Regulation of the pancreatic KATP channel

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# [ch:1-intro]Introduction

## General introduction

## Pancreatic islets and the b-cell

Pancreatic islets are endocrine cells which are responsible for maintaining glucose homeostasis. There are roughly one million islets in a human pancreas, constituting 1-2% of the total pancreatic mass. Islets consist of three principal cell types; insulin secreting b-cells, glucagon secreting a-cells and somatostatin secreting d-cells. Islets respond to increases in blood glucose by releasing insulin, which acts on peripheral tissues to increase glucose uptake and reduce blood glucose levels. Conversely, decreases in blood glucose leads to the release of glucagon, which acts on those tissues to stimulate glucose production and increase blood glucose.

## Architecture of the pancreatic KATP channel

ATP-sensitive potassium (KATP) channels are present in many tissues, where they couple the metabolic state of a cell to its electrical activity by regulating the flow of K+ across the membrane. KATP channels are an octameric complex, comprised of four inwardly-rectifying potassium channel subunits (Kir6.1 or Kir6.2), each of which is associated with a sulphonylurea receptor subunit (SUR1, SUR2A or SUR2B). In pancreatic b-cells, the KATP channel isoform is composed of Kir6.2 and SUR1. Together, Kir6.2 and SUR1 form a complex nearly a megadalton in size and over 15 nanometres across (Figure [1.6](#ch1fig:katp_cartoon), [1.7](#ch1fig:sur_topdown)).

Inwardly-rectifying potassium channels are so named because they allow K+ to flow more easily into the cell than out of it (Figure [1.1](#ch1fig:rectification)). This phenomenon is a consequence of voltage-dependent pore blockade by intracellular divalent cations (especially Mg2+) and polyamines. At depolarising membrane potentials, blockers are driven into the pore and K+ current is blocked, while at hyperpolarising potentials the blockers and cleared and K+ current can flow. Strongly rectifying Kir channels display drastically reduced conductance at potentials more positive than the K+ reversal potential. In contrast, Kir6.2 is a weak rectifier, and allows substantial current to flow at more positive potentials.

In addition to voltage, Kir6.2 is regulated by two endogenous ligands; phosphatidylinositol 4,5-bisphosphate (PIP2) and adenine nucleotides (Figure [1.2](#ch1fig:kir_struct)). The binding of adenine nucleotides to Kir6.2 leads to closure of the channel pore, while the binding of PIP2 promotes the opening of the pore (Figure [1.3](#ch1fig:shyng_trace)). Activation by PIP2 is a mechanism common to the whole Kir family, wherease inhibition by nucleotides is unique to the Kir6 subfamily.

SUR1 is a member of the ATP-binding cassette (ABC) family of transporters. While other ABC proteins transport substrate across the membrane, SUR1 does not appear to do so; instead it acts to modulate the function of its associated ion channel. The cystic fibrosis transmembrane conductance regulator (CFTR) is another member of the ABC family, and is an ion channel in its own right, capable of conducting chloride across the membrane. Like other ABC proteins, SUR1 contains two sets of transmembrane domains (TMD1 and TMD2) and two cytosolic nucleotide binding domains (NBD1 and NBD2). Unique to SUR is the presence of an additional transmembrane domain (TMD0) N-terminal to the core of the protein, and this domain forms the primary contact between SUR1 and Kir6.2.

The NBDs of ABC transporters are highly conserved, and consist of two subdomains: a larger RecA-like subdomain found in other P-loop ATPases, and a smaller a-helical subdomain which is unique to ABC transporters. There are three key structural motifs present in these subdomains: the RecA-like subdomain contains the Walker A (WA) and B (WB) motifs, while the a-helical subdomain contains the ABC signature motif (typically LSGGQ).

The two domains come together to form an antiparallel dimer with two nucleotide binding sites (NBS1 and NBS2) at the interface, such that NBS1 is formed from the WA and WB motifs of NBD1 and the signature motif from NBD2, whereas NBS2 is formed from the WA and WB motifs of NBD2 and the signature motif from NBD1. NBS2, also known as the consensus site as it is more similar in sequence to other ABC family members, is catalytically competent and able to hydrolyse ATP. In contrast, NBS1 is the degenerate site, with a less conserved sequence and an inability to catalyse hydrolysis of ATP.

## Ligand-independent regulation of the pancreatic KATP channel

### Assembly and trafficking

Biogenesis of KATP channels occurs in the endoplasmic reticulum (ER), and is an important checkpoint in determining surface expression and channel stoichiometry (Zerangue et al. 1999a; Gregory M. Martin et al. 2013). The precise nature of the events which occur between subunit translation and insertion of octameric KATP into the cell membrane are not fully mapped out, but studies have highlighted some important quality control steps in this process which regulate KATP channel expression. When Kir6.2 or SUR1 are expressed alone in heterologous systems, they are retained in the ER (Zerangue et al. 1999a). This mechanism is achieved through the exposure of a three amino acid ER-retention motif (RKR) in the cytoplasmic domains of both Kir6.2 and SUR1. Only upon complete assembly of the channel complex are the RKR motifs masked, allowing forward trafficking of KATP to the cell surface. Deletion of the RKR motif (Tucker et al. 1997), or mutation of the motif to AAA (Zerangue et al. 1999a), results in unregulated surface expression of individual subunits and/or partially assembled channel complexes. Addition of a GFP label to the C-terminus of Kir6.2 is also sufficient to allow trafficking of subunits to the cell surface in the absence of SUR1 (Scott A. John et al. 1998).

In addition to the RKR motif, there are two N-linked glycosylation sites on SUR1 (N10 and N1050) which are required for cell surface expression (Conti, Radeke, and Vandenberg 2002). Mutation of these sites to glutamines results in retention in the ER and drastically reduced expression of KATP on the cell surface. This mechanism is thought to be separate to that for the ER-retention motif, as mutation of RKR to AAA is not sufficient to drive surface expression of the glycosylation mutants (Conti, Radeke, and Vandenberg 2002).

A putatitive third site of trafficking regulation is in the C-terminus of SUR1. Mutation or deletion of a dileucine motif 16 amino acids distal to the C-terminal of SUR1 results in reduced surface expression of KATP channels in COSm6 cells (Sharma et al. 1999). This reduction in expression is not rescued by C-terminal truncation of Kir6.2, indicating that this result is not due to masking of the RKR retention motif. The dileucines are therefore suggested to promote forward trafficking of assembled channel complexes to the cell membrane (Sharma et al. 1999). Expression of KATP channels expressed in *Xenopus* oocytes is also dramatically reduced by truncation of the C-terminal 42 amino acids of SUR1 (Vedovato et al. 2018). However, longer deletions of the SUR1 C-terminus did not reduce surface expression of channels in HEK293 cells (Giblin, Quinn, and Tinker 2002), and other modifications of the SUR1 C-terminus do not exhibit effects on surface expression (Schwappach et al. 2000). The precise role of the dileucine motif remains unclear, and is potentially confounded by the use of expression system (Giblin, Quinn, and Tinker 2002; Gregory M. Martin et al. 2013)

Failure of the channel complex to pass these three checkpoints results in ER-associated degradation (ERAD), a common pathway shared by most membrane and secretory proteins (Bonifacino and Weissman 1998; Fei-Fei Yan et al. 2005). Both SUR1 and Kir6.2 are substrates for polyubiquitination, both when heterologously expressed and in INS-1 cells (Fei-Fei Yan et al. 2005). Application of proteasome inhibitors both reduces the rate of degradation for Kir6.2 and SUR1, and increases the surface expression of KATP channels by increasing their biogenesis efficiency (Fei-Fei Yan et al. 2005).

The surface expression of KATP channels is therefore controlled by a variety of different quality control mechanism to ensure that only correctly assembled octameric channel complex reach the cell membrane. Mutations which lead to defects in assembly and trafficking are therefore a common cause of congential hyperinsulinemia (HI). These mutations are found throughout both Kir6.2 and SUR1, although they are more commonly found in SUR1 (Gregory M. Martin et al. 2013).

Interestingly, sulphonylureas are able to act as pharmacological chaperones and rescue surface expression of several mutations which would otherwise not traffic to the cell surface (F. Yan et al. 2004; Fei-Fei Yan, Casey, and Shyng 2006; F. F. Yan et al. 2007; Fei-Fei Yan et al. 2007; Gregory M. Martin et al. 2016). Sulphonylureas bind directly to the channel during biogenesis, as mutation of residues in SUR1 which are critical for sulphonylurea binding abolished or reduced the effectiveness of expression rescue (Fei-Fei Yan, Casey, and Shyng 2006). Pharmacological chaperoning requires full assembly of the channel complex, as the presence of Kir6.2 was required to rescue expression of trafficking mutants even when the SUR1 RKR motif was mutated to AAA (Fei-Fei Yan, Casey, and Shyng 2006). In addition, reducing the temperature at which cells are cultured can rescue some trafficking defects (K. Yang et al. 2005).

### Regulation of intrinsic gating

In the absence of nucleotides, KATP channels are spontaneously active. This can be seen at a macroscopic level in excised patches. Upon excision of a patch from a cell membrane containing KATP channels, the magnitude of current dramatically increases when voltage is applied (Figure [1.3](#ch1fig:shyng_trace)), reflecting the relief from inhibition of cytoplasmic nucleotides. While this macroscopic time course is smooth and graded, it consists of hundreds or thousands of individual channels which exhibit binary behaviour; switching between a nonconducting closed state and a conducting open state [[hille\_ionic\_nodate]](#hille_ionic_nodate). The summed activity of these individual channels constitutes the large currents observed in macroscopic excised patches.

Single KATP channels exhibit bursts of brief openings, separated by long interburst closures (Alexey E. Alekseev, Brady, and Terzic 1998; Andrey P. Babenko, Gonzalez, and Bryan 1999b; L. Li, Geng, and Drain 2002; Proks and Ashcroft 2009). Thus, the open probability () of the channel is determined both by the kinetics of the bursting openings (open and closed durations within a burst) and the duration of the long interburst closures. The intrinsic gating of KATP can therefore be seperated into two separate ’gating’ processes; fast (responsible for intraburst closures) and slow (responsible for interburst closures). While it is helpful to distinguish between fast and slow gating processes to characterise channel regulation, doing so does not require the existence of seperate structural gates (Proks and Ashcroft 2009; Hille, n.d.).

Gating is a property intrinsic to Kir6.2, which is able to open and close in the absence of SUR1 (Tucker et al. 1997; Enkvetchakul et al. 2000b) (Figure [1.9](#ch1fig:singles_sur)). The open and intraburst closed time of single channels is voltage dependent, with increasing hyperpolarisation decreasing the amount of time channels remain in the open state and increasing the amount of time channels remain in the closed state within bursts (A. E. Alekseev et al. 1997; Stefan Trapp et al. 1998). This is a characteristic feature shared by other inwardly-rectifying K+ channels (Sakmann and Trube 1984; A. E. Alekseev et al. 1997). In addition, KATP fast gating is dependent on the K+ gradient across the membrane (Zilberter et al. 1988; Benz, Haverkampf, and Kohlhardt 1998). Benz, Haverkampf, and Kohlhardt (1998) unified these findings and proposed that it is the electrochemical gradient, or K+ driving force, which regulates the fast gating kinetics.

There are a number of domains within Kir6.2 that regulate the intrinsic gating of the channel. Firstly, the P-loop is a conserved feature across K+ channels (amongst others) (Kuang, Purhonen, and Hebert 2015). In Kir channels, the P-loop connects the two transmembrane domains, and dips into the plasma membrane to form the K+ selectivity filter. While the P-loop is broadly conserved between Kir family members, there are key residues which differ. One particularly interesting residue is V127, which is unique to Kir6.1 and Kir6.2 - all other Kir channels posess a tyrosine at this location. Mutation of this residue to the conserved tyrosine (V127T) dramatically increases the open time of KATP, while also increasing the intraburst closed time (Proks et al. 2001). There is also some suggestion of an additional open state existing in this mutant construct, evidenced by the appearance of a second peak in the open time histograms. In addition, substitutions at other residues in the P-loop of Kir6.2 lead to a range of effects on the intraburst kinetics of KATP. Crucially, none of the substitutions affected the slow gating of the channel; i.e. burst duration and interburst closed times remained similar despite the varied alterations in the intraburst kinetics. Proks et al. (2001) concluded that the P-loop is instrumental in regulating the fast gating of KATP, and suggested that the lack of correlation between perturbations of inter- and intra-burst kinetics is evidence for independence between the fast and slow gating processes.

Other domains of Kir6.2 are involved in the regulation of slow gating. The cytosolic end of the second transmembrane domain of Kir6.2 has been implicated in regulation of KATP slow gating by a number of mutational studies (Shyng, Ferrigni, and Nichols 1997a; Tucker et al. 1998; Stefan Trapp et al. 1998; Loussouarn et al. 2000). Substitution of C166 with a more bulky or hydrophobic residue dramatically reduces the frequency of the channel entering the long, closed interburst state, and increases the open time of the channel in the bursts (Stefan Trapp et al. 1998). However, no effect is seen on the length of the intraburst closed times, which is additional evidence for the independence of the fast and slow gating processes. Substitutions at N160 (Shyng, Ferrigni, and Nichols 1997a), I167, and T171 (Tucker et al. 1998) also increase channel open time and decrease the rate of entry into the interburst closed state, further implicating this region of Kir6.2 in modulating the slow gating of KATP.

While Kir6.2 is able to gate intrinsically when expressed alone, coassembly with SUR1 alters the intrinsic gating of the channel in a number of ways. Compared to single channel kinetics of Kir6.2dC or Kir6.2-GFP alone, coexpression of Kir6.2 with SUR1 increases the open time of the channel within the bursting openings, and increases the duration of the bursts, while the intraburst closed times are unaffected (Stefan Trapp et al. 1998; Scott A. John et al. 1998; Kim W. Chan, Zhang, and Logothetis 2003). This suggests that interactions of SUR1 with Kir6.2 serve to regulate the slow gating of the channel, rather than the fast gating. The mechanisms by which SUR1 regulates intrinsic gating of the KATP channel are complex and not yet fully understood. Structurally, the primary contacts between the two subunits are formed between the N-terminus and first transmembrane domain of Kir6.2 and TMD0 and L0 of SUR1 (Figure [1.4](#ch1fig:sur_struct)) (G. M. Martin et al. 2017; Kenneth Pak Kin Lee, Chen, and MacKinnon 2017; N. Li et al. 2017a). The contributions of the interactions of these regions have been studied in a variety of ways.

Andrey P. Babenko, Gonzalez, and Bryan (1999b) constructed a series of SUR1/SUR2A chimeras and characterised the changes in single channel kinetics that resulted from swapping different domains between the two isoforms of SUR. They found that Kir6.2+SUR2A channels exhibited a far higher single channel than Kir6.2+SUR1 channels (0.91 and 0.64 respectively). This difference could be attributed to increased burst durations and decreased interburst periods, while fast gating is indistinguishable. They found that a chimerical construct replacing the N-terminal 291 amino acids of SUR1 with those of SUR2A was sufficient to recapitulate the single channel kinetics of full-length SUR2A, suggesting that this region is critical for specifying the intrinsic gating of KATP.

Later work established that truncations of SUR1 to TMD0 or TMD0-L0 fragements allowed expression of "mini-KATP" channels at the cell membrane (A. P. Babenko and Bryan 2003; K. W. Chan, Zhang, and Logothetis 2003; Fang, Csanady, and Chan 2006). The first two studies showed that expression of Kir6.2 with TMD0 alone (residues 1-195 or 1-196 of SUR1) essentially recapitulates the intrinsic gating characteristics of Kir6.2 expressed with full-length SUR1, restoring the increased open time duration and burst duration as compared to expression of Kir6.2 alone (A. P. Babenko and Bryan 2003; K. W. Chan, Zhang, and Logothetis 2003). Fang, Csanady, and Chan (2006) later found that in their hands, mini-KATP channels formed from Kir6.2dC and SUR1-TMD0 were similar to full-length KATP but they consistently observed differences in the burst durations. This discrepency may be, at least in part, due to differences in the heterologous expression system (COSm6 cells in (A. P. Babenko and Bryan 2003), *Xenopus* oocytes in (Fang, Csanady, and Chan 2006)). Otherwise, the remaining difference between KATP and mini-KATP channels could either be due to differences in structural interactions due to the truncation, or could implicate a role for the ABC core domain in regulating slow gating (Fang, Csanady, and Chan 2006).

Increasing the length of the SUR1 fragment to include the first section of the L0 linker (residues 1-232 of SUR1) results in a nearly constitutively open channel, with dramatically increased open time duration and few observable interburst closures (A. P. Babenko and Bryan 2003). The resulting of 0.93 reflects a near saturation of the slow gating process; as without changes to the fast gating there can be limited further increases in due to the flickery closure. Increasing the length of the L0 linker included in the SUR1 truncation fragment results in a progressive decrease in the open time duration, burst length and , although it never regresses to the kinetics observed in Kir6.2 expressed alone (A. P. Babenko and Bryan 2003). These findings suggest that while the TMD0 and the initial segment of L0 help to stabilise the open state of KATP channels, sections of the L0 linker act to destabilise the open state in some fashion (A. P. Babenko and Bryan 2003; M. C. Puljung 2018).

One hypothesis for this destablisation is that parts of the L0 linker interact with the N-terminus of Kir6.2 to regulate intrinsic gating of KATP channels (Koster et al. 1999; Andrey P. Babenko, Gonzalez, and Bryan 1999a; Reimann et al. 1999; Andrey P. Babenko and Bryan 2002). When Kir6.2dC is expressed alone, deletion of the first 14 amino acids of the N-terminus of Kir6.2 does not affect single channel kinetics (Reimann et al. 1999). However, in the presence of SUR1, truncations of up to the first 44 amino acids of the N-terminus reduces the frequency of transitions to the long closed state, increasing the (Reimann et al. 1999; Koster et al. 1999; Andrey P. Babenko, Gonzalez, and Bryan 1999a). This effect increases with progressive truncations from dN4 to dN30, but increasing the truncation past this point does not appear to have additional effects. Application of a synthetic peptide which contains the first 33 amino acids of the N-terminus of Kir6.2 to full-length KATP channels decreases the frequency of transitions to the closed state, in a manner comparable to truncation of the N-terminus (Andrey P. Babenko and Bryan 2002). This effect was dependent on the presence of SUR1, as with the N-terminal truncation experiments. This finding suggests that the synthetic peptide competes with the endogenous N-terminal of Kir6.2 for an interaction within the KATP channel complex. Together, these results provide evidence for interactions between SUR1 and the N-terminal of Kir6.2 which facilitate transitions to the long closed state of the channel (A. P. Babenko and Bryan 2003).

Of course, when measuring currents from hundreds or thousands of KATP channels, it is not possible to distinguish between perturbations which alter fast gating and perturbations which alter slow gating; the current measured reflects the sum of both of these processes. At a macroscopic level, anything which increases single channel open time or burst duration, or decreases the intraburst closed time or frequency of entering the interburst state will appear as an increase in .

## Ligand dependent regulation of the pancreatic KATP channel

KATP channels are regulated by two classes of endogenous ligands (nucleotides and phosphoinositides) and a range of exogenous ligands (predominantly sulphonylureas and glinides) (Figure [[ch1fig:regulation\_diagram]](#ch1fig:regulation_diagram)). Thus far, the action of each of these ligands appears to exclusively affect the slow gating of channel (Proks and Ashcroft 2009). While the binding of adenine nucleotides to the Kir6.2 binding site leads to closure of the pore, binding of nucleotides to the NBSs of SUR1 in the presence of Mg2+ activates the channel (Vedovato, Ashcroft, and Puljung 2015). The interplay between the action of nucleotides at these distinct sites (Figure [1.8](#ch1fig:sur_ctd)) determines the response of the KATP channel to metabolic changes, and therefore even subtle mutations or modifications to these sites can lead to diseases of insulin secretion. In addition to allowing activation of the channel by Mg-nucleotides, proper assembly of Kir6.2 and SUR1 allows for highly sensitive inhibition of currents by sulphonylureas and glinides (Fiona M. Gribble, Tucker, and Ashcroft 1997a; Frances M. Ashcroft 2010).

### Nucleotide regulation of the pancreatic KATP channel

The physiological regulation of channel activity by nucleotides is the summed contribution of activation by Mg-nucleotides binding to the NBSs of SUR1, and inhibition by nucleotides binding to Kir6.2. To study these contributions experimentally, most research to date has relied on electrophysiological recordings of KATP currents. Separating the contributions of the different classes of site has been achieved throug a variety of methods. Firstly, activation of the channel by Mg-nucleotides can be eliminated by removing Mg2+ ions from the solutions used to perfuse excised patches (Fiona M. Gribble et al. 1998; Proks, Wet, and Ashcroft 2010).

### PIP2 regulation of the pancreatic KATP channel

### Sulphonylurea regulation of the pancreatic KATP channel

## Fluorescence methods in ion channel research

### Fluorescence as a tool

### Forster resonance energy transfer

### Unnatural amino acid incorporation

## Functional modelling of ion channels

### Why?

### A model that fits

# [ch:2-methods]Methods

## Molecular biology.

Human Kir6.2 and SUR1 were subcloned into pcDNA4/TO and pCGFP\_EU vectors for expression of wild-type and GFP-tagged constructs, respectively. pcDNA4/TO and pANAP were obtained from Addgene. peRF1-E55D and pCGFP\_EU were kind gifts from the Chin Laboratory (MRC Laboratory of Molecular Biology, Cambridge, UK) and the Gouaux Laboratory (Vollum Institute, Oregon, USA) respectively. Amber stop codons and point mutations were introduced using the QuikChange XL system (Stratagene; San Diego, CA). All constructs were confirmed by DNA sequencing (DNA Sequencing and Services, University of Dundee, Scotland).

## Cell culture and channel expression

HEK-293T cells were obtained from and verified/tested for mycoplasma by LGC standards (ATTC CRL-3216, Middlesex, UK). Our working stock tested negative for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza Bioscience; Burton on Trent, UK). Cells were plated onto either poly-L-lysine coated borosilicate glass coverslips (VWR International; Radnor, PA) or poly-D-lysine coated glass-bottomed FluoroDishes (FD35-PDL-100, World Precision Instruments). ANAP-tagged Kir6.2 constructs were labelled using amber stop codon suppression as described by Chatterjee et al. Transfections were carried out 24 hours after plating using TransIT-LT1 (Mirus Bio LLC; Madison, WI) at a ratio of 3 μl per μg of DNA. Unless specified otherwise, all transfections included a Kir6.2 construct with an amber stop codon (TAG) at position 311 (Kir6.2-W311TAG), SUR1, pANAP and eRF1-E55D in the ratio 0.5:1.5:1:1. Transfected cells cultured in Dulbecco’s Modified Eagle Medium (Sigma; St. Louis, MO) + 10% foetal bovine serum, 100 /ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific; Waltham, MA) supplemented with 20 m ANAP (free acid, AsisChem; Waltham, MA). Cells were incubated at 33 °C and in the presence of 300 μ tolbutamide to enhance protein expression and channel trafficking to the plasma membrane. eRF1-E55D was included to increase efficiency of ANAP incorporation. Experiments were carried out 2-4 days after transfection. We also expressed constructs labelled with ANAP at positions I182, F183, F198, and I210. Kir6.2-F183\*, Kir6.2-F198\*, and Kir6.2-I210\* co-expressed with SUR1 did not produce sufficient currents for subsequent experimentation. Mutations at I182 are known to produce profound effects on nucleotide inhibition of KATP. Thus, we did not consider this site for further experimentation.

## Western blots

Transfected HEK-293T cells grown in 6-well plates were harvested in cold PBS (Life Technologies Limited; Paisley, UK), pelleted at 0.2 x g for 2.5 minutes and resuspended in lysis buffer containing 0.5% Triton X-100, 100 m potassium acetate, and a cOmplete protease inhibitor tablet (1 tablet/50 ml, Roche; Basel, Switzerland), buffered to pH 7.4. After a 30-minute benzonase (Sigma) treatment at room temperature, samples were mixed with a DTT containing reducing agent and loading buffer (NuPAGE, Invitrogen; Carlsbad, CA) and run on a precast Bis-Tris 4-12% poly-acrylamide gel at 200 V for 40 minutes. Proteins were wet transferred overnight onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Merck Millipore; Burlington, VT) in 25 m Tris, 192 m glycine, 20% methanol, and 0.1% SDS at 10 V on ice. Membranes were blocked with 5% milk in TBS-Tw (150 m NaCl, 0.05% Tween 20, 25 m Tris, pH 7.2) before staining for 30 minutes with a 1:1000 dilution of rat anti-HA monoclonal antibody in TBS-Tw (clone 3F10, Roche). After washing with TBS-Tw, membranes were incubated for 30 minutes with a 1:20,000 dilution of HRP-conjugated goat anti-rat polyclonal antibodies in TBS-Tw (Jackson ImmunoResearch; Ely, UK). Detection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher) and a C-DiGit Blot Scanner (Licor Biosciences; Lincoln, NE). Analysis was performed using custom code written in Python.

To confirm our ability to express full-length Kir6.2\*-GFP, we performed western blots for HA-tagged Kir6.2 constructs in detergent-solubilized HEK-293T cells (Figure 1—Figure supplement 1C). The HA tag plus a short linker (YAYMEKGITDLAYPYDVPDY) was inserted in the extracellular region following helix M1 of Kir6.2 between L100 and A101. Transfection of wild-type Kir6.2-HA or Kir6.2-HA-GFP resulted in two bands on the western blots. The upper bands were close to the expected sizes for full-length Kir6.2-HA and Kir6.2-HA-GFP (46 kDa and 77 kDa, respectively).

We consistently observed a lower molecular weight band as well. This band must correspond to an N-terminally truncated Kir6.2 product, as the apparent molecular weight shifted with addition of the C-terminal GFP tag. Based on the molecular weight, we predict that the truncated protein product initiated from a start codon in the first transmembrane domain. Therefore, we believe it is unlikely that this protein would form functional channels or traffic to the plasma membrane. When Kir6.2-W311TAG-HA or Kir6.2-W311TAG-HA-GFP were co-transfected with SUR1, pANAP, and eRF1-E55D, and cells were cultured in the presence of ANAP, the western blots were similar to wild-type Kir6.2-HA or Kir6.2-HA-GFP. Over 90% full-length Kir6.2\*-HA-GFP was produced under these conditions (Figure 1—Figure supplement 1D). We were unable to quantify the percentage of full-length Kir6.2\*-HA produced as the C-terminally truncated band resulting from termination at the TAG codon was very similar in size to the N-terminally truncated band. Co-expression with SUR1 increased the percentage of full-length Kir6.2\*-HA-GFP produced (Figure 1—Figure supplement 1D). In the absence of ANAP, we did not observe any full-length Kir6.2, indicating that there was no read-through of the amber (TAG) stop codon (Figure 1—Figure supplement 1D).

## Confocal microscopy

Confocal imaging was performed using a spinning-disk system (Ultra-VIEW VoX, PerkinElmer; Waltham, MA) mounted on an IX81 microscope (Olympus; Southend-on-Sea, UK) with a Plan Apo 60x oil immersion objective (NA = 1.4), provided by the Micron Advanced Bioimaging Unit, Oxford. Transfected HEK-293T cells were incubated for 15 minutes with 1 n CellMask Deep Red (Thermo Fisher) to stain plasma membranes before washing with PBS and imaging. ANAP was excited with a solid-state laser at 405 n. GFP and CellMask were excited with an argon laser at 488 n and 633 n respectively. Images were captured on an EMCCD camera (ImagEM; Hamamatsu Photonics; Welwyn Garden City, UK) binned at 2 x 2 pixels and analysed using Python. A median filter with a box size of 32 x 32 pixels was applied to improve the signal-to-noise ratio by reducing background fluorescence.

We examined the surface expression of our ANAP-labelled constructs using confocal microscopy (Figure 1—Figure supplement 1A,B). When Kir6.2-W311TAG-GFP was co-transfected with SUR1 along with pANAP and eRF1-E55D in the presence of ANAP, the ANAP and GFP fluorescence were co-localized at the plasma membrane. When wild-type Kir6.2-GFP was transfected under the same conditions, only GFP fluorescence was observed at the plasma membrane. ANAP fluorescence was diffuse and confined to the cytoplasm or intracellular structures. Thus, the plasma-membrane ANAP signal was specific for Kir6.2\*-GFP.

## Surface expression assays

We measured surface expression of HA-tagged Kir6.2 subunits using an approach outlined by Zerangue et al. Cells were plated on 19 mm coverslips coated with poly-L-lysine and transfected as described above. Following incubation, cells were rinsed with PBS before fixation with 10% formalin for 30 minutes at room temperature. After washing again, cells were blocked with 1% BSA in PBS for 30 minutes at 4 °C before a 1-hour incubation at 4 °C with a 1:1000 dilution (in PBS) of rat anti-HA monoclonal antibodies. Cells were then washed 5 times on ice with 1% BSA in PBS followed by a 30-minute incubation at 4 °C with a 1:2000 dilution of HRP-conjugated goat anti-rat polyclonal antibodies. Cells were washed 5 times in PBS + 1% BSA and 4 times in PBS. Coverslips were removed from the culture dishes and placed in clean, untreated dishes for measurement. 300 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher) was added to each sample and the luminescence was measured using a Glomax 20/20 Luminometer (Promega; Madison, WI) after a 10 second incubation.

HEK-293T cells were transfected with Kir6.2 constructs with or without a TAG stop codon corresponding to position 311. Cells were co-transfected with pANAP and eRF1-E55D in the presence or absence of SUR1 and cultured with or without ANAP. Wild-type Kir6.2-HA and Kir6.2-HA-GFP in the presence of SUR1 were included as positive controls. Kir6.2 constructs with no HA tag served as negative controls. In the presence of ANAP, we observed strong trafficking of Kir6.2\*-HA-GFP to the plasma membrane, but much less trafficking of Kir6.2\*-HA (Figure 1—Figure supplement 1E). When cells were cultured in the absence of ANAP, we observed little to no Kir6.2 surface expression from cells that were transfected with Kir6.2-W311TAG-HA or Kir6.2-W311TAG-HA-GFP, suggesting that prematurely truncated constructs did not traffic to the plasma membrane. In the absence of SUR1, surface expression was weak for both wild-type and tagged constructs, despite the reported ability of Kir6.2-GFP to traffic to the plasma membrane in the absence of SUR1.

## Epifluorescence imaging and spectroscopy

Epifluorescence imaging and spectroscopy were performed using a Nikon Eclipse TE2000-U microscope with a 60x water immersion objective (Plan Apo VC, NA = 1.2, Nikon; Kingston upon Thames, UK) or a 100x oil immersion objective (Nikon, Apo TIRF, NA = 1.49). Imaging of ANAP was performed using a 385 nm LED source (ThorLabs; Newton, NJ) with a 390/18 nm band-pass excitation filter, an MD416 dichroic and a 479/40 nm band-pass emission filter (all from ThorLabs). GFP was imaged using a 490 nm LED source (ThorLabs) with a 480/40 nm band-pass excitation filter, a DM505 dichroic, and a 510 nm long-pass emission filter (all from Chroma; Bellows Falls, VT). Fluorescence spectra were collected by exciting ANAP as above but using a 400 nm long-pass emission filter (ThorLabs), then passing emitted light through an IsoPlane 160 Spectrometer (Princeton Instruments; Trenton, NJ) with a 300 g/mm grating. Images were collected with 1 s exposures on a Pixis 400BR\_eXcelon CCD (Princeton Instruments).

## Electrophysiology.

Patch pipettes were pulled from thick-walled borosilicate glass capillaries (GC150F-15, Harvard Apparatus; Holliston, MA) to a resistance of 1.5 MΩ–2.5 MΩ when filled with pipette solution. Currents were recorded at −60 mV from excised inside-out patches using an Axopatch 200B amplifier equipped with a Digidata 1322A digitizer and using pClamp 10 software (Molecular Devices; San Jose, CA). Currents were low-pass filtered at 5 kHz and digitized at 20 kHz. The bath solution (intracellular) contained 140 m KCl, 10 m HEPES, 1 m EDTA and 1 m EGTA (pH 7.3 with KOH). The pipette solution (extracellular) contained 140 m KCl, 10 m HEPES and 1 m EDTA (pH 7.4 with KOH). All experiments were carried out in Mg2+-free conditions. Currents were leak corrected using the current remaining in bath solution containing 5 m barium acetate at 60 mV, assuming a linear leak with a reversal potential of 0 mV. Inhibition was calculated and corrected for rundown by alternating test concentrations of nucleotide solution with nucleotide-free solution, then expressing the test currents as a fraction of the average of the control currents before and after the test solution as described previously.

## FRET calculations

We calculated the expected FRET efficiency between ANAP incorporated at amino acid position 311 and a docked TNP-ATP molecule as described previously. The equivalency between FRET efficiency (measured as ANAP quenching) and nucleotide binding is based on two main assumptions. Firstly, we assume that the observed quenching from a bound nucleotide does not differ dramatically between open and closed states of the channel. As there is no open-state structure of KATP, we do not know exactly how much relative movement would occur between a bound TNP-ATP and Kir6.2-W311. However, based on cryo-EM structures of apo and nucleotide-bound Kir6.2 we do not expect to see a change in the distance between these two positions.

Secondly, we assume that the ANAP and TNP-ATP molecules on each subunit do not undergo energy transfer with those on other subunits to an extent which would dramatically change the observed quenching. At saturating TNP-ATP concentrations, where each ANAP-labelled site on Kir6.2 is occupied, FRET between ANAP and the closest acceptor will be kinetically favoured and the overall FRET efficiency will not be affected by cross-talk between neighbouring sites. In the limiting case, at low TNP-ATP concentrations, one would expect a large proportion of Kir6.2 tetramers (with four ANAP-labelled binding sites) bound to only a single TNP-ATP molecule. In this case, we expect a 4% overestimation of nucleotide binding as calculated using a numerical method to simulate a single TNP-ATP acceptor with multiple ANAP donors based on the distances calculated from our docking. This may have resulted in our binding curves becoming artifically shallow at low concentrations. However, this difference is not significant in the context of our measurements as it is smaller than the observed error of our measurements at low TNP-ATP concentrations.

## Unroofed binding measurements.

Unroofed membranes were prepared as described previously. A coverslip plated with transfected HEK-293T cells was removed from the culture media and rinsed with PBS. The coverslip was then briefly sonicated using a probe sonicator (Vibra-cell; Newtown, CT) leaving behind adherent plasma membrane fragments. Cells cultured on FluoroDishes were rinsed and sonicated directly in the dish. Unroofed membrane fragments were nearly invisible in bright-field images and identified by their GFP and ANAP fluorescence. Fluorescent TNP-nucleotides (Jena Bioscience; Jena, Germany) were diluted in bath solution and perfused onto unroofed membranes using a valve controlled microvolume superfusion system (μFlow, ALA Scientific Instruments; Farmingdale, NY).

Fluorescence spectra were collected as described above. A region of interest corresponding to the membrane fragment was manually selected and line-averaged for each wavelength. A similarly sized region of background was selected and averaged, then subtracted from the spectrum of interest. After subtraction, ANAP intensity was calculated by averaging the fluorescence intensity measured between 469.5 nm and 474.5 nm. Bleaching was corrected by fitting the normalised ANAP intensity of exposures taken during perfusion with nucleotide-free solution to a single exponential decay of the form

then using the fit to correct the intensity of exposures taken during perfusion with test nucleotide solutions.

Some experiments were excluded from further analysis due to obvious cross-contamination between different solutions within the μFlow superfusion system. These were identified by noticeable colour changes in the solution in the delivery tubes.

## Patch-clamp fluorometry.

The tip of the patch pipette was centred on the slit of the spectrometer immediately after patch excision. Currents were measured as described above. Fluorescence emission spectra from the excised patch were acquired concurrently with current measurements, both during test solution application as well as nucleotide-free solution. Background subtraction was slightly imperfect due to the exclusion of TNP-ATP from volume of the glass of the pipette, resulting in spectra that have negative intensities at the TNP-ATP peak at high nucleotide concentrations. However, this over-subtraction does not affect the size of the ANAP peak, which we used to quantify nucleotide binding.

Some experiments were excluded from further analysis due to low fluorescence intensity, as we were concerned about a low signal to noise ratio influencing our results.

## Data processing and presentation.

Raw spectrographic images and current traces were pre-processed in Python and Clampfit (Axon) before analysis with R. Where applicable, all experimental data points are displayed in each figure. The number of experiments is reported in the figure legends and tables. To help visualise uncertainty and prevent some data points being hidden, they are arranged with a small amount of horizontal jitter; vertical position remains unaffected. Unless otherwise stated, summary statistics are overlaid as the mean with error bars representing the standard error of the mean. Where these error bars are not visible, they are smaller than the size of the point used for the mean.

Hill fits to fluorescence quenching were nonlinear least-squares fits to the following equation:

where represents normalised fluorescence intensity and and are values. Current inhibition data were fit to the same equation but with representing normalised current magnitude, instead of , and instead of .

## Computational docking.

Computational docking of TNP-ATP into the nucleotide binding site of Kir6.2 was performed using AutoDock-Vina and Pymol (Schrödinger, LLC; New York, NY). 11 TNP-ATP structures from the Protein Data Bank (PDB accession #s 1I5D, 3AR7, 5NCQ, 5SVQ, 5XW6, 2GVD, 5A3S, 2PMK, and 3B5J) were used as starting poses and a 15x11.25x15 Å box was centred on the ATP bound to Kir6.2 in PDB accession #6BAA. Protonation states for each residue were assigned using PDB2PQR and PROPKA 3.0. The modal highest-scoring pose from the docking run was selected (PDB accession #5XW6) and distances were measured from a pseudo atom at the centre of the fluorescent moiety. TNP-ATP (PDB #3AR7) was positioned into the first nucleotide binding domain of SUR1 (PDB #6PZI) using the alignment tool in Pymol.

## Chemicals and stock solutions.

Unless otherwise noted, all chemicals were obtained from Sigma. TNP-ATP was obtained as a 10 m aqueous stock from Jena Bioscience and stored at −20 °C. 1 m aqueous stocks of ANAP-TFA were prepared by dissolving the free acid in 30 m NaOH, and were stored at −20 °C. Tolbutamide stocks (50 m) were prepared in 100 m KOH and stored at −20 °C.

# [ch:3]Measuring nucleotide binding to KATP

## Designing a nucleotide binding assay

### Criteria for a useful assay for nucleotide binding to Kir6.2

Previous approaches to measuring nucleotide binding directly to the different binding sites of KATP have relied on isolating binding to individual classes of site by disrupting protein function; either by introducing mutations which abolish binding to a particular site, by measuring binding to Kir6.2 or SUR1 alone, or by measuring binding to fragments of the two subunits.

Two key studies have attempted to measure nucleotide binding to the inhibitory site on Kir6.2 directly. The first relied on photoaffinity labelling of Kir6.2 by the radionucleotide 8-azido-[g-32P]-ATP (Tanabe et al. 1999). In these experiments, Kir6.2 with an N-terminal FLAG-tag was expressed in COS-7 cells, and membranes were separated by centrifugation. After incubating the membrane fractions with 8-azido-[g-32P]-ATP, application of UV light results in a covalent linkage between the bound 8-azido-[g-32P]-ATP and Kir6.2. After separation of the membrane fraction proteins on a gel, the quantity of bound radionucleotide can then by quantified by counting the radioactivity of the band corresponding to Kir6.2. These experiments were able to definitively establish that the inhibitory nucleotide binding site of KATP was on Kir6.2, and suggested that the Kir6.2 binding site possessed a lower affinity toward the radionucleotide than the SUR1 binding sites.

The second made use of a fluorescent congener for ATP, trinitrophenyl (TNP)-ATP. TNP-ATP had previously been used in binding measurements of purified proteins due to it’s increased quantum yield (and thus increase in observed fluorescence) upon binding (need a ref). TNP-ATP is most commonly used as an antogonist of P2X receptors, which are also sensitive to endogenous ATP. The authors measured binding of TNP-ATP to the purified carboxyl terminal of Kir6.2 (residues 169 to 354) solubilised by linking it to mannose binding protein (MBP) (Vanoye et al. 2002). The increased fluoresence of TNP-ATP when bound to the Kir6.2-MBP construct could be measured in a spectrometer, and allowed for equlibrium measurements of nucleotide binding. These experiments were able to establish an initial estimate for the binding affinity of the Kir6.2 site for TNP-ATP at 5 μ. These findings were replicated in a similar study, which used fusion proteins constructed from residues 170 to 390 of Kir6.2 fused to glutathione-S-transferase (GST) and estimated a binding affinity of 5 μ (Wang et al. 2006).

These studies were hampered by the need to isolate the Kir6.2 binding site from the two SUR1 binding sites, which leads to unphysiological experimental conditions. To improve on these methods, an ideal assay measuring nucleotide binding to the KATP channel neeeds to fulfill a number of criteria.

1. We need sufficient spatial sensitivity to distinguish between different classes of binding site; i.e. the assay should be capable of distingushing binding to Kir6.2 from binding to NBS1 or NBS2.
2. We should be able to measure binding to a channel which we know is functional, so our experimental conditions cannot be drastically different from those used to measure channel function.
3. There should be minimal perturbation of the channel in order for binding measurements to be physiologically relevant.
4. For accurate measures of affinity, binding should be at equilibrium so we cannot use covalent interactions or other forms of non-equilibrium labelling.
5. We should be able to achieve a higher temporal resolution.

TO fulfill these criteria, we used an approach involving a fluorescent unnatural amino acid, ANAP. ANAP has been used increasing widely in the study of ion channel structure and function due to several desirable qualities.

1. It is smaller than traditional fluorescent labels such as fluorescent proteins or rhodamine derivatives. Therefore, it should be less perturbing to the function of the protein it labels.
2. As it is an amino acid, it can be site-specifically inserted into any protein. This avoids the issues of other small chemical dyes which are targeted to a site via post-translational covalent modifications, typically by reacting with a cysteine residue. While this can be avoided in some proteins by mutating each cysteine residue to an alternative residue to avoid off-target labelling, there are functionally important cysteines in the KATP channel which cannot be mutated. In addition, this does not solve the problem of off-target labelling of other membrane proteins
3. ANAP is environmentally sensitive, which has been used to great effect in other studies. Notably, the peak emission ranges from ~450nm to ~490nm depending on the hydrophobicity of the surrounding environment.

Initially, we hoped that the environmental sensitivity of ANAP fluorescence might be sufficient for the peak fluorescence of an ANAP residue inserted into an ATP binding site to measureably change when ATP was bound. Unfortunately, when we introduced ANAP directly into the Kir6.2 binding site in place of residues I182 or F183 we were not able to observe any functional KATP channels at the cell membrane.

Instead, we turned to FRET as a reporter for ATP binding. As ATP itself is not fluorescent, and has no intrinsic fluorescence quenching, we turned to TNP-ATP (Figure [3.1](#ch3fig:chemical_structures)). TNP-ATP is an excellent FRET partner of ANAP, as evidenced by the good overlap in the TNP emission spectra and the ANAP extinction spectra (Figure [3.2](#ch3fig:spectral_overlap)). This leads to a theoretical distance-dependency of FRET which is most sensitive between 20 Å–60 Å (Figure [3.3](#ch3fig:fret_efficiency)) with a calculated R0 of 38.4 Å.

### Choosing a site to incorporate ANAP

THe theoretical R0 of 38.4 Å for FRET between ANAP and TNP-ATP allowed for flexibility when choosing a site to incorporate ANAP. Ideally, a residue should be chosen to maximise the following aims:

1. The incorporated ANAP needs to be close enough to the nucleotide binding site of interest to report a quantifiable change in FRET when TNP-ATP is bound. This would not have to be close enough for 100 % FRET to occur, but the greater the efficiency achieved the higher the signal-to-noise ratio would be for measuring binding.
2. It also needs to be far enough from each other class of nucleotide binding site to avoid quenching by TNP-ATP bound to other sites.
3. In addition to avoiding interference from other classes of binding site, we also need to avoid cross-talk between nucleotide binding sites of the same class on different subunits, as this would lead to difficulty interpreting the measured quenching. The ideal theoretical solution would be labelling only one nucleotide binding site per ion channel, but without using a concatemer this is not so easy in practise.
4. More practically, incorporation of ANAP should not lead to drastic changes in nucleotide binding or channel gating properties, and the complete KATP channel needs to be expressed on the membrane.

To narrow down which residues could be candidates for ANAP incorporation to measure binding at Kir6.2, we took three cryo-EM structures of KATP with ATP bound and computationally docked TNP-ATP into the nucleotide binding pocket (Figure [[ch3fig:docking]](#ch3fig:docking)). To assess the validity of computationally docking a ligand to each structure, we first attempted to dock ATP into the inhibitory binding pocket of Kir6.2 to check that the highest-scoring binding poses were similar to those observed in the cryo-EM structures. Docking ATP to both #6C3P and #6C3O yielded binding poses which were very similar to the pose found in the cryo-EM structures (Figures [3.5](#ch3fig:6c3p_docking), [3.6](#ch3fig:6c3o_docking)). However, docking ATP to #6BAA resulted in binding poses which were in a flipped orientation relative to the pose found in the cryo-EM structure (Figure [3.4](#ch3fig:6baa_docking)).

We then took TNP-nucleotide structures from eleven different X-ray diffraction and cryo-EM structures published on RCSB to dock to the Kir6.2 binding site of KATP. For both #6BAA and #6C3P we observed that the three highest scoring binding poses for TNP-nucleotides closely resemble those of the ATP solved in complex with the channel (Figures [3.4](#ch3fig:6baa_docking), [3.5](#ch3fig:6c3p_docking)). It is not so clear for #6C3O, for which the highest scoring poses are not in agreement with each other or the solved structure of ATP.

Based on the predicted TNP-ATP poses for #6BAA and #6C3P, we could narrow down potential ANAP incorporation sites to within 25 Å of the centre of the TNP-moiety, at which distance we would expect to see over 90 % FRET efficiency when TNP-ATP is bound to Kir6.2. In addition, we excluded residues which fell within 45 Å of NBS1 or NBS2, as this restricts the potential FRET between TNP-ATP bound at these sites and our chosen residue to roughly 25 % or less. While we can exclude residues which fall too close to the NBS’s of SUR1, the close proximity of the Kir6.2 nucleotide binding sites to each other means that we cannot exclude intersubunit FRET occuring; i.e. TNP-ATP binding to a neighbouring subunit will also be able to quench ANAP to a certain extent. However, this occurs in a predictable way that we can measure and account for.

We ended up with one residue which fulfilled these criteria and for which membrane expression of the ANAP-incorporated channel could be detected: W311. It is a bulky hydrophobic residue similar to ANAP, and no mutations at this residue have been previously identified to alter KATP function. To determine the extent to which crosstalk between neighbouring subunits would affect the measure FRET efficiency when ANAP is incorporated at position 311, we simulated the expected FRET for a single KATP channel bound to 0-4 molecules of TNP-ATP in an idealised scenario with no crosstalk, and compared the observed fluorescence quenching to a scenario including the possibility of crosstalk (Figure [3.9](#ch3fig:exifret_out)). Due to the much shorter fluorescence lifetime of TNP-ATP compared to ANAP, we observe a deviation from the ideal scenario. We can correct for this systematic deviation by transforming our observed ANAP quenching () into .

## Incorporating ANAP into the Kir6.2 binding site

### Amber stop codon expression system

ANAP can be introduced into a protein of interest by essentially expanding the genetic code to incorporate a noncanconical amino acid. The amber stop codon (TAG) is the least frequently occuring stop codon in eukaryotic cells, and can be repurposed to encode ANAP. An orthologous set of tRNA and its aminoacyl-tRNA synthetase are mutated and screened through directed evolution in order to charge the tRNA which recognises the amber stop codon with ANAP. Thus, in cells expressing the ANAP charged tRNA, the amber stop codon introduced into the protein of interest is suppressed and ANAP is translated instead (Figure [3.10](#ch3fig:amber_codon)).

### ANAP incorporation into amber stop codon containing constructs

The nature of the amber stop codon suppression system requires a number of careful controls to ensure the following:

1. Stop codon recognition is not perfect, and there is a chance of read-through. Instead of incorporating ANAP, it is possible that the translation machinery can insert endogenous amino acids instead, leading to production of full length,unlabelled Kir6.2. However, we found that cells transfected with W311TAG constructs and pANAP which were not cultured in the presence of ANAP did not produce full length Kir6.2 (Figure [3.11](#ch3fig:western_1), [3.12](#ch3fig:western_2)), suggesting there is minimal read-through of the stop codon in our experiments.
2. Introducing a stop codon creates a risk that truncated Kir6.2 will be produced instead of ANAP labelled Kir6.2. This risk can be reduced by transfecting a dominant negative engineered version of eukaryotic translation termination factor 1(eRF1-E55D), which does not efficiently terminate protein synthesis in response to the amber stop codon (but leaves opal and ochre stop codons nearly unaffected) and thus increases the incorporation of ANAP. We found that transfection of W311TAG constructs with a C-terminal GFP tag produced minimal truncated Kir6.2 (less than 10 % of the total density observed in Figure [3.12](#ch3fig:western_2)).
3. Despite being the least frequent eukaryotic stop codon, the amber stop codon is still present in a significant number of proetin sequences. We must be careful that ANAP is not incorporated into a protein which localises to the plasma membrane to an extent which would affect our ability to assign ANAP fluorescence to Kir6.2. We found that in cells transfected with GFP-tagged Kir6.2 without an amber stop codon, there was no increase in ANAP fluorescence at the cell membrane (Figure [3.13](#ch3fig:wt_confocal), [3.14](#ch3fig:wt_confocal_profiles)). By contrast, when W311TAG-GFP was transfected, we saw a clear increase in ANAP fluorescence at the cell membrane (Figure [3.15](#ch3fig:w311_confocal), [3.16](#ch3fig:w311_confocal_profiles)), suggesting that any observed ANAP fluorescence at the cell membrane originates from our labelled Kir6.2 construct.

## Testing for functional membrane expression

### Surface expression of HA-epitope labelled Kir6.2 constructs

To assess the ability of ANAP-incorporating constructs to traffic to the plasma membrane, we used a luminence-based surface expression assay. This assay relies on the recognition of an HA-epitope introduced into an extracellular region of the protein of interest (in this case, the N-terminal region of Kir6.2) by an anti-HA primary antibody followed by an HRP-conjugated secondary antibody. The luminescence after applying HRP substrate is then proportional to the amount of protein at the plasma membrane of the cells.

We assessed the membrane expression of N-terminally HA-tagged Kir6.2 (nHA-Kir6.2) in the presence or absence of ANAP in the culture media and in the presence or absence of cotransfected SUR1. We also measured how the addition of a C-terminal GFP tag affected membrane expression under these conditions. We used untagged Kir6.2 as a control for nonspecific luminesence.

We find that for wild-type Kir6.2 (WT) there is roughly a 20-fold increase in observed luminescence when coexpressed with SUR1 over background, and roughly a 100-fold increase for the C-terminally GFP tagged Kir6.2 (WT-GFP, Figure [3.17](#ch3fig:surface_expression_1), [3.19](#ch3fig:surface_expression_3)). There is no difference in surface expression of these constructs when ANAP is present in the culture medium (Figure [3.17](#ch3fig:surface_expression_1), [3.20](#ch3fig:surface_expression_4)). When ANAP is incorporated at either residue 183 or 311 (F183\* and W311\* respectively) we see an increase in luminescence over background when coexpressed with SUR1 and with ANAP present in the culture medium (Figure [3.17](#ch3fig:surface_expression_1), [3.19](#ch3fig:surface_expression_3)). The presence of the C-terminal GFP tag increases luminescence further for both constructs, dramatically so for W311\*. However, when F183\* is transfected and ANAP is not present in the culture media we still see a similar increase in fluorescence over background when compared to the luminescence when ANAP is present (Figure [3.17](#ch3fig:surface_expression_1), [3.20](#ch3fig:surface_expression_4)), suggesting that a large proportion of the protein reaching the membrane does not have ANAP incorporated. In contrast, when W311\*-GFP is transfected with SUR1 in the presence of ANAP, we see a 10-fold increase in luminescence compared to when ANAP is not present, consitent with the majority of surface expressed protein incorporating ANAP. We also see a consistent increase in luminescence for all constructs aside from W311\* due to cotransfection with SUR1 (Figure [3.18](#ch3fig:surface_expression_2)), suggesting that the incorporation of ANAP and the addition of a C-terminal GFP tag do not affect the role of SUR1 in forming the full KATP complex and trafficking to the membrane.

### Electrophysiology of Kir6.2 constructs

To establish whether W311\*-GFP formed KATP channels with similar function to wild-type, we excised patches from cells transfected with either WT-GFP or W311\*-GFP cotransfected with SUR1. Excision was performed in Mg2+-free solution to reduce rundown and to prevent activation of the channel by nucleotides. We observed similar magnitudes of current for both WT-GFP and W311\*-GFP, and currents ran own at similar rates.

We fit our inhibition data with equation [[eq:hill]](#eq:hill) (Figure [3.21](#ch3fig:atp_inhibition_1)) as described in the methods. Briefly, our fitting procedure assumes that there is a population parameter for , and , and an additional ’random’ effect on that can differ between experiments (shown in Figure [3.23](#ch3fig:atp_inhibition_2)). Our fits result in posterior probability distributions for the population parameter shown in blue in Figure [3.25](#ch3fig:ec50_fits_1). These distributions reflect our confidence in the population parameter for the , marginalising over the random effect of different experiments. For all and values fitted this way, in the text we will report the 95 % intervals of the posterior probability distribution for the fitted population parameter.

Perfusion of ATP resulted in current inhibition with an IC50 of 24 μ–45 μ for WT-GFP+SUR1 and 75 μ–124 μ for W311\*-GFP+SUR1. Thus, despite the distance from the ATP binding site, the incorporation of ANAP at W311 clearly affects some aspect of nucleotide inhibition. However, we assume that insights into the function of the ANAP-incorporating channel will still be applicable to wild-type channels despite the change in nucleotide inhibition.

Next, we established that TNP-ATP inhibits KATP (Figure [3.22](#ch3fig:tnpatp_inhibition_1), [3.24](#ch3fig:tnpatp_inhibition_2)). We observed current inhibition with an IC50 of 0.7 μ–1.8 μ for WT-GFP+SUR1 and 2.9 μ–10 μ for W311\*-GFP+SUR1. KATP thus appears to be more sensitive to inhibition by TNP-ATP than by ATP. This could potentially be due to extra contacts made by the TNP moiety with Kir6.2, seen in our computational docking (Figure [[ch3fig:docking]](#ch3fig:docking)).

### Unroofed membrane binding assay of Kir6.2 constructs

We then directly measured nucleotide binding to W311\*-GFP in unroofed membranes. Briefly sonicating transfected cells adhered to coverslips results in the lower membrane of the cell remaining stuck to the coverslip while the rest of the cell contents is disrupted and perfused away. This leaves the cytoplasmic domains of expressed KATP channels open to perfusion of TNP-ATP. These patches of membrane are barely visible under brightfield illumination, but due to the presence of the C-terminal GFP tag and the incorporated ANAP, we can see patches of membrane expressing KATP channels light up when we excite either fluorophore (Figure [3.26](#ch3fig:unroofed_images)). By measuring the fluorescence spectra of patches of unroofed membrane, we can separate the fluorescence emission peaks of the C-terminal GFP tag and the incorporated ANAP (Figure [3.27](#ch3fig:unroofed_spectral_images)). The peak at 472 nm corresponds to ANAP emission, while the peak at 508 nm corresponds to GFP emission. We observed no change in the locations of those peaks in the presence of ATP or TNP-ATP.

Perfusing TNP-ATP results in a decrease in the peak corresponding to ANAP fluorescence, and a concomittant increase in a fluorescence peak which corresponds to the TNP-ATP (Figure [3.28](#ch3fig:unroofed_spectral_traces)). This phenomenon is the fresult of FRET between TNP-ATP bound to the channel at the Kir6.2 binding site. The decrease in ANAP fluorescence is almost directly correlated to an increase in bound nucleotide. We chose to measure the decrease in ANAP fluorescence rather than the increase in TNP-ATP fluorescence or the change in the ratio of ANAP:TNP-ATP fluorescence as we know that the ANAP fluorescence is specific to the Kir6.2 binding site. Increases in TNP-ATP fluorescence could in part be due to direct excitation of TNP-ATP bound to other membrane proteins. We can plot the quenching of ANAP fluorescence as a concentration-response curve as in Figure [3.29](#ch3fig:unroofed_intensities).

Before analysis, ANAP bleaching was corrected as shown in Figure [3.30](#ch3fig:bleaching_plots_2). ANAP intensities of spectra imaged during bath perfusion in between applications of TNP-ATP were fit with Equation [[eq:bleaching]](#eq:bleaching). We found that all unroofed experiments showed bleaching well described by the single exponential fit to equation [[eq:bleaching]](#eq:bleaching). In each experiment, there was a mean proportion of 49 % ANAP fluorescence remaining by the last exposure (Figure [3.32](#ch3fig:bleaching_terms_4)), maintaining a good signal-to-noise ratio for each spectra imaged.

To equivocate ANAP quenching and nucleotide binding, first we need to adjust for the FRET between adjacent subunits of Kir6.2. The difference between the ideal 1:1 subunit/ligand FRET efficiency and the simulated experimental FRET efficiency resulted in a correction factor of .

Secondly, we need to correct for incomplete FRET due to the distance between the donor and acceptor. Based on the computational docking, we predicted a maximal FRET efficiency of 91 % when every Kir6.2 subunit is bound by TNP-ATP. Fitting our adjusted data to a Hill equation results in a maximum observed FRET efficiency of 90 %, agreeing well with our prediction. We can then normalise our observed fluorescence data by this value.

Overall, these two corrections do not dramatically alter our results (Figure [3.33](#ch3fig:tnpatp_quenching_1)), and we performed our analysis on both corrected and uncorrected results to ensure that there was no potential difference in interpration which derived from treating our data in this way.

We observed quenching of ANAP fluorescence over a concentration range of TNP-ATP similar to the range in which we observed inhibition of current in W311\*-GFP (Figure [3.34](#ch3fig:ec50_fits_3), [3.35](#ch3fig:tnpatp_quenching_2)). When fit to a Hill equation, quenching () was fit with an EC50 of 21 μ–31 μ, while the corrected binding data (adjusted ) gave an EC50 of 30 μ–45 μ.

### Patch-clamp fluorometry of Kir6.2 constructs

To ensure that the ANAP fluorescence we observe in the unroofed membranes is emitted by functional channels, we measured fluorescence quenching and current inhibition from the same excised patches (Figure [3.39](#ch3fig:atp_tnpatp_trace), [3.40](#ch3fig:atp_tnpatp_spectra_1), [3.41](#ch3fig:atp_tnpatp_spectra_2)).

This experimental paradigm leads to two complications compared to performing the measurements separately. Firstly, the number of channels in an excised patch are far smaller than the number of channels in an unroofed membrane patch. This results in a much dimmer fluorescence readout, and a lower signal-to-noise ratio. Secondly, the presence of the pipette glass in the images results in some abnormalities in the background subtraction procedure. This is not due to the glass itself, but results from the occlusion of TNP-ATP from the image surrounding the patch. This leads to oversubtraction of the background TNP-ATP spectra, leading to an apparent negative peak in our corrected images. However, we find that there is no overlap of this peak and the ANAP peak, so our fluorescence quenching measurements are unaffected by this phenomenon. We were able to correct for ANAP bleaching in the same manner as we did for unroofed membranes (Figure [[ch3fig:pcf\_bleaching]](#ch3fig:pcf_bleaching)).

Our fluoresence measurements from excised patches are right-shifted when compared to our measurements for unroofed membranes (Figure [3.42](#ch3fig:pcf_1)), with an value of 76 μ–144 μ. Our finding that the EC50 for TNP-ATP binding is right-shifted compared to the IC50 for TNP-ATP inhibition is consistent between each experimental paradigm (Figure [3.43](#ch3fig:ec50_fits_4)). This finding has implications for how exactly the binding of nucleotides to Kir6.2 leads to closure of the KATP channel pore.

## Discussion

We have demonstrated that we can measure nucleotide binding to the inhibitory nucleotide binding site of Kir6.2 in intact, functional KATP channels in their native membrane environment. Measuring binding directly in either unroofed membrane patches or in excised patches simultaneously with current recordings reveals that nucleotide binding is right-shifted compared to nucleotide inhibition; i.e. KATP channels begin to close at nucleotide concentrations where there is very little binding. This observation rules out certain models of ion channel function, which will be explored further in chapter [[ch4]](#ch4).

These findings come with some important caveats. Firstly, the introduction of ANAP into Kir6.2 at residue 311 clearly impacts nucleotide inhibition of the channel, increasing the observed values for ATP. Our analysis of nucleotide binding and inhibition is therefore predicated on this decrease in sensitivity to ATP inhibition not reflecting a disruption of the normal physiological mechanism of ATP inhibition. As all of our binding experiments are performed in the W311\* background by necessity, we hope that measurements of relative changes in binding and inhibition will still be meaningfuly interpretable as they will mirror similar relative changes in inhibition observed in the WT background.

Secondly, KATP channels are more sensitive to inhibition by TNP-ATP than by ATP. Again, this means that any conclusions we draw from experiments measuring relative changes in binding and inhibition rely on those relative changes affecting ATP binding and inhibition to a similar extent. To try and ameliorate these caveats as best as we can, where possible we have performed control experiments in the WT background with ATP to ensure that introduced mutations result in similar relative effects on nucleotide inhibition despite the background of the construct or the identitity of the nucleotide. As control experiments of this sort are not possible in unroofed membranes, where it is impossible to measure current inhibition, we have focused on patch-clamp fluorometry for constructs where expression is good enough to measure sufficient fluorescence.

# [ch:4]MWC modelling

## Modelling of ion channel function

### Restricting the subset of possible models

### Considerations for fitting a model

### Determining open probability

Noise analysis - you can get a correct answer the wrong way (Puljung).

Measuring the open probability of an ion channel is most accurately accomplished by single-channel electrophysiological recordings, which allows direct measurement of the time a channel spends in an open state. This approach does not allow for the determination of the open probability of a population of channels great than 2/3 at a time, as it becomes increasingly difficult to separate the openings of different channels in the population. Thus it would not be possible to determine single channel open probability simultaneously with nucleotide binding, as the fluorescence signal from a small number of channels would be impossible to resolve.

Another approach is noise analysis of currents from large populations of channels. The ’noise’ in noise analysis refers to current fluctuations which occur when recording from a population of ion channels due to the stochastic channel gating of individual channels. If there are a constant number of channels () which are gated independently from each other and share a homogenous open probability () and a single open conductance level (), the observed macroscopic current level can be described by equation [[eq:inpo]](#eq:inpo):

and the observed variance of the macroscopic current can be described by the variance of the binomial distribution, equation [[eq:bin\_1]](#eq:bin_1):

where the single channel current is essentially a scaling factor. If we assume that in a given recording and remain constant, and it is which changes in response to any given stimuli, then we can combine equations [[eq:inpo]](#eq:inpo) and [[eq:bin\_1]](#eq:bin_1) to yield equation [[eq:bin\_2]](#eq:bin_2):

This equation yields a parabola from to . Intuitively, there can be no variance when is exactly 0 or 1, as there will be no opening or closing events which can give rise to current fluctuations. Once and have been determined for a given experiment, the observed current magnitude can be converted into the for the population of channels by rearranging equation [[eq:inpo]](#eq:inpo) as follows:

Equation [[eq:bin\_2]](#eq:bin_2) can be fit to experimental data by calculating the variance of observed current at different current magnitudes. This calculation is not exactly trivial, and has been accomplished a number of different ways for different purposes. For channels with fast inactivation such as the NaV family, non-stationary noise analysis involves repeating a stimulus multiple times and measuring variance as the squared sum of deviations from the mean of the current magnitude calculated at the same time point across multiple stimuli, referred to in the literature as an ’isochrone’. For channels which do not inactivate, stationary noise analysis is possible, and variance can be measured as the squared sum of deviations from the mean current magnitude over a period of time for which is ’stationary’(Figure [4.1](#ch4fig:noise_example_1), [4.2](#ch4fig:noise_example_2), [4.3](#ch4fig:noise_example_3)).

Stationary noise analysis has been described for KATP channels before by a number of different researchers (Shyng, Paolo, Peter). Unfortunately, in most of the published research the exact procedure for extracting the parameters in equation [[eq:bin\_1]](#eq:bin_1) is described in the methods section, but the quality of the fits and the value of the fitted parameters besides the final calculated is not discussed. A notable exception to this rule is (Paolo), in which two findings are discussed. Firstly, fitting equation [[eq:bin\_2]](#eq:bin_2) to the mean and variance of 200 ms sections of macroscopic currents from wild-type Kir6.2+SUR2A resulted in a systematic underestimation of the single channel current . From single channel experiments, the single channel current was determined to be 4 pA, while the value obtained from fitting macroscopic currents was only 2 pA. In the case of WT-GFP+SUR1, we see a similar understimation of single channel current (Figure [4.4](#ch4fig:noise_example_fits_1), [4.5](#ch4fig:noise_example_fits_2), [4.6](#ch4fig:noise_example_fits_3)), with fits yielding estimates of 1.66 pA–2.64 pA, while measured single channel currents are at least 4 pA. This underestimate of is most likely due to an underestimate of channel current variance as increases, which could be due to two main reasons.

Firstly, the process of filtering and digitising channel currents can lead to underestimates of variance depending on the relationship between the open time of the measured channel and the cut-off frequency of the filter used. It is unlikely that this phenomenon is responsible for our findings, as the KATP mean open time duration is close to 1 ms and filtering at 5 kHz would lead to less than a 5 % underestimation of . Even if the mean open time of WT-GFP+SUR1 was closer to 0.1 ms, we would expect a 20 % reduction rather than the 50 % we actually observe. Empirically, we can use the frequency power spectrum of our measured current fluctuations to determine whether there may be high frequency channel openings we are missing (Figure [4.7](#ch4fig:spectra_converge)). For WT-GFP+SUR1, we observe that at frequencies approaching our filter cut-off at 5 kHz there is very little observed amplitude in active channels when compared to fully inhibited channels, suggesting we are not missing high frequency current fluctuations.

Secondly, an underestimation of could occur due to violations in the underlying assumptions of the binomial distribution. The first two assumptions are that and are constant throughout a recording. We know that does not change on nucleotide inhibition of KATP channels, noris it affected by PIP2 and rundown. Additionally, in excised patches it is improbable that there will be any change in during the course of a recording. The third assumption is that the channels in a patch share a homogenous , which exhibits graded changes in response to stimuli (in our case, application of nucleotide). This assumption is far harder to justify for our experimental condition, in which channel rundown due to loss of PIP2 results in a complicated mixture of channel populations with different s.

An extreme case in which channels transition between two states, one where and one where can be approximated by equation [[eq:bin\_1]](#eq:bin_1), with a channel transitioning to the state essentially considered to be no longer available to open, thus reducing . Thus, fitting the observed current-variance data with [[eq:bin\_1]](#eq:bin_1) would yield a straight line where the slope of the line is equal to . This formulation of equation [[eq:bin\_1]](#eq:bin_1) has been used successfully in the analysis of currents from CRAC channels, VSOA channels, and in the analysis of a specific cardiac KATP channel mutation. Unfortunately, in our case channel rundown does not render the KATP channel completely unable to open, with fully rundown channels displaying single channel open probabilities in the range of 0.05–0.25. Instead of each current measurement being a draw from a single binomial distribution, we are instead drawing from a mixture of binomial distributions with different s. We can demonstrate how this leads to an underestimation of by simulating a simple case where there are two populations of channels, and , one with a tenfold lower than the other.

## Implementing an MWC model

### A simple case

The simplest case of an allosteric MWC model for an ion channel is shown as Scheme I in Figure [4.13](#ch4fig:mwc_model_diagrams). This simple case assumes a channel composed of a single monomer with a single binding site for ligand . The channel is restricted to two states, open and closed. These two states exist in an equilibrium described by L, which is equivalent to . Ligand binds to the protein with a microscopic affinity constant . The ligand differentially stabilises the open and closed states by a constant . When is unity, the ligand binds equally to both states and so does not influence the conformational changes of the channel. When , the ligand preferentially stabilises the open state, while when the ligand instead preferentially stabilises the closed state.

### The role of PIP2

If we consider introducing a second ligand B which binds to a distinct site on the same monomer and does not directly interact with ligand A, we introduce the states shown in Scheme II of Figure [4.13](#ch4fig:mwc_model_diagrams). Each ligand has its own microscopic association constant ( or ) and its own preference for the open or closed states ( or ). Importantly, there is no interaction term between ligand and ligand ; the only way the binding of the ligands can impact each other is through effects on . Scheme II is therefore a restricted form of scheme III, which explicity introduces a term for local interaction () between binding sites for ligands and on the same monomer. When is unity, Scheme III becomes Scheme II. When , binding of one ligand reduces the ability of the other ligand to bind on the same monomer. When , binding of one ligand enhances the ability of the other ligand to bind on the same monomer.

To study nucleotide binding to Kir6.2, I have used Scheme I (expanded to incorporate four identical monomers) as an approximation of the KATP channel, with ligand representing nucleotides. To determine whether this approximation is appropriate, I generated data using each of the three schemes as the underlying model of channel function and then fit the generated observations to Scheme I (Figure [4.14](#ch4fig:mwc_scheme_1_fits), [4.15](#ch4fig:mwc_scheme_2_fits), [4.16](#ch4fig:mwc_scheme_3_fits)). Ten individual sets of observations were generated using the inputs shown above each figure panel as the centre of a lognormal distribution with a standard deviation of 0.25. These observations were then fit to Scheme I (as done previously throughout the thesis) and the values of the three free parameters (, and ) were estimated (Figure [4.17](#ch4fig:mwc_params_1)).

We know that Scheme I is only an approximation of nucleotide binding as it does not explicitly include PIP2. The question is, if the underlying data generating model is Scheme II which explicitly includes a second ligand, are we still able to extract meaningful parameter estimates by fitting the observed data to Scheme I? In addition,to date it remains unclear whether there is local allostery between the nucleotide and PIP2 binding sites. The existence of local allostery would mean that Scheme III, which includes an explicit term for this interaction, would best represent the true data generating model. We can show that even when Scheme II or Scheme III are the underlying data generating model, with ligand representing PIP2, we are still able to extract the true values of and by fitting the generated data to Scheme I (Figure [[ch4fig:mwc\_models]](#ch4fig:mwc_models)). Parameter choices for Scheme II and III are such that the open probability of the channel at 0 [ATP] is still 50%, equivalent to in Scheme I. I really need to redo this with the true set to instead of as that is closer to post rundown open proability...

We can also show that when Scheme I is the underlying data generating model, changes in any of the three parameters are easily identified and retrieved by fitting the observed data to Scheme I (Figure [[ch4fig:scheme\_1\_shifts]](#ch4fig:scheme_1_shifts)). This suggests that introducing mutations which directly effect any of the three parameters of this model would be easily identifiable if Scheme I was the true underlying model.

What if Scheme II or III were the underlying model? We would still expect changes in the three parameters which exist in Scheme I to be identifiable (I should probably check this), although would not represent the true unliganded open/closed equilibrium as we would be estimating an modified by the resting PIP2 concentration, , and - in this case, the estimated parameter in fact represents the ATP-unbound open/closed equilibrium.

However, it is unclear how changes in parameters which are not explicitly modelled in Scheme I will affect the generated data and the parameter estimates obtained by fitting the data to Scheme I. Figure [[ch4fig:scheme\_2\_3\_shifts]](#ch4fig:scheme_2_3_shifts) shows the results of increasing by tenfold on data generated from Scheme II (Figure [4.22](#ch4fig:scheme_2_kb_shift)) or Scheme III (Figure [4.23](#ch4fig:scheme_3_kb_shift)). The first observation of note is that the generated data closely resemble those generated from Scheme I when is increased (Figure [4.20](#ch4fig:scheme_1_l_shift)), and indeed when the parameter estimates for a tenfold shift in in Scheme II/III and tenfold shift in for Scheme I are compared (Figure [4.24](#ch4fig:mwc_params_3), right panel) are compared they appear to be similar. So far so good, as an observed increase in when fit with Scheme I would lead us to draw the correct inferences about changes in the underlying model (i.e. the open probability of the cnall has indeed increased). However, changes in are not perfectly captured by changes in when fit to scheme I. Notably, if local allostery exists between the nucleotide and PIP2 binding site - if Scheme III is the true underlying model - then fitting the observed data to Scheme I would lead us to estimate an incorrect value for (Figure [4.22](#ch4fig:scheme_2_kb_shift)). Thus, if there is local allostery between the sites, then a mutation which induces an increase in the binding affinity for PIP2 would not just increase our estimate of (which would lead to a correct inference) but it would also decrease our estimate of by a not-insignificant amount, which could lead to the incorrect inference that a mutation is causing a direct change in nucleotide binding when it is in fact causing a direct change in PIP2 binding, which through local allostery is influencing our estimates of .

# [ch:5]Nucleotide regulation of Kir6.2

## Introduction

There are variety of ways in which mutations can lead to altered KATP channel function. These can be divided into two broad categories; mutations which have a ligand-independent effect, and those which affect the ligand-dependent functions of the channel. Each of these categories can be further subdivided. Mutations which exhibit ligand-independent effects do so via two major mechanistic routes. Firstly, mutations can affect channel production, trafficking, and more generally expression of KATP at the cell membrane (Thomas, Ye, and Lightner 1996; Tornovsky et al. 2004; Gregory M. Martin et al. 2013). Secondly, mutations can lead to alterations in the open-closed equilibrium of the channel (i.e. by altering in Figure [[ch5fig:regulation\_diagram]](#ch5fig:regulation_diagram)). Reductions in the stability of the open state leads to decreased intrinsic (Shyng and Nichols 1998; Cartier et al. 2001; Marthinet et al. 2005; Lin et al. 2006), while increases in open state stability leads to increased intrinisc (Tucker et al. 1998).

Reduction in activity can be caused by either reductions in stability of the open state of the channel (i.e. a reduction in the resting ) (Shyng and Nichols 1998)), or reductions in the ability of Mg2+-nucleotides or PIP2 to stimulate channel currents (C. G. Nichols et al. 1996; Fiona M. Gribble, Tucker, and Ashcroft 1997b; Shyng, Ferrigni, and Nichols 1997b). Inactivating mutations *in vivo* result in hyperinsulinism (HI), with the reduction in KATP channel activity leading to persistent depolarisation of pancreatic b-cells and permanently increased insulin secretion (Colin G. Nichols 2006; Frances M. Ashcroft and Rorsman 2013).

Activating mutations cause gain of function by increasing the intrinsic stability of the open state of the channel, by reducing the sensitivity of the channel to inhibition by nucleotides, or both. Mutations which increase the intrinsic stability of the open state result in an increase in the resting of the channel. A distinction then has to be made between the apparent nucleotide affinity of the channel

It is possible to reduce sensitivity to ATP inhibition by two separate mechanistic routes.

The wide variety of KATP channel mutations, their functional effects and their involvement in diseases of insulin secretion are detailed across extensive reviews: (Hattersley and Ashcroft 2005; Colin G. Nichols 2006; Aguilar-Bryan and Bryan 2008; Flanagan et al. 2009; Frances M. Ashcroft and Rorsman 2012; F. M. Ashcroft and Rorsman 2013; Frances M. Ashcroft, Puljung, and Vedovato 2017; Rorsman and Ashcroft 2017; Pipatpolkai, Usher, et al. 2020)

Electrophysiological characterisation of KATP mutations tend to assign one of two roles to individual residues on Kir6.2. Firstly, if a change in the IC50 value for nucleotide inhibition is observed in the absence of a change in intrinsic (or nucleotide-independent) gating, typically measured as a change in single channel open probability (although thorough studies also tend to examine the bursting characteristics of the channel), then the residue is assumed to form part of the inhibitory nucleotide binding site but not affect transduction of binding to the channel pore. Secondly, if a change in the IC50 value for nucleotide inhibition is observed in addition to a change in intrinsic gating, the residue is assumed to regulate the relative stability of the closed and open states of the channel. The effects on nucleotide inhibition are taken to secondary to this main effect, due to the estalished preference of nucleotides for the closed state. Further interrogation of residues in this second category is very difficult using electrophysiological measures alone, as without measuirng binding of nucleotides directly it is impossible to truly separate effects on open probability from effects on binding and transduction. In this chapter, we aim to clarify the role of several residues implicated in regulating the inhibitory effect of nucleotides on Kir6.2 by measuring TNP-ATP binding directly to the inhibitory nucleotide binding site, where possible in conjunction with simultaneous current measurements.

## Nucleotide binding

### G334D abolishes nucleotide binding

Residue G334 of Kir6.2 is located in the C-terminal region (Figure [[ch5fig:g334d]](#ch5fig:g334d)) and has been hypothesised to form part of the ATP binding site since electrophysiological studies demonstrated a dramatic reduction in nucleotide sensitivity upon mutation of the residue (Drain, Li, and Wang 1998; L. Li, Geng, and Drain 2002; L. Li et al. 2005). In addition, mutation of this residue to aspartic acid (G334D) results in severe permanent neonatal diabetes mellitus (Masia, Koster, et al. 2007). This hypothesis was confirmed by the solving of cryo-EM structures of KATP in the presence of ATP, which revealed the close proximity of residue G334 to the bound ATP (K. P. K. Lee, Chen, and MacKinnon 2017; G. M. Martin et al. 2017; N. Li et al. 2017b; M. C. Puljung 2018). Mutating G334 to a total of 13 different amino acid substitutions led to a increase in the IC50 for ATP by over an order of magnitude in excised patches (L. Li et al. 2005). However, only two of those substitutions (R and K) resulted in any changes in nucleotide-independent channel gating when examined at the single-channel level, with unliganded remaining constant. It has therefore been suggested that while G334 forms part of the ATP binding site of Kir6.2, it does not participate in channel gating or transduction of ligand binding to the channel pore.

We sought to test this directly by measuring the binding of TNP-ATP in unroofed membranes to W311\*,G334D-GFP+SUR1. Fluorescence spectra captured from unroofed membrane patches expressing W311\*,G334D-GFP+SUR1 were indistinguishable from those expressing W311\*-GFP+SUR1. The location of the ANAP peak and the bleaching characteristics were also identical. We found that ANAP fluorescence from W311\*,G334D-GFP+SUR1 was barely quenched by even 1 m TNP-ATP, reducing the apparant binding EC50 from 30 μ–45 μ to at least 2.8 m. Unfortunately, we were unable to resolve macroscopic currents from W311\*,G334D-GFP+SUR1 in excised patches despite seeing fluorescence in unroofed membranes. Thus, we were unable to measure nucleotide inhibition of this construct to determine whether the G334D substitution affected transduction in addition to this binding effect.

## Channel gating

### C166S alters inhibition without affecting binding

Residue C166 of Kir6.2 is located at the cytosolic end of the second transmembrane domain (Figure [5.5](#ch5fig:c166s_loc), (K. P. K. Lee, Chen, and MacKinnon 2017; G. M. Martin et al. 2017; N. Li et al. 2017b; M. C. Puljung 2018)), and has been suggested to play a role in regulating the intrinsic gating of the channel (Gloyn et al. 2006; S. Trapp et al. 1998; Ribalet et al. 2006; H.-Q. Yang et al. 2020; Loussouarn et al. 2000; Enkvetchakul et al. 2000a). Mutations at this residue lead to dramatically increased unliganded in single-channel experiments (S. Trapp et al. 1998; Enkvetchakul et al. 2000a; Ribalet et al. 2006), and a reduction in sensitivity to nucleotide inhibition at both single-channel and the macroscopic level (S. Trapp et al. 1998; Enkvetchakul et al. 2000a; Ribalet et al. 2006; J. B. W. Li et al. 2013; H.-Q. Yang et al. 2020). In addition, two substitutions at this residue (F and Y) have been found to cause severe neonatal diabetes (Gloyn et al. 2006). Electrophysiological measurements alone are not sufficient to distinguish between the reduction in sensitivity to nucleotide inhibition being caused by the increase in intrinsic alone, or whether there is an additional disregulation of transduction.

We measured TNP-ATP binding to W311\*,C166S-GFP+SUR1 in unroofed membranes to determine the how mutations at C166 reduce sensitivity to nucleotide inhibition (Figure [[ch5fig:c166s\_unroofed]](#ch5fig:c166s_unroofed)). We observed no real change in binding of TNP-ATP to the channel, with an EC50 of 44 μ–74 μ. If the C166S mutation solely increases the of the channel, we would expect an increase in the apparent EC50 of nucleotide binding due to the preference of nucleotides for the closed state of the channel. This finding suggested a role for C166 in the transduction of nucleotide binding to the channel pore.

To investigate this further, we excised patches expressing W311\*,C166S-GFP+SUR1 and measure current inhibition and fluorescence quenching by TNP-ATP simultaneously (Figure [5.9](#ch5fig:c166s_traces)). We found that the apprent affinity for nucleotide binding was indistinguishable from that for W311\*-GFP+SUR1, and similar to our observations in unroofed membranes (Figure [[ch5fig:c166s\_mwc\_fit\_1]](#ch5fig:c166s_mwc_fit_1), EC50 of 26 μ–218 μ). Consistent with the literature, we did observe a large reduction in the apparent sensitivity of W311\*,C166S-GFP+SUR1 currents to inhibition by TNP-ATP (IC50 of at least 155 μ. Intuitively, a change in nucleotide-dependent channel gating which is not accompanied by a change in nucleotide binding must be due (at least in part) to a change in the transduction of nucleotide binding to channel gating.

Fitting our data to the MWC-type model described previously, we found that in addition to the effects of the C166S mutation on the intrinsic open probability of KATP, there is a striking shift in (Figure [5.14](#ch5fig:c166s_mwc_params_1)). This shift to a value much closer to unity indicates that binding of TNP-ATP to W311\*,C166S-GFP+SUR1 favours the closed state far less than binding of TNP-ATP to W311\*-GFP+SUR1. Equivalently, binding of TNP-ATP to the mutant channel is less able to induce closure of the pore. Thus, even at millimolar concentrations of TNP-ATP when all of the Kir6.2 subunits are predicted to be bound, the KATP channels are still able to open.

This finding makes the prediction that nucleotide inhibition of KATP channels with the C166S mutation will exhibit a plateau of inhibition. Unfortunately, we were not able to test this directly with higher concentrations of TNP-ATP due to its purification as a TEA+ salt. High m concentrations of TEA+ inhibit KATP channels, and we determined that for W311\*-GFP+SUR1 and W311\*,C166S-GFP+SUR1 concentrations of above 1 m TEA+ began to inhibit currents to an extent that would interfere with our measurements (Figure [5.16](#ch5fig:tea_trace), [5.17](#ch5fig:tea_drc)). The precise ratio of TEA+ to TNP-ATP in our solutions is unknown, but is assumed to be between 1:1 and 3:1. Any additional inhibition observed at TNP-ATP concentrations greater than 1 m for W311\*,C166S-GFP+SUR1 will therefore be (at least in part) due to the presence of TEA+. However, we do see that even at concentrations of 10 m ATP, W311\*,C166S-GFP+SUR1 is not fully inhibited (Figure [[ch5fig:c166s\_mwc\_fit\_1]](#ch5fig:c166s_mwc_fit_1), open circle). The literature is divided on the existence of a current plateau for mutations at C166, although these experiments have been performed under a variety of different conditions and with various different Kir6.2 backgrounds.

### Mutations at E179 alter both inhibition and binding

Residue E179 of Kir6.2 is located in the C-terminal region of Kir6.2 between the inhibitory nucleotide binding site and the proposed PIP2 binding site. In one early predicted structures of Kir6.2, it was theorised that E179 would form part of the nucleotide binding pocket directly, potentially coordinating the adenine ring of ATP directly through hydrogen bonding (Antcliff et al. 2005). In another, it was hypothesised to form part of the PIP2 binding pocket instead (Haider et al. 2007). Electrophysiological experiments painted a confusing picture of the residues role (Antcliff et al. 2005). Mutation to an amino acid capable of forming hydrogen bonds (Q) resulted in no change in the IC50 for nucleotide inhibition (although a separate study found that Q increased the IC50 (Proks et al. 1999)), while only one of two amino acids incapable of forming hydrogen bonds tested (M and L) resulted in an increased IC50. In addition, mutation of the residue to asparagine (which is not capable of forming hydrogen bonds) not only dramatically increased the nucleotide IC50, but increased the intrinsic open probability of the channel (Antcliff et al. 2005).

The cryo-EM structures of KATP in complex with ATP revealed that bound ATP adopted a radically different conformation to that proposed in early models, and the E179 side chain actually lies over 8 Å away from bound ATP (K. P. K. Lee, Chen, and MacKinnon 2017; G. M. Martin et al. 2017; N. Li et al. 2017b; M. C. Puljung 2018). Unfortunately, no structure has been resolved in the presence of PIP2 to date. However, coarse-grained molecular dynamics simulations using the cryo-EM structures as a starting point indicate that E179 may form part of the PIP2 binding pocket (Pipatpolkai, Corey, et al. 2020). In addition, mutation to E179K results in reduced inhibition of the channel by the sequestering agent neomycin - potentially due to an increased affinity of the mutated residue for PIP2 (Pipatpolkai, Corey, et al. 2020).

To attempt to resolve the precise role of E179 in nucleotide binding and inhibition, we first determined how ATP and TNP-ATP inhibiton of KATP channels was affected by mutation of E179 to A or K. For E179A-GFP+SUR1 and E179K-GFP+SUR1, we observed an increase in IC50 for both ATP and TNP-ATP inhibition. ATP inhibition did not seem to be influenced by the identity of the replacement amino acid (x and x respectively), while TNP-ATP inhibition was more reduced by mutation to a K rather than an A ( x and x respectively). Introducing the mutations into the ANAP-labelled construct did not affect the relative changes in inhibition by either nucleotide, with ATP inhibition occurring at similar IC50s for A and K (x and x respectively) and with K increasing the IC50 for TNP-ATP more than A (x and x respectively). Measurements of TNP-ATP binding mirrored our observations for current inhibition by TNP-ATP, with mutation to both A and K resulting in an increased apparant binding EC50, with K having more of an effect than A (x and x respectively). Fitting the combined data to the MWC-type model, we found that both mutations resulted in a decreased estimate, with no apparent change in . In addition, mutation to a K led to a value closer to unity than for E or A.

We believe there are two possible ways to interpret these findings. The first is to accept the shift in at face value - a decrease in the apparent TNP-ATP binding affinity would suggest a role for residue E179 in forming the nucleotide binding pocket, and this function is abrogated by our mutations. Despite the distance of the residue from the bound ATP, there could be interactions between E179 and the sidechains of residues which do form the pocket (e.g. R54), such that mutation of E179 leads to alterations in the binding pocket which reduce nucleotide binding affinity and therefore our estimate of . The additional effect on caused by mutating the residue to K suggests a dysregulation of the transduction of nucleotide binding to the channel pore, making nucleotides less selective for the close state.

The second interpretation is possible due to the simplification of the role of PIP2 in our MWC model as discussed previously. Briefly, if there is an additional allosteric interaction between nucleotide and PIP2 binding to Kir6.2 which is separate to the channels open/closed state, then changes in may reflect alterations in the affinity for PIP2 binding in addition to or instead of alterations in the affinity for nucleotide binding. Thus, the decrease in upon mutation of E179 may reflect an increase in PIP2 affinity and demonstrate the presence of local allostery between nucleotide and lipid.

Distinguishing between these two interpretations is difficult given our current evidence, and essentially depends on the weight you place on the assumptions of each, but should be possible with one or two further experiments. Firstly, an increase in PIP2 affinity should lead to an increase in channel open probability on excision (barring an effect on the relative preference of PIP2 for the open state). Our inability to accurately determine the open probability of the macroscopic experiments described so far could be supplemented by single channel analysis of the mutants to test this directly. In addition, we could measure the affinity of PIP2 directly in macroscopic patches. Finally, to definitively test the existence of local allostery between the nucleotide and PIP2 binding sites, we could introduce PIP2 binding mutants into the C166S background. C166S channels exhibit almost no nucleotide-dependent gating; i.e. nucleotide binding is uncoupled from gating of the channel pore. Thus, any changes observed in nucleotide binding in the C166S background when PIP2 affinity is changed must be due to a local allosteric interaction which does not involve the pore.

### Mutations at K39 alter both inhibition and binding

Residue K39 of Kir6.2 is located in the N-terminal region of Kir6.2, and is positioned between the inhibitory nucleotide binding site and the proposed PIP2 binding site. In previous studies, the mutation K39A has shown a small reduction in open probability (Cukras, Jeliazkova, and Nichols 2002), and a small reduction in sensitivity to nucleotide inhibition (Cukras, Jeliazkova, and Nichols 2002; Tucker et al. 1998). These effects are somewhat contradictory, as mutations which reduce open probability tend also to increase sensitivity to nucleotide inhibition. In each of the cryo-EM structures of KATP, the K39 side chain appears to coordinate the bound ATP molecule (K. P. K. Lee, Chen, and MacKinnon 2017; G. M. Martin et al. 2017; N. Li et al. 2017b; M. C. Puljung 2018). These structures are presumed to represent the closed state of the channel, and no PIP2 bound structure of the channel has yet been solved. However, molecular dynamics simulations using the ATP-bound structure as a starting point and introducing PIP2 suggest that the K39 residue is able to contact both ligands. This suggests a role for K39 in the binding sites of both ATP and PIP2, which may explain the contradictory findings on open probability and nucleotide inhibition changes when the residue is mutated.

We tested three mutations at K39 (K39A, K39E, K39R) to examine the effects of changing the side chain characteristics on nucleotide binding and inhibition. Mutation to E (opposite charge) or R (same charge) results in an increase in IC50 for ATP inhibition for both WT and W311\* backgrounds (x for each). We did not see an increase in the IC50 for ATP inhibition when K39 was mutated to A (neutral) in either background (x and x respectively). Inhibition by TNP-ATP displayed a different profile depending on the mutant residue. In both WT and W311\* backgrounds, inhibition by TNP-ATP exhibited higher IC50 values for K39A and K39E than we observed for K39R, which was not really distinguishable from K39 (x for each). Our docked conformation for TNP-ATP suggests that the TNP-moiety of the nucleotide may result in extra contacts with K39 compared to ATP, which may be the cause of the different sensitivity to inhibition between the two nucleotides when this residue is mutated. Measurements of TNP-ATP binding showed increases in the EC50 estimates for each of the three mutations (x for each).

Fits of the combined data to the MWC model gave parameter estimates for that decreased from K>R>E>A. In addition, mutation to an E or an A resulted in values closer to unity. Interpretation of these parameters for the R and A mutations is frustrated by the differences in inhibition between TNP-ATP and ATP; we cannot be sure that these differences in binding and inhibition are due to the identity of the nucleotide rather than the identity of the residue. However, the K39E mutation displayed similar inhibition for both TNP-ATP and ATP. The increase in our estimate for when K39 is mutated to an A or an E, but not for R, may indicate a positive charge at the sidechain of this residue being important for transduction of nucleotide binding to the channel pore.

## Discussion

# [ch:6]Regulation of Kir6.2 by SUR1

## Introduction

The SUR1 subunit exerts a number of different regulatory effects on the KATP channel. Firstly, it dramatically enhances trafficking of Kir6.2 to the cell membrane by masking the endoplasmic retention motif in Kir6.2 (RKR). Without coexpression with SUR1, Kir6.2 is confined to the endoplasmic reticulum. Truncating the C-terminal by deleting the last 26 (Kir6.2-DC26) or 36 (Kir6.2-DC36) amino acids (Tucker et al. 1997), mutation of the RKR motif to AAA (Zerangue et al. 1999b), or addition of a C-terminal GFP tag (S. A. John et al. 1998) are sufficient to allow expression of Kir6.2 at the membrane alone without the presence of SUR1. Comparing the function of these modified Kir6.2 subunits alone to the function of octameric KATP channels makes it possible to discern the multifaceted roles of SUR1. Crucially, these C-terminal modifications do not appear to alter KATP function when they are coexpressed with SUR1 (Tucker et al. 1997; S. A. John et al. 1998; Ribalet et al. 2006) and the cryo-EM structure solved for C-terminally GFP labelled Kir6.2 (N. Li et al. 2017b) was highly similar to those solved without the GFP label (Gregory M. Martin et al. 2017; Kenneth Pak Kin Lee, Chen, and MacKinnon 2017).

Coexpression of SUR1 has two effects on KATP channel function. Firstly, SUR1 increases the of the channel (Tucker et al. 1997; S. A. John et al. 1998; Kim W. Chan, Zhang, and Logothetis 2003). Expressing the TMD0 region of SUR1 (residues 1 - 195) alone is sufficient to recapitulate the increase in observed when full-length SUR1 is coexpressed(Andrey P. Babenko and Bryan 2003; Kim W. Chan, Zhang, and Logothetis 2003). When TMD0 is coexpressed with Kir6.2, there is additionally a decrease in the sensitivity of Kir6.2 to nucleotide inhibition - allosterically, an increase in would result in a decrease in apparent ATP affinity due to the reduction in stability of the closed state. However, when full length SUR1 is coexpressed with Kir6.2, there is a marked increase in sensitivity to ATP inhibition (Tucker et al. 1997; S. A. John et al. 1998; Kim W. Chan, Zhang, and Logothetis 2003; Ribalet et al. 2006). This increase in sensitivity is not due to the L0 linker, the other domain of SUR1 postulated to make contacts with Kir6.2. Expression of TMD0-L0 (residues 1 - 232) with Kir6.2 increases the to nearly saturating, and reduces ATP inhibition even further (Andrey P. Babenko and Bryan 2003). Increasing the fraction of L0 (up to residue number 256 or 288) attenuates this increase in , but there is not the dramatic increase in ATP sensitivity observed from expression of full-length SUR1, implicating a role for the core region of SUR1 in regulating nuclelotide binding and inhibition (Michael C. Puljung 2018).

## Intrinsic effects of SUR1

### SUR1 alters nucleotide inhibition, but not binding

Expressing WT-GFP alone without SUR1 results in smaller, noisier currents than when coexpressed with SUR1. Currents are less sensitive to ATP and TNP-ATP by an order of magnitude (x and x). Our surface expression assay suggested that while WT-GFP was able to reach the membrane in the absence of SUR1, W311\*-GFP was not, and when we excised patches from cells expressing W311\*-GFP alone, we were not able to resolve any currents. We were still able to resolved fluorescence in unroofed membranes expressing W311\*-GFP alone, and so we measured binding of TNP-ATP to W311\*-GFP alone in unroofed membranes. We observed very minimal differences in the EC50 for binding. However, given that we did not observe currents under these experimental conditions, we cannot determine the functional state of these channels and so this finding may not be representative for KATP channels physiologically.

Given that we were able to observe currents in the absence of SUR1, we confirmed that when SUR1 was cotransfected with our constructs we were measuring currents and fluorescence from correctly assembled KATP channels. Firstly, we used tolbutamide to inhibit excised patches from cells expressing either WT-GFP alone, WT-GFP+SUR1 or W311\*-GFP+SUR1. Tolbutamide inhibition occurs at two sites on the KATP channel; a high affinity site on SUR1 and a low affinity site on Kir6.2 (F. M. Gribble et al. 1998; Ashfield et al. 1999). Inhibition occurring at these two sites can be well separated, with the high affinity site saturating at 100 μ tolbutamide at 50 % fractional inhibition. Tolbutamide inhibition of Kir6.2 expressed alone does not display inhibition until concentrations of over 100 μ. When we expressed WT-GFP alone, we saw no inhibition of currents by 100 μ, whereas when we expressed WT-GFP+SUR1 or W311\*-GFP+SUR1, we observed roughly a 50 % fractional inhibition of current as expected for proper associated of Kir6.2 and SUR1.

In addition, we labelled the C-terminus of SUR1 with the fluorophore mOrange (SUR1-mO), and measured FRET between the GFP on WT-GFP or W311\*-GFP and the mOrange on SUR1 in unroofed membranes. The cryo-EM structures suggest a distance between the C-termini of Kir6.2 and SUR1 of roughly 60 Å, while the GFP-mOrange FRET pair has a theoretical R0 of 54 Å. We would therefore expect to see FRET if our Kir6.2 and SUR1 contructs are coassembling. To measure FRET, we used an approach described in (Zheng, Trudeau, and Zagotta 2002) whereby FRET is measured as an increase in the emission of the acceptor fluorophore (mOrange) on excitation of the donor fluorophore (GFP) (Figure [[sur\_assays]](#sur_assays)). We observed FRET between WT-GFP and SUR1-mO, consistent with the two subunits being in close proximity. Less FRET was observed when W311\*-GFP was coexpressed with SUR1-mO (although still much higher than background). This is probably due to the reduced expression of W311\*-GFP compared to WT-GFP, i.e. there is less donor fluorescence to transfer to mOrange. Correcting for the magnitude of the GFP peak results in observed ratios for WT-GFP and W311\*-GFP that are very similar.

### Presence of SUR1-TMD0 alone does not alter apparent nucleotide binding

## SUR1 and nucleotide regulation

### Mutations at SUR-K205 alter nucleotide binding and inhibition

Residue K205 of SUR1 is located in the L0 region which links TMD0 and TMD1. While expression of Kir6.2 and TMD0-L0 have shown that the region is important in modulating the of KATP channels (A. P. Babenko and Bryan 2003; Kim W. Chan, Zhang, and Logothetis 2003; Pratt et al. 2011), it does not confer the high sensitivity to ATP inhibition seen in Kir6.2+SUR1 channels. It has therefore been suggested that the elements of SUR1 which contribute to the higher sensitivity of KATP channels to ATP inhibition lie outside of this region (A. P. Babenko and Bryan 2003; Pratt et al. 2012). However, the cryo-EM structures of KATP suggest a close proximity between L0 and the ATP binding pocket (Gregory M. Martin et al. 2017; Kenneth Pak Kin Lee, Chen, and MacKinnon 2017; N. Li et al. 2017b) and mutations in this region reduce the sensitivity of KATP to nucleotide inhibition (Ding et al. 2019; Pratt et al. 2012; Masia, Leon, et al. 2007). Mutation of K205 to A (Ding et al. 2019) or E (Ding et al. 2019) have resulted in marked reduction of KATP channel sensitivity to nucleotide inhibition. Why would mutation of this residue in L0 affect nucleotide inhibition given that expression of the L0 region does not in itself affect nucleotide inhibition?

We excised patches expressing W311\*-GFP+SUR1-K205A or W311\*-GFP+SUR1-K205E and measured current inhibition and fluorescence quenching by TNP-ATP simultaneously. We found that both substitutions resulted in an increased IC50 for TNP-ATP inhibition and an increased EC50 for TNP-ATP binding, with K205E exhibiting a more pronounced effect than K205A. Fitting the data to our MWC model gave parameter estimates for which were reduced when compared to wild-type SUR1; with the neutral mutation K205A not affecting quite as much as the charge reversal mutation K205E. In addition, both mutations led to similar increases in . Thus, the reduced sensitivity to nucleotide inhibition is due to a combination of reduced apparent binding affinity in addition to reduced stabilisation of the closed state of the channel by nucleotides.

## Discussion

Expression of W311\*-GFP in the absence of SUR1, or in the presence of SUR1 truncated to the TMD0 or TMD0-L0 regions alone does not appear to affect nucleotide binding directly. When current inhibition is measured, we see TMD0-232 decreases KATP senesitivity to nucleotide inhibition as seen in previous studies. So why do mutations in L0 reduce sensitivity to nucleotide inhibition and cause neonatal diabetes? Measuring binding directly allows us to address this problem. We found that K205, a residue in L0, does in fact contribute to the nucleotide binding site. This alone is not enough to explain the change in nucleotide inhibition. Our novel finding is that mutating this residue alters the ability of TNP-ATP to close the channel. This is consistent with the hypothesis that L0 plays a key role in tuning the modulatory function of nucleotides on Kir6.2. The wild-type residue K205 is at least partly responsible for the strong coupling of nucleotide binding to channel inhibition. But this does not explain why TMD0-L0 expression is not enough to restore full-length SUR1 like nucleotide inhibition. Future experiments - residue E128 potentially plays a similar role for PIP2.

# [ch:7]Discussion

# [ch:8-appendix]Appendices

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