homomeric channel. However, the lack of an extracellularly tagged version of this channel subunit makes immunocytochemistry less informative, since cells need to be permeabilized [66]. Luckily, we were able to take advantage of an extracellularly Flag-tagged Kir3.1 channel construct in our laboratory, and an anti-Flag antibody to help us study Kir3.2 channel trafficking. Since the Kir3.1 subunit can only reach the cell surface when co-expressed with the Kir3.2 or Kir3.4 targeting subunits [3, 64, 66], we used the trafficking of tagged Kir3.1 as a surrogate for trafficking of Kir3.2 in the context of a heteromultimer. On its own, the Kir3.1 subunit fails to arrive at the plasma membrane, and thus, remains undetectable in non-permeablized cells [3, 64, 66].

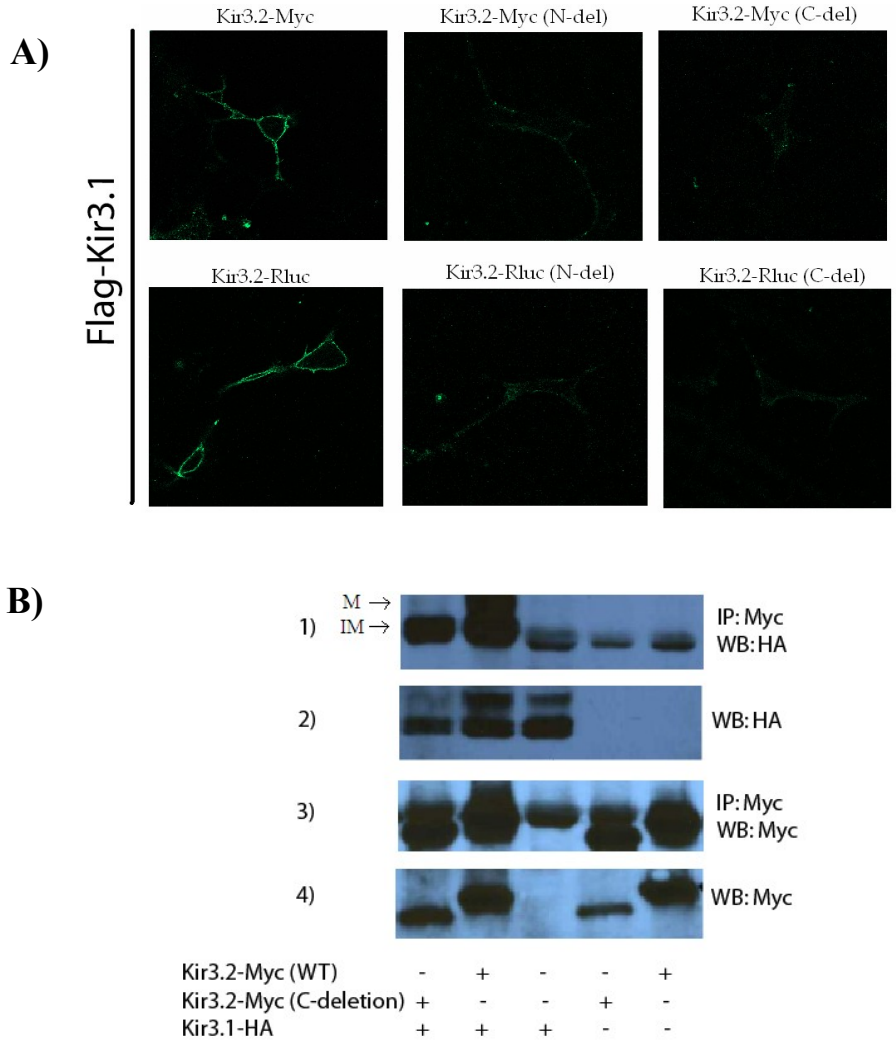


Figure 9. Trafficking of the deletion channels. (A) HEK293 cells were transfected with Kir3.2-Myc or Kir3.2-Rluc (wildtype or deletion channels) and extracellularly Flag-tagged Kir3.1 subunits. Confocal imaging showed that the wildtype Kir3.2 channels allowed the Kir3.1 subunit to reach the cell surface (alone, the Kir3.1 subunit cannot reach the cell surface). However, neither of the Kir3.2 channels containing the Gβγ binding site deletions was able to reach the cell surface. Anti-Flag antibodies were used on non-permeabilized HEK293 cells. Data are representative of three independent experiments. (B) Co-immunoprecipitation experiments showed that the wildtype Kir3.2-Myc channel could interact with Kir3.1-HA. However, Kir3.2-Myc containing the C-terminal deletion could not interact with Kir3.1-HA. "M" indicates bands depicting the glycosylated, mature form of Kir3.1-HA. "IM" indicates the immature Kir3.1-HA channel. Panels 1 and 3 show immunoprecipitated proteins. Panels 2 and 4 show cell lysates (n=2).

Cells transfected with the wildtype Myc- or Rluc-tagged Kir3.2 channel and the Flag-Kir3.1 subunit, were clearly seen at the cell surface as revealed by the green staining of the Kir3.1 subunit (Figure 9A). Since the Kir3.1 subunit only reaches the surface in the presence of the Kir3.2 subunit, any Kir3.1 surface labeling also reflected the presence of the Kir3.2 channel at the plasma membrane. When the N- or C-terminal deletion channels were co-expressed with the Flag-Kir3.1 subunit however, the intensity of Kir3.1 staining at the cell surface significantly reduced even in the face of similar overall expression levels (Figure 9A, but also see western blot in Figure 5A and 5B). In some cases, Kir3.1 channel staining at the cell surface was barely detectable showing that unlike the wildtype channels, neither of the deletion channels was able to traffic to the cell surface. This was true for both the Myc-tagged and Rluc-tagged deletion constructs but was more pronounced for the C-terminal deletion.

Since the deletion channels did not traffic to the cell surface, we sought to determine as to why this was the case. We have already shown, through BRET saturation experiments, that the deletion channels could in fact interact with the G protein (Figure 6). We next determined whether the Kir3.2 deletion constructs were unable to reach the cell surface due to their inability to interact with the Kir3.1 channel subunit. To address this, we performed co-immunoprecipitation experiments to detect interactions between the Kir3.2-Myc and Kir3.1-HA channel subunits. Detergent extracts from cells co-expressing Kir3.2-Myc and Kir3.1-HA were immunoprecipitated with anti-Myc antibodies and then probed on a Western blot with either anti-myc or anti-HA antibodies. These experiments showed that

the wildtype Kir3.2 channel subunit could interact with both the immature and mature glycosylated forms of Kir3.1-HA (Figure 9B). When individual wildtype Kir3.2 channels and Kir3.1-HA channels were expressed in different cell populations and mixed prior to immunoprecipitation, this interaction did not occur (data not shown), indicating that this interaction was not an artifact of the experimental protocol, thus reflecting a real biosynthetic event.

In contrast to the wildtype Kir3.2-Myc channel subunit, we could not detect an interaction between the Kir3.2-Myc channel containing the C-terminal deletion and the mature Kir3.1-HA channel subunit (Figure 9B), suggesting that these channels which remained trapped in the ER, do not form complexes. The same co-immunoprecipitation experiments were also repeated between the Kir3.2-Myc channel containing the N-terminal deletion and the Kir3.1-HA channel subunit. However, since we were unable to detect the expression of the N-terminal deletion construct on a Western blot, we were unable to fully assess its ability to interact with the Kir3.1 subunit (data not shown).

When confocal experiments were performed with the Myc- and Rluc tagged point mutation constructs, we observed clear and dramatic differences in their trafficking patterns compared to the deletion channels. As shown in our earlier experiments, we observed strong cell surface labeling of the Kir3.1 channel subunit, when co-expressed with the wildtype Kir3.2-Myc or Kir3.2-Rluc channels (Figure 10A). Interestingly, the H67F mutant also displayed strong plasma membrane localization, unlike when the N-terminus was deleted completely.

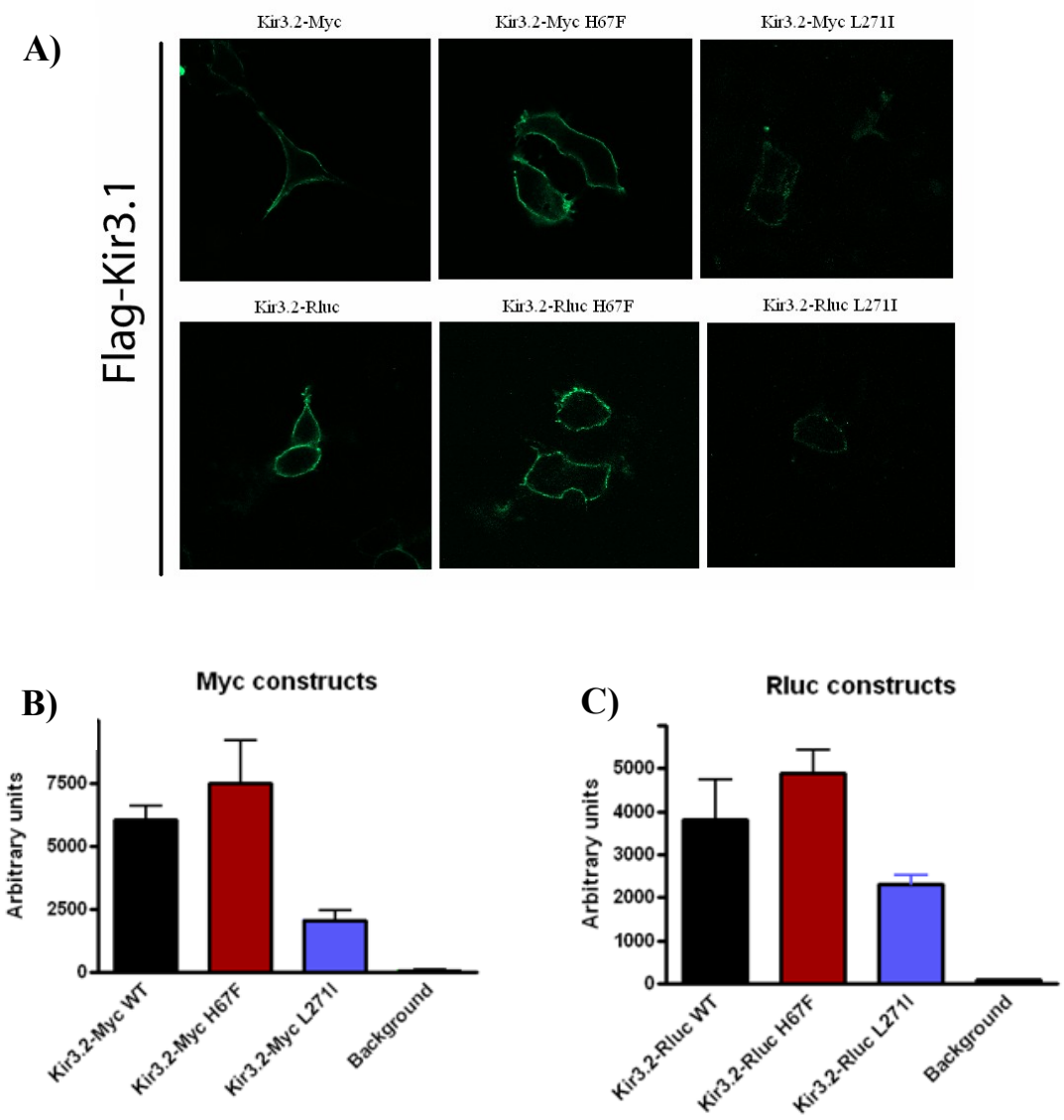


Figure 10. Trafficking of the point mutation channels. HEK293 cells were transfected with Kir3.2-Myc or Kir3.2-Rluc (wildtype or mutant channels) and extracellularly Flag-tagged Kir3.1 subunits. (A) Confocal imaging showed that the wildtype Kir3.2 channels allowed the Kir3.1 subunit to reach the cell surface (alone, the Kir3.1 subunit cannot reach the cell surface). The Kir3.2 channels containing the N-terminal mutation (H67F) were also able to traffic to the cell surface. The Kir3.2 channels containing the C-terminal mutation (L271I) could not reach the cell surface, however. Anti-Flag antibodies were used on non-permeabilized HEK293 cells. Data are representative of two independent experiments. Quantification of the cell surface fluorescence levels for both the (B) Myc-tagged and (C) Rluc-tagged proteins verify that the wildtype and H67F mutant channels were present at the cell surface, as opposed to the L271I mutant channel which failed to arrive at the cell surface. Data represent mean +/- SEM of two separate experiments.

However, in contrast to both the wildtype and H67F mutant channels, the L271I mutant failed to efficiently traffic to the cell surface as demonstrated by a substantial decrease in cell surface staining for Kir3.1.

Quantification of cell surface fluorescence levels for both the Myc-tagged (Figure 10B) and Rluc-tagged (Figure 10C) proteins verified that the wildtype and H67F mutant channels were present at the cell surface given their high fluorescence, as opposed to the L271I mutant, which exhibited low fluorescence levels. Thus, the two point mutation constructs exhibited distinct trafficking abilities, with the N-terminal mutant being able to arrive at the cell surface associated with Kir3.1, and the C-terminal mutant failing to do so. These confocal experiments were also repeated using permeabilized cells, in which a large proportion of wildtype Kir3.2 channels were localized at the cell surface, even though some channels resided inside the cell (data not shown). Since the deletion constructs not only had difficulty expressing in HEK293 cells, but also lacked the ability to interact with the mature Kir3.1 channel subunit, we decided to pursue the rest of our experiments focusing exclusively on the point mutation constructs.

Ligand-stimulated changes in the Gβγ-channel interaction

We next wished to assess the effects of GPCR ligands on the activation of the Kir3.2 channel constructs. This was first done using BRET experiments, in which we measured the BRET ratio between the channel and the G protein, both before and after ligand stimulation of a GPCR.

We first applied carbachol, a muscarinic cholingeric receptor agonist, to cells expressing Kir3.2-Rluc (wildtype or mutant channels), the G protein and the Gαi-coupled muscarinic M_2 receptor. The wildtype channel responded to the ligand with a large increase in BRET as demonstrated by the large change in BRET ratio after compared to before the ligand was added (Figure 11A). However, the H67F mutant channel was less responsive to the ligand given the 50% decrease in the change in BRET ratio relative to the wildtype channel. The L271I mutant on the other hand, was completely insensitive to the ligand as there was little change in the BRET ratio upon the addition of the ligand.

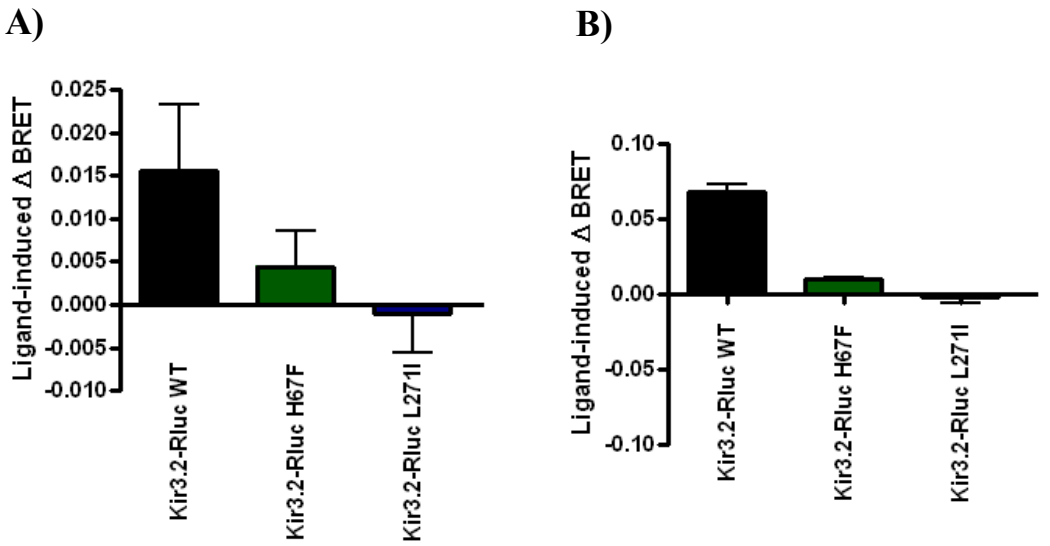


Figure 11. Ligand-induced changes in the G protein-channel interaction. HEK293 cells were transfected with Kir3.2-Rluc (wildtype or mutants) as well as $G\gamma_2$-GFP_{10}, Flag-$G\beta_1$, Gαi and either (A) the muscarinic M_2-HA receptor or (B) the dopamine Flag-D4 receptor. Cells were stimulated with (A) carbachol (1μM) for 2 minutes or (B) dopamine (1μM) for 2 minutes, prior to assaying the samples. BRET ratios were measured before and after addition of the ligand, and the difference in these values was plotted as the ligand-induced change in BRET. In both cases, addition of the ligand promoted increases in BRET values for the wildtype channel, reflecting conformational changes in the Gβγ-channel interaction. However, the N-terminal mutant (H67F) was less sensitive to the ligand, and the C-terminal mutant (L271I) was completely insensitive to the ligand. Data represent mean +/- SEM of three separate experiments.

These results were then verified with another Gαi-coupled receptor. When dopamine was applied to cells expressing the channel, the G protein and the dopamine D_4 receptor, we observed a similar trend in which the wildtype channel underwent the largest change in BRET ratio, followed by the H67F mutant and the L271I mutant (Figure 11B). Thus, once again, both mutant channels were less sensitive to receptor activation compared to the wildtype channel, with the C-terminal mutant being the least sensitive.

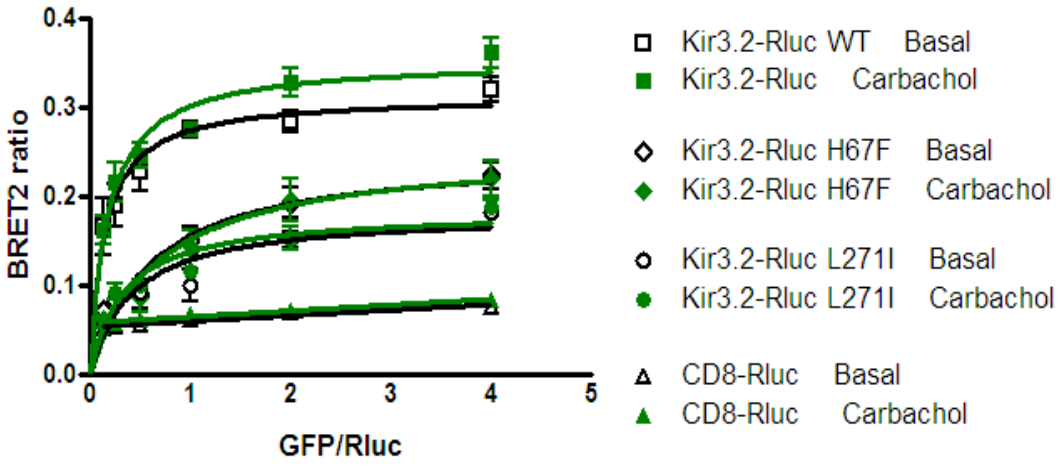


Figure 12. BRET saturation experiments confirm the ligand-induced changes in the G protein-channel interaction. HEK293 cells were transfected with Kir3.2-Rluc (wild-type or mutants) as well as $G\gamma_2$-GFP_{10}, Flag-$G\beta_1$, Gαi and the muscarinic M_2-HA receptor. Cells were transfected with a constant amount of the donor and increasing concentrations of the acceptor protein. Prior to assaying the samples, cells were stimulated with carbachol (1μM) for 2 minutes. BRET ratios were measured before and after addition of the ligand. Application of carbachol promoted hyperbolic increases in BRET values for the wildtype channel, reflecting conformational changes in the Gβγ-channel interaction upon the addition of the ligand. However, the N-terminal mutant (H67F) and the C-terminal mutant (L271I) conferred no changes in response to the ligand. CD8-Rluc was used as a negative control. Data represent mean +/- SEM of three separate experiments.

We then wished to extend measurements of these ligand-induced changes in the G protein-channel interaction by performing BRET saturation experiments,

this time, with and without the ligand. As before, the wildtype channel responded to the ligand by promoting large increases in the BRET ratio (Figure 12). These increases lead to a large change in the BRET signal given the Bmax of 0.28 in the absence of the ligand, compared to 0.35 in the presence of the ligand. In contrast to the wildtype channel, neither of the mutant channels was affected by the ligand since there were no changes detected in BRET ratios upon addition of the ligand. As with previous BRET experiments, CD8-Rluc was used as a negative control.

Receptor-mediated activation of the Kir3.2 channels

We then turned to electrophysiology to further study Kir3.2 channel activation when we modified the two Gβγ binding sites. However, prior to doing so, we wanted to ensure that our channel constructs could be expressed in HEK293 cells. Current recordings from cells expressing the wildtype Kir3.2-Myc channel gave rise to inwardly rectifying currents, as depicted by the presence of smaller outward currents with increasing depolarizing voltage commands (Figure 13A and 13B). Current-voltage plots indicated that the currents were in fact characteristic of Kir3 channels (Figure 13C). Recordings from cells expressing the wildtype Rluc-tagged Kir3.2 channels also yielded similar results (data not shown). As a negative control, recordings were made from cells expressing the Kir3.1 channel subunit (data not shown). As mentioned before, when expressed alone, this channel subunit does not reach the plasma membrane [66] and as such, no inwardly rectifying currents could be observed and the resulting current-voltage plot portrayed a linear

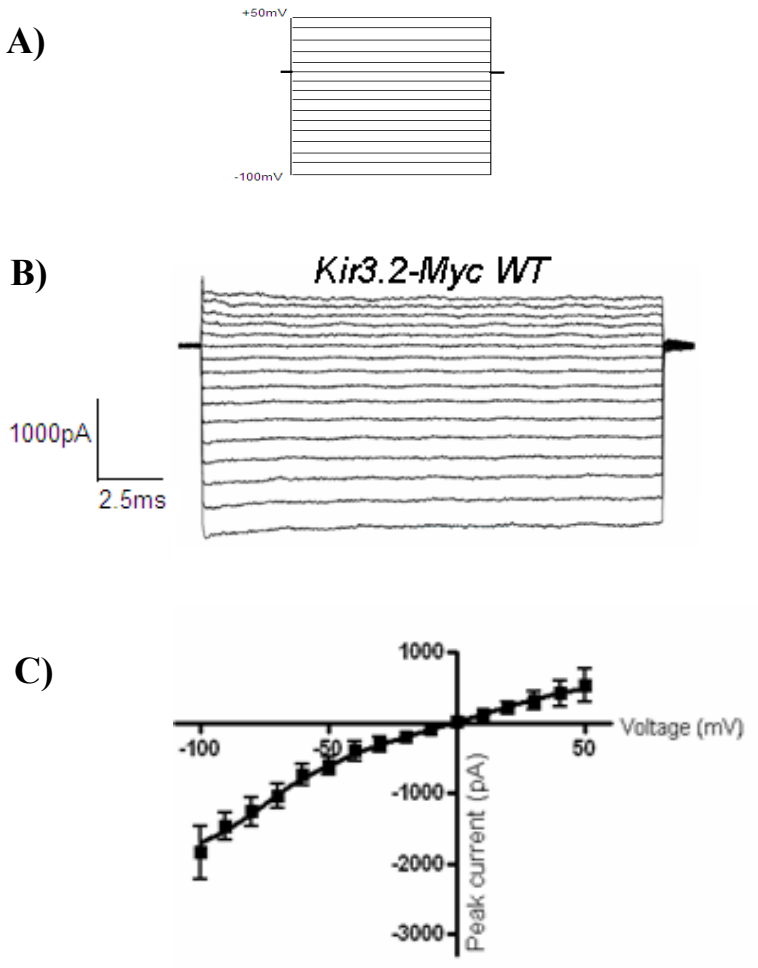


Figure 13. Expression of the inwardly rectifying Kir3.2 channels. (A) Currents were evoked by 20ms voltage commands between -100mV and +50mV in 10mV increments. (B) Currents recorded from HEK293 cells transfected with the wildtype Kir3.2-Myc channel resulted in the expression of inwardly rectifying potassium channels. Data are representative of measurements in five separate cells. (C) The current-voltage relationship of the trace in (B) depicts the inward rectification properties of the wildtype Kir3.2 channels due to smaller outward currents with increasing voltage. Data represent mean +/- SEM of three separate experiments.

line, characteristic of untransfected HEK293 cells.

Electrophysiology was also used to further verify our BRET data in which we had previously observed changes in the Gβγ-channel interaction upon ligand stimulation of a GPCR. Whole-cell patch recordings from cells transfected with the

wildtype Kir3.2-Myc channel and the dopamine D_4 receptor showed that this channel was capable of being activated upon stimulation with dopamine, as depicted by the increased inward current following agonist addition (Figure 14A). These inward currents could be washed out within seconds of removing the ligand. Unlike the wildtype channel however, neither the H67F mutant (Figure 14B) nor the L271I mutant (Figure 14C) could be activated by dopamine. Reflecting their surface expression patterns, the H67F mutant showed a basal current, while the L271I mutant did not.

According to our hypothesis, we proposed that each Gβγ binding site on the channel would serve a different role. That is, one binding site would be required for the "precocious" interactions leading to complex formation, while the other binding site would be required for signalling interactions following receptor activation. If this hypothesis is true, then one would expect to be able to co-express both mutants together, and thus be able to re-constitute a wildtype channel phenotype. As such, each mutant would be able to complement the other so as to yield a channel that is both able to assemble and traffic to the plasma membrane, and be capable of activation upon stimulation with a ligand. Surprisingly, when both mutants were co-expressed, they were still unable to mediate channel activation (Figure 14D). Further, the basal currents reflected what was seen with the L271I mutant suggesting that it either prevented the trafficking of the H67F mutant or acted as a dominant negative in terms of agonist-independent channel function (Figure 14D, Figure 15).

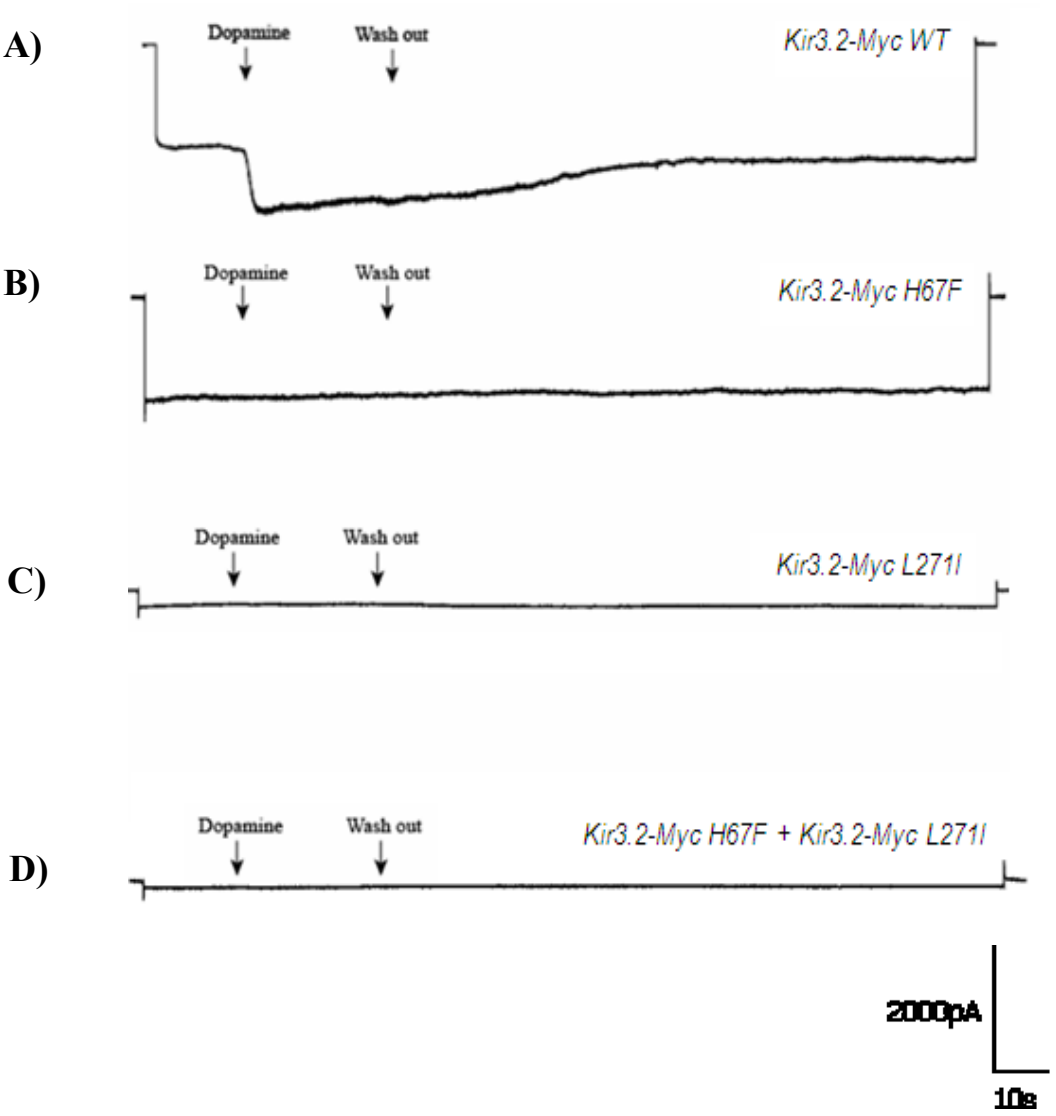


Figure 14. Receptor-activated Kir3.2 currents. Whole-cell patch clamp recordings were used to measure Kir3.2 channel currents upon activation of a GPCR. HEK293 cells were transfected with the Kir3.2-Myc channel (wildtype or mutants) and the Flag-D4 receptor. Cells were held at -60mV and the Flag-D4 receptor was stimulated with dopamine (1μM). (A) Representative traces show that the wildtype channel could be activated upon the addition of dopamine. However, neither the N-terminal mutant (B) nor the C-terminal mutant (C) could be activated, even though they displayed basal channel currents. (D) Co-expression of the two mutants together still renders the channel incapable of activation. Data are representative of measurements in least four separate cells.

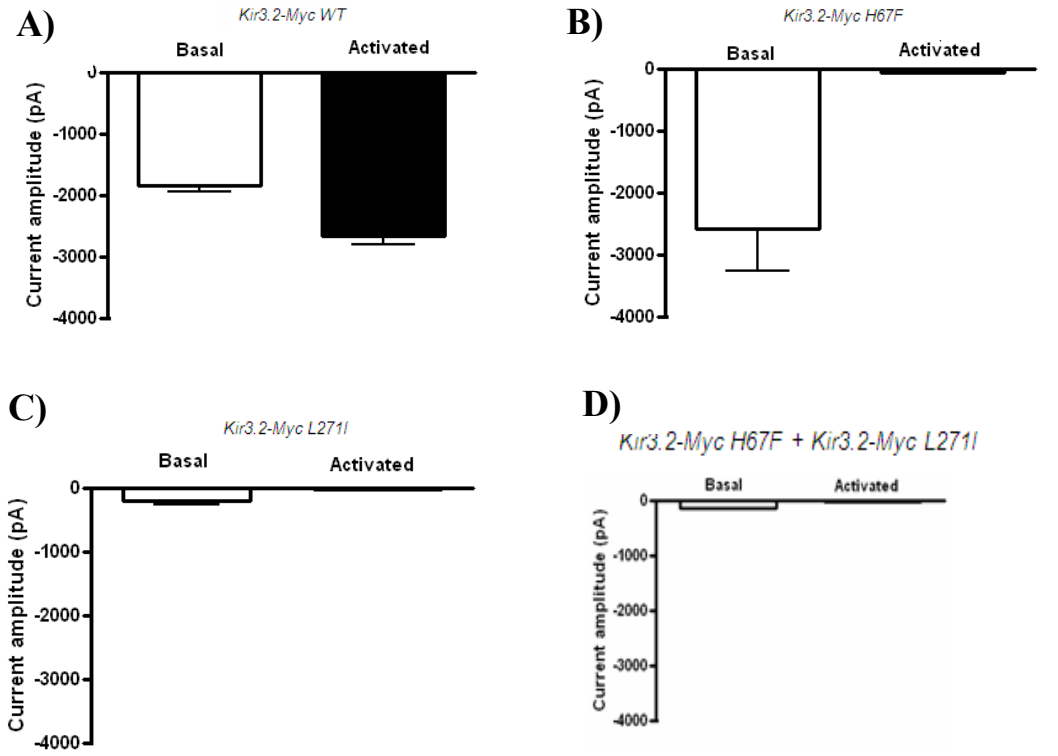


Figure 15. Basal and receptor-activated Kir3.2 channel currents. Plotting the basal versus receptor-activated currents from the Kir3.2 channels when stimulated with dopamine (from Figure 14) showed that the (A) wildtype channel exhibited large basal currents which were further increased in the presence of the ligand. (B) The Kir3.2-Myc H67F mutant also exhibited large basal currents, but was not capable of channel activation in the presence of the ligand. (C) The basal currents of cells expressing the Kir3.2-Myc L271I mutant were significantly smaller than those of the wildtype or N-terminal mutant channels. However, like the N-terminal mutant channels, these channels could not respond to the ligand with channel activation. (D) Cells co-expressing the two mutants exhibited little basal or agonist-induced activation. Data represent mean +/- SEM of three separate experiments.

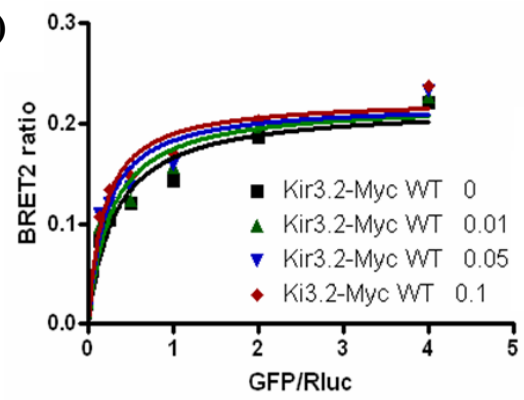
Rescue of the mutant channels

Since co-expression of the two mutants did not re-constitute the wildtype channel phenotype as expected (ie. the heterotetramer should have possessed both classes of Gβγ binding sites and thus produced both basal currents as well as agonist-induced channel activation), we next attempted to rescue the mutants by

titrating their levels of expression with a view towards favouring the expression of heterotetramers with both types of mutant channels. Thus, we co-expressed each of the Rluc-tagged mutant channels with increasing concentrations of either the Myc-tagged wildtype channel or Myc-tagged mutant channel counterpart. We then performed BRET saturation experiments in order to assess as to whether we could rescue the BRET interaction between the channel and the Gβγ subunit.

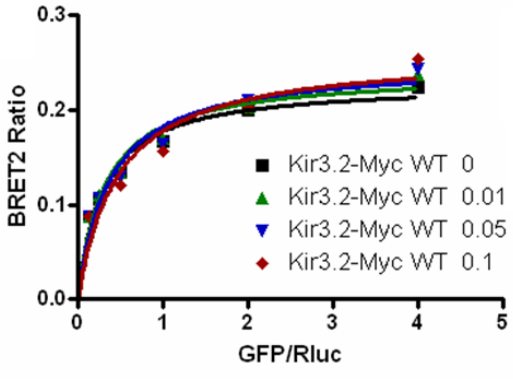
When the H67F mutant-Gβγ BRET pair was co-expressed with the wildtype channel, we observed sequential increases in the BRETmax and $BRET_{50}$ values with increasing concentrations of the wildtype channel, indicating a partial rescue of the channel-Gβγ interaction (Figure 16A). However, when the L271I mutant was co-expressed with the wildtype channel, we only observed sequential increases in the BRETmax values, but no changes in the $BRET_{50}$ values (Figure 16B). In contrast to this, when the Rluc-tagged H67F mutant was co-expressed with the L271I mutant (Figure 16C) or when the Rluc-tagged L271I mutant was co-expressed with the H67F mutant (Figure 16D), we found no definitive changes or trends in the BRETmax or $BRET_{50}$ values in response to increasing concentrations of the mutant channels. Thus, only when the N-terminal mutant was co-expressed with the wildtype channel, did we observe a partial rescue of this mutant channel with respect to the Gβγ interaction suggesting that in a tetramer containing a number of wildtype subunits, the interaction of Gβγ with the mutant was similar to the interaction of Gβγ with the wildtype channel.

A)



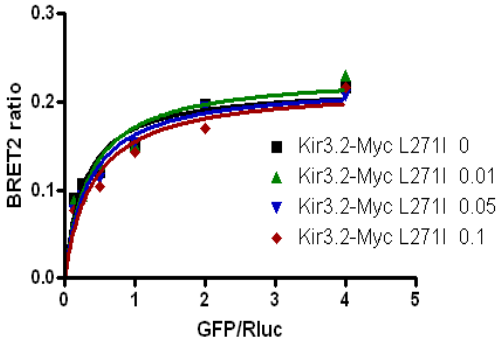
	$BRET_{50}$	std error	Bmax	std error
Kir3.2-Rluc WT 0	0.32	0.06	0.21	0.01
Kir3.2-Rluc WT 0.01	0.27	0.07	0.22	0.01
Kir3.2-Rluc WT 0.05	0.21	0.06	0.23	0.01
Kir3.2-Rluc WT 0.1	0.19	0.05	0.25	0.01

B)



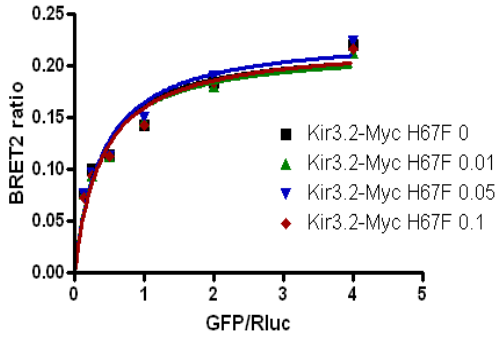
	$BRET_{50}$	std error	Bmax	std error
Kir3.2-Rluc WT 0	0.28	0.03	0.22	0.00
Kir3.2-Rluc WT 0.01	0.32	0.05	0.23	0.01
Kir3.2-Rluc WT 0.05	0.37	0.06	0.24	0.01
Kir3.2-Rluc WT 0.1	0.39	0.09	0.26	0.02

C)



	$BRET_{50}$	std error	Bmax	std error
Kir3.2-Rluc WT 0	0.28	0.05	0.22	0.01
Kir3.2-Rluc WT 0.01	0.36	0.07	0.23	0.01
Kir3.2-Rluc WT 0.05	0.35	0.04	0.22	0.00
Kir3.2-Rluc WT 0.1	0.40	0.08	0.21	0.01

D)



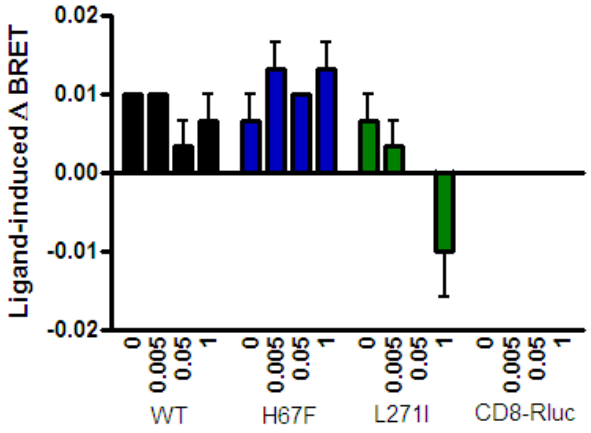
	$BRET_{50}$	std error	Bmax	std error
Kir3.2-Rluc WT 0	0.36	0.06	0.22	0.01
Kir3.2-Rluc WT 0.01	0.36	0.05	0.21	0.00
Kir3.2-Rluc WT 0.05	0.40	0.05	0.23	0.00
Kir3.2-Rluc WT 0.1	0.39	0.05	0.22	0.00

Figure 16. Rescue of the mutant channels with BRET saturation experiments. HEK293 cells were transfected with the Kir3.2-Rluc N-terminal mutant (A and C) or C-terminal mutant (B and D), as well as $G\gamma_2$-GFP_{10} and Flag-$G\beta_1$. Increasing concentrations (0, 0.01, 0.05 or 0.1μg DNA) of the wildtype Kir3.2-Myc channel or the Myc-tagged mutant counterpart was added to both Rluc-tagged mutant channels. (A) In the case of the N-terminal mutant, increasing concentrations of the wildtype Kir3.2-Myc channel was seen to sequentially increase the BRETmax and the $BRET_{50}$ values, indicating a partial rescue of the mutant phenotype. A table of $BRET_{50}$ and BRETmax values as well as their standard errors are listed on the right of each panel. No significant patterns were observed in all other channel combinations. CD8-Rluc was used as a negative control (data not shown). Data represent mean +/- SEM of three separate experiments.

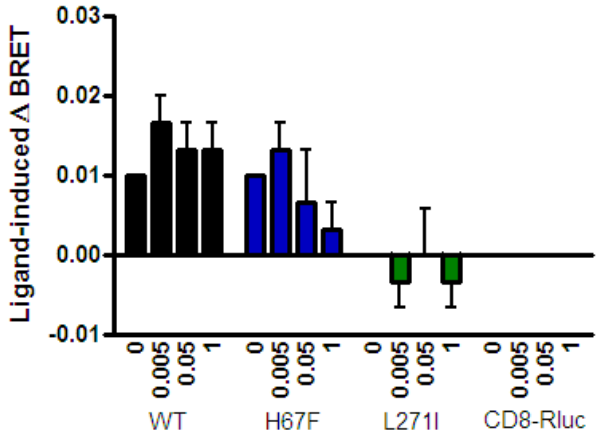
We also transfected the dopamine D_4 receptor into HEK293 cells, and could thus, stimulate the cells with dopamine and measure BRET changes in response to the ligand. As seen in Figure 17A, the H67F mutant could again be partially rescued by increasing concentrations of the wildtype channel as seen by the increases in BRET ratios. However, the H67F mutant did not appear to be rescued by the L271I mutant (Figure 17C). In contrast, the L271I mutant could not be rescued by either the wildtype channel or the H67F mutant channel (Figure 17A and 17B). Thus, as we observed in Figure 16, only the N-terminal mutant could be partially rescued by the wildtype channel. Neither of the mutants could rescue or complement one another in order to re-constitute the wildtype channel phenotype.

In order to confirm that the Myc-tagged channel proteins were in fact expressed in increasing concentrations in the above BRET experiments, we performed a Western blot on each of the samples used in the BRET experiments. Figure 17D shows a sample blot from cells expressing the wildtype Kir3.2-Rluc channel and increasing amounts of the wildtype Kir3.2-Myc channel, in which the

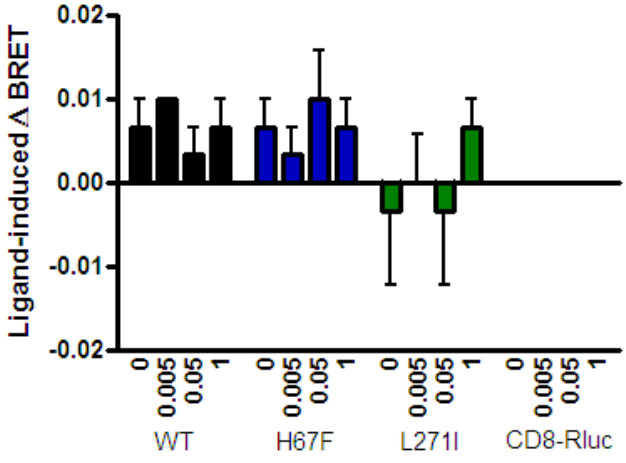
A) Increasing concentrations of Kir3.2-Myc WT



B) Increasing concentrations of Kir3.2-Myc H67F



C) Increasing concentrations of Kir3.2-Myc L271I



D)

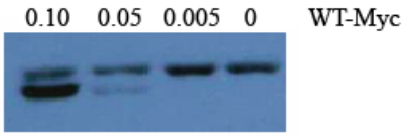


Figure 17. Rescue of channel mutants in the presence of ligand. HEK293 cells were transfected with the Kir3.2-Rluc channel (wildtype, N-terminal mutant or C-terminal mutant), as well as increasing concentrations (0, 0.005, 0.05 or 0.10μg of DNA) of (A) the wildtype Myc channel, (B) the N-terminal mutant, or (C) the C-terminal mutant. In addition, the cells were also transfected with $G\gamma_2$-GFP_{10}, Flag-$G\beta_1$ and the Flag-D_4 dopamine receptor. Dopamine (1μM) was applied to the cells for two minutes prior to assaying the samples. (A) Increasing concentrations of the Myc-tagged wildtype channel did not affect the wildtype or CD8-Rluc channels as expected. However, while it did not affect the C-terminal mutant, increasing concentrations of the Myc-tagged wildtype channel, appeared to increase the BRET ratio of cells expressing the N-terminal mutant. (B) Increasing concentrations of the Myc-tagged N-terminal mutant (A) or the Myc-tagged C-terminal mutant (B), had no effect on any of the channels. CD8-Rluc was used as a negative control. Data represent mean +/- SEM of three separate experiments. (D) A western blot confirms that all cells were in fact expressing increasing concentrations of the Myc-tagged channels. An example of cells expressing the wildtype Kir3.2-Myc channel is shown in this blot.

increasing intensity of the bands demonstrates increasing expression levels of the Myc-tagged protein.

In an attempt to develop another complementation strategy, we tried to rescue Kir3.2 channel mutants with Kir3.1 and vice versa. Tap-tagging experiments were performed by Peter Zylbergold (a student in the Hébert lab) in order to study the effects of the Kir3.2 channel mutants on Kir3.1 maturation. As seen in Figure 18A, the TAP-Kir3.1 construct alone interacted weakly with the Gβγ subunit, and was mainly detected in its immature form. However, when co-expressed with the wildtype Kir3.2-Myc channel, TAP-Kir3.1 was able to co-purify greater amounts of Gβγ and undergo channel maturation. When co-

expressed with either of the Kir3.2-Myc deletion constructs, we found that the TAP-Kir3.1 interaction with Gβγ was not improved, and the channels remained in their immature form.

The point mutation constructs were then co-expressed with the TAP-Kir3.1 construct and these experiments were repeated. The H67F mutant fostered the interaction of Kir3.1 with Gβγ, as well as its maturation. However, to our surprise, the L271I mutant, which as previously shown by confocal experiments, could not reach the cell surface on its own, now appeared to allow the Kir3.1 subunit to not only interact with Gβγ, but also allow for its maturation. Thus, the point mutants appeared to facilitate Kir3.1 maturation, suggesting that they themselves might be able to be rescued by the Kir3.1 subunit, i.e. the trafficking block of the L271I mutant or the loss of function in both point mutations might be overcome by co-expression with Kir3.1.

To follow up on this idea, we then measured ligand-induced BRET, in order to see if the Kir3.1 subunit could in fact rescue the point mutants. We co-transfected each mutant channel with increasing concentrations of the extracellularly-tagged Flag-Kir3.1 subunit, and then measured changes in the BRET between Gβγ and Kir3.2 upon the addition of dopamine. As seen in Figure 18B, Flag-Kir3.1 had no effect on the wildtype channel or the H67F mutant upon agonist addition, but appeared to increase the BRET between the L271I mutant as the Kir3.1 subunit concentration increased. Thus, the Kir3.1 subunit appeared to be able to rescue the L271I mutant.

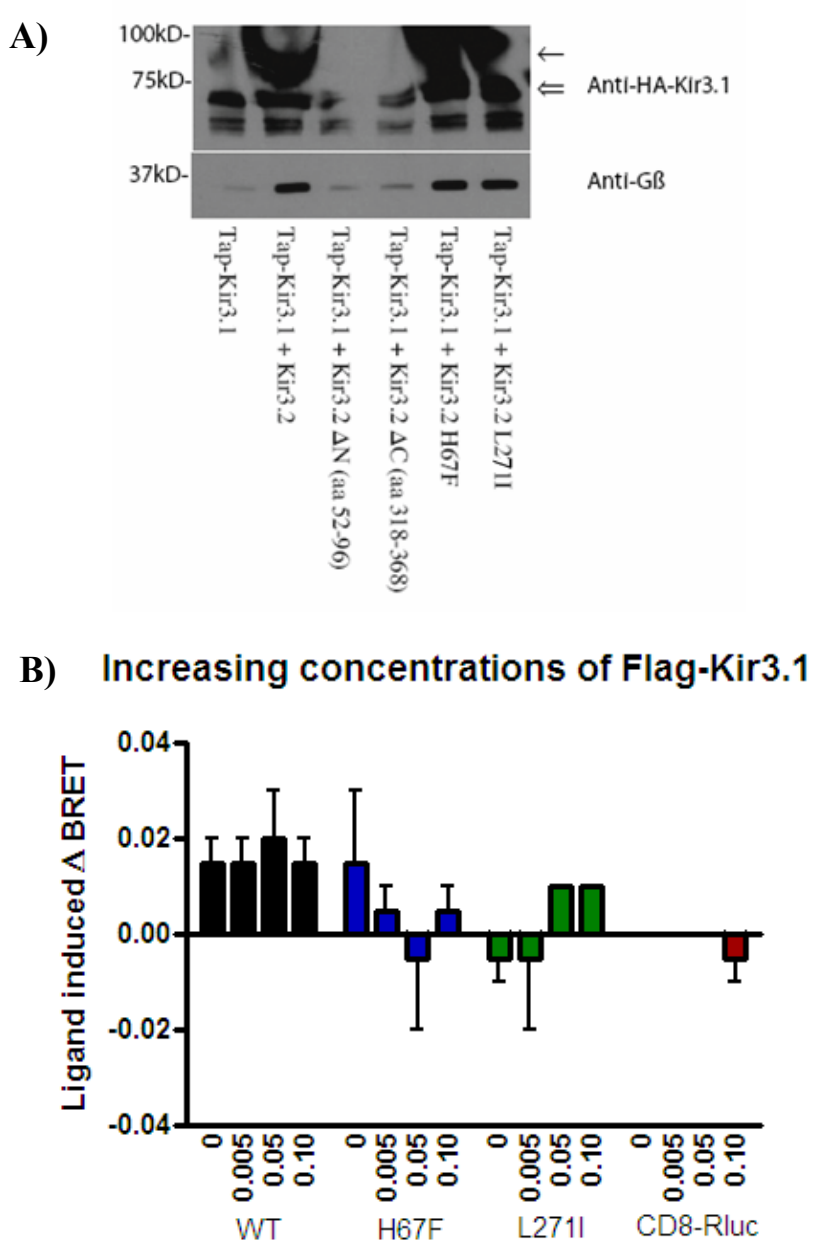


Figure 18. Rescue of the mutant channels by Kir3.1. (A) HEK293 cells were transfected with the TAP-Kir3.1 subunit and either the wildtype Kir3.2-Myc channel or the deletion or point mutation channels. Anti-HA antibodies were used to detect the Kir3.1 channel and anti-Gβ antibodies were used to detect channel-Gβγ interactions. While the Kir3.1 subunit did not mature on its own or bind to Gβγ, it appeared to be able to do both in the presence of the Kir3.2 channel. The Kir3.1 subunit also matured and bound to Gβγ in the presence of the point mutation constructs, but not the deletion constructs. Data represent at least two separate experiments. (B) HEK293 cells were transfected with the wildtype Kir3.2-Rluc channel, or either of the point mutation constructs, as well as the Flag-D_4 dopamine receptor and increasing concentrations (0, 0.005, 0.05 or 0.10μg of DNA) of the Flag-Kir3.1 subunit. Cells were stimulated with dopamine (1 μM) for

two minutes, prior to assaying the samples. The presence of the Flag-Kir3.1 subunit had no effect on the wildtype channel or the N-terminal point mutant. However, higher concentrations of the Flag-Kir3.1 subunit appeared to increase the BRET between Gβγ and Kir3.2 in cells expressing the C-terminal mutant. CD8-Rluc was used as a negative control. Data represent mean +/- SEM of three separate experiments.

DISCUSSION

In a previous paper by Robitaille et al., we studied the specificity of the Kir3.2 channel-G protein interaction using a series of BRET experiments [3]. Caution must be exercised when interpreting BRET data since a BRET signal may not necessarily indicate a true protein-protein interaction [94]. The proteins of interest may simply be within 10 nm of one another, and yet not interact directly [94, 97]. Alternatively, the proteins may not interact but could be forced into an orientation that causes for a BRET signal to be generated [94]. Thus, ideally, BRET experiments should be followed up with co-immunoprecipitation or GST-pulldown assays in order to confirm the presence of a true physiological protein-protein interaction.

Our results revealed the presence of a BRET interaction when cells expressed the channel and $G\beta_1$ or $G\beta_2$, but not $G\beta_3$, $G\beta_4$ or $G\beta_5$, suggesting that the Gβ subunits showed specificity in their interactions with the Kir3.2 channel (Figure 4). Thus, the Kir3.2 channel could interact with the G protein inside the cell, during the assembly of signalling complexes.

When these BRET experiments were repeated with DN Sar1 and Rab1 GTPases which are known to block the ER-golgi step of the exocytic trafficking pathway, we found that neither of the DN GTPases affected the channel-G protein interaction compared to the wildtype GTPases. This suggested that the "precocious" channel-G protein interactions occurred in the ER, after which the complex could likely be trafficked out to the cell surface.

From the data in that initial article, it was clear that the Kir3.2 channel could interact with the Gβγ dimer. Furthermore, given that the Kir3.2 channel can traffic to the cell surface as a homomultimer, the channel would both be able to interact with G proteins while at the cell surface and also while inside the cell.

Over the recent years, electrophysiological and structural studies have indicated that there are in fact multiple binding sites for the Gβγ subunits on the Kir3 channels [31, 40, 43]. These binding sites have been localized to the intracellular N- and C-terminal domains of the channel [31, 98] and each channel subunit contains 2-3 putative Gβγ binding sites, giving each channel tetramer a possible of 8-12 binding sites [3, 31, 43]. Studies have shown that the Kir3 channel opens maximally when only a subset of these Gβγ binding sites are occupied, suggesting that some sites may be required for agonist-dependent interactions [3, 91]. Yet, distinct roles for the different Gβγ binding sites on the channel have not been identified.

In the present study, we wished to further our understanding of the Kir3 channel-G protein interaction by studying the various Gβγ binding sites on the channel and delineating their distinct roles in channel modulation. Therefore, we engineered deletion constructs in which either the N- or the C-terminal Gβγ binding sites were removed from the Kir3.2 channel. The Kir3.2 channel served as a good model to use since it could be studied on its own, without having to co-express it with another Kir3 channel subunit in order to target it to the cell surface.

Prior to using the deletion constructs, we wanted to ensure that they could be expressed in HEK293 cells, our heterologous expression system of choice for the study. While we were able to detect expression of most constructs by Western blotting, we were unable to detect the expression of the Kir3.2-MycΔ52-96 (N-terminal deletion) channel (Figure 5).

Each of the Gβγ binding sites on the N- and C-terminal domains of the channel spans a length of 44-50 amino acids [40]. Thus, the removal of such a large number of amino acids could have modified the underlying structure of the protein, which could have in turn, altered its folding so as to mask the epitope recognized by the antibody that would normally bind to the channel. Alternatively, the N-terminal deletion construct may have degraded spontaneously in the cell.

However, since the Rluc-tagged deletion constructs could be detected by Western blotting, we decided to perform BRET saturation experiments to examine the relative affinities of the channel for the G protein. In such an experiment, the expression of the Rluc-tagged protein is maintained as the acceptor-tagged protein is increased. If the BRET interaction is saturable, in that it approaches a maximum value asymptotically as the concentration of the acceptor protein increases, a $BRET_{50}$ can be determined [9]. The $BRET_{50}$ then allows us to compare the affinities of the two proteins for one another [9].

When the N-terminal Gβγ binding site was removed from the Kir3.2 channel, we observed a marginal change in the affinity of the channel for the G protein relative to the wildtype Kir3.2 channel (Figure 6). However, when the C-

terminal Gβγ binding site was removed, we observed a 10-fold decrease in the affinity of the channel for the G protein, compared to the wildtype channel. Since the BRET pairs are different, it is difficult to compare and directly draw conclusions about the affinities of the different channel-G protein interactions, however.

Caution must also be used when interpreting the Bmax. The Bmax cannot be used to assess the specificity of a protein-protein interaction [14]. It merely depends on both the distance and relative orientation of the donor and acceptor proteins [14, 95]. As such, a larger Bmax signal may not necessarily mean that the channel has recruited more Gβγ subunits, since the channel may simply have bound to Gβγ in a different conformational state.

Nevertheless, these results suggest that the Kir3.2 channel possesses Gβγ binding sites of differing affinities. Similar observations have in fact also been observed by He et al., in which it is proposed that there are likely two sites for Kir3 channel regulation by the Gβγ subunits [39]. In their model, the high affinity site would be required for agonist-independent basal currents which depend on free Gβγ subunits, whereas the low affinity site would respond to Gβγ subunits that are liberated from G protein heterotrimer activation [39].

We next wished to ask whether the deletion constructs could effectively reach the cell surface. From our confocal experiments in non-permeabilized cells, we were able to detect the trafficking of the wildtype Kir3.2-Myc or Kir3.2-Rluc channels to the cell surface. When the same experiment was repeated with

permeabilized cells, we discovered that a large number of Kir3.2 channels resided inside the cell, even though a certain proportion of them were trafficked to the cell membrane. These findings are in fact in agreement with other groups who have studied Kir3.2 channel trafficking and confirmed its localization both at the cell surface and inside the cell [3, 49, 66].

In contrast to the wildtype channels, cells expressing either of the deletion constructs exhibited dramatic decreases in cell surface fluorescence levels, indicating that the deletion constructs were unable to traffic as efficiently to the cell surface, as the wildtype channels (Figure 9A). We initially wondered whether this was due to the inability of the deletion constructs to interact with the G protein, as this would be required for the assembly of the complex, and subsequent trafficking of the complex to the cell surface. However, we soon ruled out this possibility since our BRET saturation experiments had clearly established that the deletion constructs could in fact interact with the G protein in a specific and saturable manner. From our experiments, it would seem that the C-terminal Gβγ binding site is absolutely required for trafficking of the channel to the cell surface, something not generally considered in models of Kir3 function. Deletion of the N-terminus also compromised cell surface trafficking, but not to the same extent. Thus, both sites are important for channel assembly and function.

We then wondered if the deletion constructs were able to interact with the Kir3.1 subunit with which they were co-expressed in our confocal experiments, as a lack of this channel-channel interaction, would result in the inability of the channel to traffic to the cell surface. Our co-immunoprecipitation data established

that the C-terminal deletion construct was unable to interact with the mature form of the Kir3.1 subunit (Figure 9B). Thus, the interaction of the Kir3.1 subunits with the Kir3.2 deletion constructs was insufficient to allow the hetero-multimeric channels to exit the ER for correct glycosylation of Kir3.1 in the Golgi apparatus. When co-immunoprecipitation experiments were repeated with the N-terminal construct, we could not detect the expression of this channel on a Western blot (data not shown). Thus, we were unable to determine as to whether the N-terminal deletion construct could interact with mature or immature forms of the Kir3.1 subunit. Repeating these co-immunoprecipitation experiments with the Rluc-tagged channel could have answered this question, as the Rluc-tagged deletion channels were always able to express on a Western blot. Alternatively, it is possible that the Myc-tagged N-terminal deletion construct in our experiments interacted with the Kir3.1 subunit, but that this interaction had just not been stable enough to survive the conditions required for co-immunoprecipitation.

Our confocal experiments required that we co-expressed the Kir3.2 channel with the Kir3.1 subunit in order to study Kir3.2 channel trafficking. This was due to the lack of suitable antibodies to study the Kir3.2 channel on its own. Matters were made worse by the fact the Kir3.2 channels could reside both inside the cell and at the cell surface, which made it difficult to identify its localization within the cell. Had we not used the Kir3.1 channel subunit to study Kir3.2 channel trafficking, and had we still reported that the deletion constructs had been unable to reach the cell surface, one obvious question to ask is, why have the deletion constructs lost their ability to traffic efficiently?

One possibility is that the amino acids within the Gβγ binding region on the channel overlap with a sequence of amino acids which also contain a signal required for channel transport or stabilization at the membrane. In fact, the intracellular domains of Kir3 channels are essential for the regulation of the channel by various modulators [31, 58, 61]. As such, there are specific motifs within these domains which contain phosphorylation sites, and other sites to which intracellular proteins might bind [30]. These sites would essentially control the trafficking itineraries of the channel. The intracellular domains of the channel also contain distinct trafficking signals such as the ER export motif or the post-Golgi surface promoting motif, which allow for the expression of the channel at the cell surface [64, 66-67]. When as much as 50 amino acids from the intracellular domains of the channel have been deleted on each domain, it is likely that the channel is now lacking critical sequences which are essential for its function. Given the recent emerging roles of the Gβγ subunit as an organizational chaperone required for the assembly and trafficking of signalling complexes, it is also intriguing to postulate that preventing the Gβγ-channel interaction could have prevented channel trafficking [16].

Since the deletion constructs failed to traffic to the cell surface, and also could not interact with the mature Kir3.1 channel subunits, we refrained from using the deletion constructs in further experiments, given the caveats discussed above. In hindsight, we could have engineered fragments of the channel, each harboring incremental deletions of 25 amino acids or so, until Gβγ binding was

completely abolished. This would have helped and can still help identify minimal regions on the channel which maximally reduced Gβγ binding.

We then decided on a similar strategy used by He et al. The authors of this paper identified crucial residues on the Kir3.1 and Kir3.4 channels, which mediated functional channel-Gβγ interactions. In the Kir3.1 channel subunit for example, an N-terminal histidine-64 residue and a C-terminal leucine-268 residue were identified as critical residues controlling Gβγ binding since point mutations at these residues reduced the binding of Gβγ to the channel by ~70% [39]. Thus, we engineered the equivalent point mutations in the Kir3.2 channel and wished to perform experiments through this less perturbing, and more subtle approach.

Unlike our deletion constructs, we were successful in expressing our point mutation constructs as detected by Western blotting (Figure 5). BRET saturation experiments were then performed to measure the affinity of the G protein for the mutant channels. As with the deletion constructs, we observed two classes of binding affinities on the Kir3.2 channel for the Gβγ subunits (Figure 7). The N-terminal mutant, like the wildtype channel, exhibited a higher affinity for binding to Gβγ than the C-terminal mutant. These results not only confirm our data with the deletion constructs, but also provide more evidence towards the notion of two different affinity states of Gβγ binding to the channel. We did not systematically test the relative reduction in Gβγ binding to the mutant channels indicated in the BRET experiments, however. This could have ideally been done through GST-

pulldown experiments in order to definitively attribute our data to the lack of Gβγ binding to the channel.

Through confocal experiments in non-permeablized cells, we found that the wildtype Kir3.2-Myc and Kir3.2-Rluc channels could traffic to the cell surface. In contrast to our observations with the deletion channels, the N-terminal mutant, like the wildtype channel, was also able to reach the cell surface (Figure 10A). The C-terminal mutant, however, failed to traffic efficiently to the cell surface. Similar differences between the deletion and point mutation constructs have also been observed by Clancy et al [73]. For example, constructs containing several point mutations within the Gα binding site of the Kir3.2 channel, failed to express at the cell surface [73]. In comparison, constructs containing only a single point mutation within the Gα binding site, were shown to efficiently express at the cell surface [73]. Although these constructs were intended to study Gα binding, but not Gβγ binding to the Kir3.2 channel as with our study, it still serves to illustrate that differently engineered mutants do yield strikingly different results.

Nevertheless, that the N-terminal, but not the C-terminal point mutant could traffic to the plasma membrane, suggests that each of the Gβγ binding regions in the N- and C-terminal domains of the channel likely serve distinct trafficking roles. The C-terminal mutant may be required for the early or 'precocious' interactions, which include the assembly and trafficking events needed to bring the Gβγ-channel complex out to the cell surface. Since the N-terminal mutant was able to reach the cell surface, this Gβγ binding site may not be

required for the 'precocious' assembly or trafficking events, but instead may be required for the signalling events which occur at the cell surface following receptor activation.

To further study the involvement of the different Gβγ binding sites in channel activation, we next conducted BRET experiments to assess the effects of GPCR ligands on the Gβγ-induced activation of the channel. When cells expressing the wildtype channel were stimulated with carbachol, a Gαi-coupled muscarinic M_2 receptor agonist, we observed increases in the BRET signal following agonist stimulation (Figure 11A). Agonist-induced changes in BRET ratios are normally indicative of conformational changes that accompany G protein activation [9]. The increase in BRET ratio in the presence of an agonist may be attributable to either a change in the number of protein complexes due to a change in the affinity of the proteins for one another, or a change in the relative orientation of the donor and acceptor proteins due to a change in their conformation [9]. To study the basis of these agonist-induced changes in BRET between the channel and the G protein, we performed BRET saturation experiments with the muscarinic M_2 receptor (Figure 12). As previously shown in Figure 11, the wildtype channel exhibited increases in the BRET ratio upon the addition of carbachol. For the mutant channels, we observed either marginal or no changes in the BRET signal after the addition of the agonist. Interestingly, the $BRET_{50}$ values for the mutant channels were the same whether or not the cells had the agonist, suggesting that the activation of the G protein did not change the affinity of these proteins for one another. Furthermore, the agonist also did not shift the Bmax values for the mutant

channels, providing additional evidence that there was no agonist-induced conformational change between the Gβγ subunit and the channel. Thus, the increases in Bmax for the wildtype channel in the absence of changes to $BRET_{50}$ upon agonist stimulation likely reflect conformational changes in the Gβγ-channel interaction rather than additional recruitment *per se*.

In contrast to the wildtype channel, we found that the N-terminal mutant was less sensitive to the agonist as seen by the smaller increase in BRET ratio upon the addition of carbachol. The C-terminal mutant was however, completely insensitive to the ligand. To confirm these BRET results, we also repeated these experiments with the dopamine D_4 receptor, another Gαi-coupled receptor (Figure 11B). As with carbachol, dopamine receptor stimulation resulted in conformational changes between the Gβγ subunit and the wildtype Kir3.2 channel. This effect was again less pronounced with the N-terminal mutant, and even to a smaller extent with the C-terminal mutant. This may have been due to the low affinity of the C-terminal mutant for Gβγ, or more likely the fact that this mutant was unable to efficiently traffic to the cell surface, in order to be available at the membrane to become activated upon receptor activation. Experiments could be conducted using lipid soluble ligands to evaluate which of these (or both) possibilities is more likely. Taken together, these data demonstrate that both the N-terminal and C-terminal mutants alone are not sufficient to mediate channel activation. Thus, both Gβγ binding sites of the channel appear to be required for optimal channel function.

As an additional measure of channel activation, we also turned to electrophysiology and performed whole cell patch recordings in cells transfected with the wildtype Kir3.2 channels. As seen in Figure 13, we showed that the wildtype channels were functional since they were able to exhibit the hallmark properties of inward rectification which characterize the Kir3 channels.

We then stimulated the cells with dopamine and attempted to measure channel activation upon stimulation of the dopamine D_4 receptor. When dopamine was added to cells expressing the wildtype channel, we observed an immediate and robust inward current, which reflected channel activation (Figure 14). This observation is in line with our previous BRET experiments, in which a conformational change between the wildtype channel and the Gβγ subunit was detected upon ligand activation.

With the mutant channels however, we recorded basal channel currents with the H67F mutant and even smaller basal currents with the L271I mutant. Furthermore, neither manifested agonist-induced currents (Figure 14 and 15). The effects of these mutations appeared to be less severe than those identified from another study, in which some chimeric mutants resulted in the complete loss of Kir3 channel currents [92]. On the other hand, similar results have also been reported by He et al., whom when working with the Kir3.4 channel subunit discovered a leucine residue in the C-terminal Gβγ binding region, which when mutated, consistently exhibited only basal, but not GPCR-induced currents [39]. Collectively, these data show that basal channel activity is a requirement for agonist-induced activity.

Since both point mutant channels responded weakly to the ligand in our earlier BRET experiments, and since neither one of them was capable of channel activation as seen from our patch recordings, it appears that both Gβγ sites on the channel are required for the functional activation of the tetrameric channel. This observation is actually in agreement with the current model of Kir3 channel activation. According to Mirshahi et al, both the N- and C-termini of the channel are postulated to bind to distinct surfaces of Gβγ in order to mediate channel activation [79]. Thus, the N-terminus would bind to the heterotrimeric G protein through the Gβ subunit and upon G protein activation and the subsequent dissociation of the Gα subunit from the Gβγ subunit, the Gβγ subunit would now also be able to bind to the C-terminus of the channel, through residues on Gβγ that were previously masked by the Gα subunit [79, 81]. As such, only when both the N- and C-termini of the channel are bound to Gβγ, would the channel be activated [44].

Based on our confocal experiments, we had originally proposed that the N-terminal mutant, while being able to reach the cell surface, would lack the ability to signal following receptor activation. This proposition was supported through our patch recordings in which we showed that the N-terminal mutant, while retaining basal channel activity, was unable to undergo the necessary requirements for channel activation.

The C-terminal mutant which could not traffic to the cell surface displayed very little or essentially no basal channel currents, unlike the N-terminal mutant. This is in fact analogous to a C-terminal mutant in the Kir3.1 (L262I) and Kir3.4

(L268I) channel subunits, in which basal channel currents were greatly reduced, though not completely abolished [39]. One possibility is that the C-terminus contains additional Gβγ interaction sites with the channel that contribute to Gβγ-mediated basal currents. In fact, previous studies have already indicated that the C-terminal domain of the Kir3.1 channel contains one to possibly two binding sites [40, 43]. One way in which this could have been tested for the Kir3.2 channel is by using a Gβγ-binding molecule, such as βARK-CT or phosducin. Such a molecule would act as a Gβγ scavenger that can sequester Gβγ away from the channel [39]. If βARK-CT inhibited the basal currents exhibited by the C-terminal mutant, this would have confirmed that additional residues on the C-terminal domain may have contributed to the Gβγ-mediated basal currents.

Knowing that both of the domains of the channel were essential for channel activation, we wondered whether co-expression of the mutant channels would rescue channel function and re-constitute the wildtype channel phenotype. Unfortunately, when both mutants were transfected into HEK293 cells, we were unable to measure any channel currents upon GPCR activation, even though basal channel currents were present in small amplitudes (Figure 14D).

We next aimed to titrate the levels of mutant channel expression, in hopes of being able to rescue the mutants by forcing the formation of heterotetramers containing both classes of Gβγ binding sites. To this end, we performed BRET saturation experiments, in which we co-expressed the N- or C-terminal mutants with increasing amounts of the wildtype or counterpart channels. Similar

experiments have also been performed by He et al., in which each of their N- and C-terminal point mutants was paired with a chimeric mutant, resulting with the mutants being able to now display both basal and agonist-induced currents [39]. In this case, the double mutants rescued function by enhancing the channel-PIP_2 interactions which were sufficient enough to allow gating of the channel, even in the absence of Gβγ [39].

When pairing mutants in order to titrate their levels of expression in a BRET experiment, there are a number of possible outcomes that can be expected. In one scenario, the wildtype channel might be expected to sequester Gβγ away from the mutant channel, which could reduce the BRET between the mutant channel and the G protein. Alternatively, if the two mutant channels are able to interact with one another, there may be an increase in the BRET signal. Conversely, the BRET ratio may still increase if the orientation of the donors and acceptors changes so as to favor a BRET signal even though the two channel subunits are not interacting as strongly as usual.

As seen in Figure 16, different pairs of mutants yielded different results. Only in the presence of the N-terminal mutant and the wildtype channel, was there an increase in the BRET signal, suggesting a partial rescue of the mutant phenotype or possibly recruitment by the "rescuing" partner of Gβγ close enough to the BRET donor such that the energy transfer is more efficient. With all other combinations, there was either very little or no complementation between the mutant channels. In contrary to our initial prediction, these data suggest that

complementation does not occur when two individual mutants are paired together. We do not know if this is due to the two mutants not being able to interact with one another. If the double mutants can interact, we also do not know whether they can reach the cell surface although since no basal currents were detected, this is unlikely. While, this could have been tested in our study, we were unable to do so without having to add the Kir3.1 channel subunit, due to the lack of appropriate antibodies. As of this writing, extracellularly tagged versions of the Kir3.2 channel are being constructed to circumvent this difficulty. Nevertheless, our data suggest that mutations at different sites on the channel were unable to complement one another, indicating that both Gβγ binding sites on each channel subunit need to be intact in order for the channel to function properly.

To begin to address this problem, pilot Tap-tagging experiments were performed in order to study Kir3.1 channel maturation. As shown in Figure 18A, the TAP-Kir3.1 construct alone remained in its immature form and bound weakly to Gβγ. This is in contrast to BRET data previously obtained in our lab, in which the Kir3.1 channel subunit could interact with many Gβγ subunit combinations, whether or not it remained in the ER or associated with other Kir3 subunits that facilitated its maturation and cell surface trafficking [3, 9]. This discrepancy between the data suggests that the interaction between the Kir3.1 channel and Gβγ may be weak, such that it is unstable and easily lost when detergents are added to the proteins during purification, but is preserved in experiments conducted in living cells (i.e. BRET depends mainly on proximity and less on affinity between interacting proteins).

Our data established that while the TAP-Kir3.1 construct could interact with the wildtype Kir3.2-Myc channel which facilitated Kir3.1 maturation, it was unable to interact with both of the deletion constructs to have the same effect. This is in fact analogous to the results obtained earlier during our co-immunoprecipitation experiments, in which the Kir3.1 subunit was found to interact with the wildtype Kir3.2 channel but not the deletion channels.

Interestingly, when the TAP-Kir3.1 subunit was co-expressed with the point mutant channels, we found that the both point mutants improved binding of Kir3.1 to Gβγ as well as its maturation. This was surprising, since confocal experiments had previously suggested that the C-terminal mutant did not efficiently traffic to the cell surface. Thus, the L271I mutant appeared to facilitate Kir3.1 maturation which should allow Kir3.1 to traffic to the cell surface, suggesting that this C-terminal mutant might in turn be able to be rescued by the Kir3.1 subunit.

To test this possibility, we performed BRET experiments following dopamine stimulation of dopamine D_4 receptors, in order to assess as to whether the Kir3.1 subunit could rescue the point mutants. As we had previously done, we again paired the mutant channels with increasing concentrations of the Flag-Kir3.1 subunit. As seen in Figure 18B, Flag-Kir3.1 had no effect on the wildtype channel or the N-terminal point mutant, but appeared to increase the BRET ratio of the C-terminal point mutant upon addition of the agonist, especially at higher concentrations of the Kir3.1 subunit. While the effects of the Kir3.1 subunit on the

BRET interaction were difficult to assess due to the small BRET values, there seemed to be a trend towards the ability of the Kir3.1 subunit to rescue the C-terminal mutant. A similar trend has also been reported by Clancy et al., in which the co-expression of a Kir3.1 subunit with a mutant Kir3.2 channel construct, partially rescued membrane expression, which the mutant construct failed to do on its own [73].

All experiments in this study were performed in a HEK293 cell line expression system. Ideally, it would have been best to study native Kir3.2 channels, from either brain detergent extracts or atrial membranes, as transfection systems usually require that your proteins of interest are over-expressed. However, due to the use of engineered deletion and mutation constructs as well as our ability to easily perform BRET experiments within the laboratory, it would have been impossible not to use a heterologous expression system. Having said this, our experiments could have been performed in at least one other heterologous expression system to verify our findings.

Implications of channel-Gβγ interactions

Due to their role in many critical physiological functions in eukaryotic cells, GPCRs have become the largest drug targets for the therapeutic treatment of a number of diseases [25, 29]. In fact, either directly or indirectly, more than 50% of the top-selling drugs on the market today target GPCRs [6, 99].

Therapeutic strategies have generally aimed at regulating effectors in order to modulate a specific outcome [6, 99]. However, drugs targeting the receptor often cannot modulate one specific effector, since a given receptor is usually coupled to multiple effectors [19]. This can in turn, produce undesirable side effects [6]. Fortunately, the presence of multiple signalling proteins and their associated isoforms [26], suggests that there may be unique structural surfaces which could serve as targets for therapeutic small molecules which would help achieve greater specificity and fewer side effects [6, 19, 80].

The first step towards such a therapeutic approach would be to identify and characterize the interactions or peptide motifs involved between individual partners in a signalling complex [6]. Once identified, we should be able to modulate specific signalling events by either disrupting or augmenting interactions which would normally lead to the formation, trafficking and functioning of these complexes, so as to favor a specific signalling outcome [6].

Interestingly, some studies have already begun to employ such an approach. For example, Gq peptides that target the receptor-Gq interface in transgenic mice have been shown to prevent the development of hypertrophy after transverse aortic constriction [2]. Thus, peptidomimetics that target specific protein interfaces may have the potential to pave the way towards a new approach of modulating cellular signalling in a number of diseases [80].

Taken together, the vast number of cellular functions of the Kir3 channels underscores their medical implications, suggesting that they are potentially

important targets for pharmaceutical agents [30, 48, 57]. Therefore, by understanding the molecular sites of interactions between Kir3 channels and the Gβγ subunits - their central activators, we should in the future, be able to discover a means to treating diseases with the Kir3 channel [52]. It should then be possible to create small molecules that can target Gβγ or the Kir3 channels, as opposed to targeting multiple downstream pathways associated with a given GPCR [19, 80].

Conclusion

Taken together, our data suggest that both the N- and C terminal Gβγ binding sites on the Kir3.2 channel harbor distinct roles, which together are required for proper channel signalling. The C-terminal Gβγ binding site may be required for the early interactions which bring the channel to the cell surface, while the N-terminal Gβγ binding site may be necessary for the signalling of the channel at the cell surface, following receptor activation.

Summary

Through a variety of experiments, we were able to show that different Gβγ binding regions on the Kir3.2 channel harbored distinct roles with regards to channel function. The C-terminal Gβγ binding site on the Kir3.2 channel could be required for 'precocious' interactions, while the N-terminal Gβγ binding site could facilitate signalling interactions at the cell surface, following receptor activation. These findings may have a significant impact on our notions of signalling

specificity and aid in the future development of novel drugs for Kir3 channel disorders.

Future Directions

There are still several questions that remain to be answered in subsequent studies:

(1) Since our confocal experiments showed that the C-terminal mutant could not reach the cell surface, one question that arises from these observations regards the subcellular location of the mutant channel. That is, does the C-terminal mutant get trapped in the Golgi or some other intracellular compartment?

(2) Given that both the Tap-tagging and BRET experiments indicated the possibility for the Kir3.1 subunit to rescue the mutant Kir3.2 channels, we would also like to pursue these experiments by functionally measuring these responses via patch clamp recordings. In this case, we would expect the mutant channels to be capable of agonist-induced currents in the presence of the Kir3.1 subunit.

(3) We would also like to repeat the Tap-tagging experiments with the Kir3.2-Rluc constructs, since all constructs used in the above experiments utilized the Kir3.2-Myc constructs. In the present study, we were unable to express the Kir3.2-Myc N-terminal deletion construct on a Western blot, and thus were never able to fully assess its ability to interact with the Kir3.1 subunit. By using the Rluc-tagged construct, we hope to be able to assess the contribution of the N-terminal deletion construct in Kir3.1 maturation.

(4) It would also be worthwhile to construct an extracellularly Flag-tagged Kir3.2 channel subunit, which would allow us to study Kir3.2 channel trafficking alone, without having to co-express it with the Kir3.1 subunit.

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