Kir6.2 mutations causing neonatal diabetes prevent endocytosis of ATP-sensitive potassium channels

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ATP-sensitive potassium (K_{ATP}) channels couple the metabolic status of a cell to its membrane potential—a property that endows pancreatic β -cells with the ability to regulate insulin secretion in accordance with changes in blood glucose. The channel comprises four subunits each of Kir6.2 and the sulphonylurea receptor (SUR1). Here, we report that K_{ATP} channels undergo rapid internalisation from the plasma membrane by clathrin-mediated endocytosis. We present several lines of evidence to demonstrate that endocytosis is mediated by a tyrosine based signal (³³⁰YSKF³³³) located in the carboxy-terminus of Kir6.2 and that SUR1 has no direct role. We show that genetic mutations, Y330C and F333I, which cause permanent neonatal diabetes mellitus, disrupt this motif and abrogate endocytosis of reconstituted mutant channels. The resultant increase in the surface density of K_{ATP} channels would predispose β -cells to hyperpolarise and may account for reduced insulin secretion in these patients. The data imply that endocytosis of K_{ATP} channels plays a crucial role in the (patho)-physiology of insulin secretion.

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Introduction

ATP-sensitive potassium (K_{ATP}) channels are involved in numerous physiological processes including the regulation of insulin secretion and the protection of neuronal and cardiovascular cells during periods of metabolic stress (Huopio *et al*, 2002; Seino and Miki, 2003; Ashcroft, 2005). Underlying these roles is their ability to couple the metabolic

state (ATP/ADP ratio) of the cell to its membrane potential. This ability is conferred by the unique property of K_{ATP} channels: they are inhibited by ATP and ADP and activated by Mg-ADP and Mg-ATP. The role of these channels in the regulation of glucose-stimulated insulin secretion has been the subject of intense research (Huopio *et al*, 2002; Seino and Miki, 2003; Dunne *et al*, 2004; Ashcroft, 2005; Haider *et al*, 2005). A rise in blood glucose increases the uptake and metabolism of glucose in pancreatic β -cells, leading to an increase in the ATP/ADP ratio, which triggers a cascade of events: K_{ATP} channels close leading to membrane depolarisation, activation of voltage-gated calcium channels and influx of Ca^{2+} into the cell. The resultant rise in intracellular Ca^{2+} triggers insulin secretion.

The mechanism by which the ATP/ADP ratio regulates the channel activity became clear after the molecular identity of the channel had been established (Inagaki *et al*, 1995). K_{ATP} channels are octameric proteins, made up of four subunits of Kir6.x, which form the central K^{+} -selective pore, and four sulphonylurea receptor (SUR) subunits, which regulate channel function (Clement *et al*, 1997; Shyng and Nichols, 1997). Pancreatic and neuronal channels are composed of Kir6.2 and SUR1, whereas cardiac K_{ATP} channels comprise Kir6.2 and SUR2A (Seino and Miki, 2003; Haider *et al*, 2005); smooth muscle channels are thought to comprise Kir6.1 and SUR2B (Yamada *et al*, 1997). Mutagenic studies suggest that ATP inhibits the channel by binding to Kir6.2, whereas Mg-nucleotides stimulate pore opening indirectly by binding to the SUR subunit and transducing the signal to the pore (Gribble *et al*, 1998).

Genetic mutations in the genes encoding Kir6.2 (*KCNJ11*) and SUR1 (*ABCC8*) have been shown to cause abnormal insulin secretion (Huopio *et al*, 2002; Seino and Miki, 2003; Dunne *et al*, 2004; Ashcroft, 2005). Mutations that lead to the loss-of-function by decreasing the affinity for Mg-nucleotides for the channel (Shyng *et al*, 1998; Matsuo *et al*, 2000) cause persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI). By suppressing channel function, these mutations appear to cause excessive insulin secretion even at substimulatory concentrations of glucose. On the other hand, gain-of-function mutations are associated with the genetic disorder represented by permanent neonatal diabetes mellitus (PNDM), characterised by severe hypoinsulinaemia and hyperglycaemia (Gloyn *et al*, 2004; Ashcroft, 2005). Many PNDM mutations reduce the ability of ATP to block K_{ATP} channels. The resultant increase in K_{ATP} current hyperpolarises the β -cell, suppressing Ca^{2+} influx and insulin secretion, thereby causing neonatal diabetes (Gloyn *et al*, 2004; Proks *et al*, 2005; Tammara *et al*, 2005). Both these clinical phenotypes have been replicated to some extent in transgenic mice engineered to express the inactive (Miki *et al*, 1997) and overactive (Koster *et al*, 2000) forms of K_{ATP} channels.

Some PHHI causing mutations do not appear to affect channel function *per se*; instead, they impair forward traffick-

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ing thereby reducing channel numbers at the cell surface (Cartier *et al*, 2001; Partridge *et al*, 2001). These findings raise the intriguing possibility that regulation of cell surface channel levels might play a role in the regulation of insulin secretion under normal physiological conditions. Three main factors determine the density of a protein at the plasma membrane: biosynthetic delivery, internalisation and recycling. K_{ATP} channel assembly and trafficking to the cell surface is controlled by the 'RKR' ER retention signals located in Kir6.x and SUR subunits (Zerangue *et al*, 1999). These signals prevent the independent exit of subunits from the ER to the cell surface, but upon assembly into hetero-oligomers, the signals are masked leading to their exit from the ER to the cell surface. Recent studies reported that K_{ATP} channels at the plasma membrane undergo internalisation when cells are treated with the phorbol ester, 4- β -phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC; Hu *et al*, 2003).

In this study, we report that K_{ATP} channels (the term K_{ATP} channels refers to Kir6.2 + SUR1 throughout this paper) undergo rapid internalisation by clathrin-mediated endocytosis (CME) in the absence of PMA and that endocytosis is inhibited when the tyrosine-based motif, ³³⁰YSKF³³³, but not the dileucine motif, ³⁵⁵LL³⁵⁶, located in the C-terminal tail of Kir6.2, was mutated. More importantly, we show that mutation of Y330 to C and F333 to I, both linked to PNDM (Sagen *et al*, 2004; Vaxillaire *et al*, 2004), impair endocytosis of the channel. The latter finding underscores the potential importance of endocytosis, and the tyrosine-based signal, in the regulation of insulin secretion, and highlights the (patho-) physiological significance of K_{ATP} channel endocytosis.

Results

Internalisation of K_{ATP} channels

Previous studies reported that PMA induces internalisation of K_{ATP} channels, and chelerythrine, an inhibitor of PKC, prevents this effect, implicating a role for PKC in determining the surface density of these channels (Hu *et al*, 2003). Here, we examined whether K_{ATP} channels can undergo endocytosis in the absence of PMA treatment. Figure 1 shows that a range of commonly used cell lines (tsA-201, COS-7 and HeLa) were indeed able to internalise K_{ATP} channels in the absence of PMA. When live cells expressing recombinant K_{ATP} channels, containing an engineered extracellular haemagglutinin (HA) epitope in the Kir6.2 subunit (Kir6.2-HA), were incubated with rat anti-HA antibodies, the cells accumulated significant amounts of these antibodies at 37°C (Figure 1A, bottom panel), but not at 4°C, where endocytosis cannot occur (Figure 1A, top panel). The effect is not a consequence of temperature shock because cells incubated in the continuous presence of anti-HA antibodies at 37°C showed internalisation (Figure 1B, left). Cells transfected with channel subunits lacking the HA epitope showed no staining (Figure 1B, right), indicating that internalisation is dependent on the binding of the antibody to the HA epitope of the channel. These data suggest that either activation of PKC is not essential or there is sufficient basal PKC activity in these cell lines to induce internalisation. Regardless of the mechanism, it allowed us to investigate the mechanism of K_{ATP} channel internalisation in the absence of pharmacological stimulation of PKC. Such an approach eliminates potential problems associated with any

Figure 1 Internalisation of K_{ATP} channels. **(A)** Cell lines of different tissue origin, as indicated, were transiently co-transfected with Kir6.2-HA and SUR1, or Kir6.2-HA alone for INS1e cells. The cells were incubated with primary rat anti-HA antibodies at 4°C for 1 h, washed and then incubated at either 4°C (top row) or at 37°C (bottom row) for 30 min. The cells were fixed, permeabilised and stained with anti-rat Cy3 conjugated antibodies. **(B)** Cells co-transfected with HA-tagged or untagged Kir6.2 and SUR1 were incubated with rat anti-HA antibodies at 37°C for 1 h before washing and staining as in (A). Inset shows the bright field image. Representative confocal images are shown ($n = 3$); bar = 10 μ m.

nonspecific effects of the drug or indirect effect on channel internalisation. The latter possibility could not be ruled out because mutation of all potential PKC sites in Kir6.2 failed to prevent PMA-induced endocytosis (Hu *et al*, 2003).

Parallel studies were carried out on the INS-1e cell line. This is a pancreatic β -cell line that resembles the native β -cell in its ability to secrete insulin in response to glucose stimulation (Sekine *et al*, 1997) and expresses Kir6.2 and SUR1. We transfected INS-1e cells with Kir6.2-HA alone, with the expectation that Kir6.2-HA will co-assemble with native subunits and reach the cell surface, thereby enabling the use of anti-HA antibodies to follow internalisation. As predicted, exogenously expressed Kir6.2-HA showed surface expression (Figure 1A, top right); in addition, channels bearing Kir6.2-HA showed internalisation (Figure 1A, bottom right). These data eliminate uncertainties associated with the other cell lines, such as HEK293 and COS-7 cells, where channels could be overexpressed leading to erroneous results. In the INS-1e cells, Kir6.2-HA levels at the cell surface will not exceed the endogenous levels, because Kir6.2 cannot reach the cell surface unless its ER retention signals are masked by the endogenous SUR1 (Zerangue *et al*, 1999). Secondly, INS1e cells are considered a good model β -cell line, so the results likely have physiological relevance. However, the presence of endogenous channel subunits makes this cell line unsuitable to study the molecular basis of internalisation using mutagenic approaches. For this reason, further studies were performed on HEK293 cells.

Figure 2 shows the time course of internalisation of HA-tagged K_{ATP} channels stably expressed in HEK293 cells. In Figure 2A, channels were coated with anti-HA antibodies at 4°C and then shifted to 37°C for various time periods before immunostaining; the results show a time-dependent increase

Figure 2 Time course of K_{ATP} channel internalisation. (A) Internalisation time course by confocal microscopy. HEK293 cells stably expressing Kir6.2-HA and SUR1 were incubated with primary rat anti-HA antibodies at 4°C for 1 h; after washing, the cells were incubated at 37°C for indicated time periods to allow the antibody-coated channels to internalise before fixing with 3% PFA. Cells were then permeabilised, stained with anti-rat Cy3-conjugated antibodies and the labelled channels visualised by confocal microscopy; representative confocal images are shown ($n=4$); bar = 10 μ m. (B) Internalisation time course by a quantitative chemiluminescence assay. The experiment was performed as in (A) up to the 3% PFA fixation step. Channels remaining at the cell surface were estimated by chemiluminescence (see Materials and methods). Data are presented as % of channels remaining at the cell surface following internalisation for the indicated time periods; data points represent means $\pm$ s.e. ($n=5$). (C) Internalisation of biotinylated channels. Biotinylated cell surface channels were allowed to internalise for the times indicated; internalised channels were detected by Western blotting for the Kir6.2-HA subunit (representative of two independent experiments).

in intracellular staining. Quantitative analysis of internalisation, using a chemiluminescence-based assay, revealed that over 50% of the antibody-coated surface channels were internalised within 5 min of temperature shift, reaching the maximum by 15 min (Figure 2B). To eliminate the possibility that antibody binding has an effect on internalisation, we performed a surface biotinylation assay (Graeve *et al*, 1989). Figure 2C shows that the intensity of the bands corresponding to the internalised Kir6.2-HA increased with time, also reaching an apparent maximum by 15 min. Internalisation rates thus do not seem to be affected by the antibody binding. Since the chemiluminescence assay is relatively easy to perform and, like the biotinylation assay, measures the density of channels as an average from a large number of cells (thereby minimising cell to cell variability), we used this assay for quantitative analysis.

Internalisation occurs via clathrin-coated vesicles

We provide several lines of evidence to demonstrate that internalisation of surface K_{ATP} channels occurs via CME. First, when the formation of clathrin-coated vesicles was inhibited by depletion of cytosolic K^+ , or exposure of the cells to hypertonic medium, little or no internalisation of K_{ATP} channels occurred (Figure 3A and B). Internalisation of Alexafluor488-transferrin (Figure 3A; green), used as an internal positive control, was also abolished under these conditions (for reasons unclear, K^+ depletion prevented surface labelling with Alexafluor488-transferrin). These perturbations prevent CME of several membrane proteins (Zhu *et al*, 1996). Second, expression of the dominant-negative form of μ 2 (D176A/W421A) (Nesterov *et al*, 1999), but not the wild-type (WT) μ 2, significantly ($P<0.01$) inhibited endocytosis (Figure 3C). μ 2 is a subunit of the AP2 adaptor complex; binding of μ 2 to sorting signals on cargo proteins is

required for subsequent addition of clathrin coat proteins and vesicle formation (Bonifacino and Traub, 2003). Third, expression of the dominant negative forms of dynamin-1 and dynamin-2 (K44A), but not their WT versions, also inhibited the extent of internalisation of surface channels (Figure 3C). Dynamins are GTPases required for pinching of the clathrin-coated vesicles from the membrane (Bonifacino and Traub, 2003). Finally, co-immunolabelling experiments (Figure 3D) show significant colocalisation (yellow) of internalised channels stained with FITC-conjugated antibodies (green) and the clathrin light chain stained with Cy3-conjugated antibodies (red). Taken together, these data indicate that endocytosis of K_{ATP} channels occurs via clathrin-coated vesicles.

Internalisation signals reside in the Kir6.2 subunit of K_{ATP} channels

We next asked which of the two subunits of K_{ATP} channels, Kir6.2 and SUR1, contain the signal(s) for endocytosis. For this, we first mutated the ER retention signal (RKR) of SUR1 to AAA, and inserted two tandem c-myc epitopes into its extracellular loop (Sharma *et al*, 1999). The resultant construct myc-SUR1(AAA), as expected, showed surface expression in the absence of Kir6.2 (Figure 4A, top middle), but no detectable internalisation of SUR1-bound anti-c-myc antibodies. Co-expression of Kir6.2, however, led to extensive internalisation of myc-SUR1(AAA) (Figure 4A, top right), indicating that the endocytic signals are either located in the Kir6.2 subunit, or that assembly with Kir6.2 is necessary to unmask any endocytic signals in SUR1. To distinguish between the two possibilities, we tested if Kir6.2-HA Δ C26, lacking the C-terminal 26 amino acids (and the RKR signal) and hence capable of independent trafficking, could undergo endocytosis. Although the level of surface expression of this construct, in the absence of co-expressed SUR1, was low we

Figure 3 K_{ATP} channel internalisation occurs via CME. **(A)** Blockade of internalisation by K⁺ depletion and hypertonicity. HEK293 cells stably expressing Kir6.2-HA and SUR1 were treated as follows. For K⁺ depletion cells were incubated in hypotonic incubation buffer (1:1 serum-free medium/water) for 5 min at 37°C, rinsed and incubated in K⁺-free buffer (100 mM NaCl, 50 mM HEPES, pH 7.4) for 30 min prior to internalisation assay in K⁺-free buffer. To induce hypertonicity, cells were pre-incubated in hypertonic buffer (0.45 M sucrose in blocking medium) for 15 min prior to internalisation assay in the same buffer. The experiment was performed as described in Figure 1B, except that during the final 15 min of the internalisation chase, Alexafluor488-labelled transferrin (25 μg ml⁻¹; green) was included; K_{ATP} channels were stained as described in Figure 1 (red). Representative images are shown (*n* ≥ 3); bar = 10 μm. **(B)** Effect of K⁺ depletion and hypertonicity on the time course of internalisation. Stable cells were subjected to K⁺ depletion and hypertonicity as in (A) and the time course of internalisation performed by the chemiluminescence assay as described in Figure 2B; (○, untreated; ●, K⁺ depletion; □, hypertonic, sucrose treated (*n* = 3)). **(C)** K_{ATP} internalisation is both dynamin and μ2 dependent. Cells were transiently co-transfected with Kir6.2-HA and SUR1 alone (Ctrl; control) or together with the indicated mediators (WT; DN, dominant negative; Dyn, dynamin) of CME. Surface channels were coated with anti-HA antibody at 4°C; after 30 min of internalisation at 37°C, remaining surface channels were quantified using chemiluminescence. The data, expressed as means ± s.e. (*n* = 3), are normalised to cells expressing K_{ATP} channels only (ctrl); **Significant difference (*P* < 0.01) from cells expressing the corresponding WT dynamin or μ2 constructs. **(D)** Internalised K_{ATP} channels co-localise with clathrin. Internalised K_{ATP} channels were stained with anti-rat FITC conjugated antibodies (green), clathrin with mouse anti-clathrin antibody and Cy3 conjugated anti-mouse antibodies (red). Images were taken close to the interface between the cell and the cover glass to view vesicles close to the cell surface; bar = 10 μm; region of interest is magnified in the inset.

were able to detect internalisation in a small number of cells (Figure 4A, bottom left), indicating that the endocytic signal resides in Kir6.2. To examine if the signal is located in the C-terminus of Kir6.2, we fused the C-terminus of Kir6.2 lacking the last 26 amino acids (residues 178–364, referred to as CT) to the membrane domain of CD4, a reporter cell surface protein, and tested if this confers the ability to endocytose on CD4. We did not include the last 26 amino acids of Kir6.2

in this construct because this region contains the ER retention signal and its presence would prevent surface expression of CD4 (Zerangue *et al*, 1999). Substitution of the Kir6.2 C-terminus (residues 178–364) did not prevent surface expression of CD4, but unlike the WT CD4 (Figure 4A, bottom middle), the CD4–Kir6.2CT hybrid protein (Figure 4A, bottom right) displayed internalisation, indicating the presence of endocytic signal in the C-terminus. The data

Figure 4 Endocytosis of K_{ATP} channels is mediated by Kir6.2. (A) tsA-201 cells transiently transfected with the indicated epitope-tagged K_{ATP} channel subunits or CD4 constructs were incubated at 4°C with appropriate antibodies: rat anti-HA antibodies for Kir6.2, mouse anti-c-myc antibodies for SUR1 and antibodies to the extracellular domain of CD4 (CD4). The constructs were allowed to internalise at 37°C for 30 min, fixed, permeabilised and stained with appropriate Cy3-conjugated secondary antibodies. Representative confocal images (*n* = 3) are shown; bar = 10 μm. (B) Pull-down of *in vitro* [³⁵S]methionine labelled μ2 subunit with GST-Kir6.2 C-terminal fusion protein (lane 2), but not with GST alone (lane 1). Input lane has 10% of the μ2 subunit used in the pull-down experiment. The figure is a phosphorimage of proteins separated by SDS-PAGE. (C). Pull-down of Kir6.2-HA from stable cell lysates with GST-μ2 fusion protein (lane 2) or GST alone (lane 1); input contained 1/40th of the lysate; bands are detected by Western blotting. The data in (B) and (C) are representative of two independent experiments.

also suggest that the C-terminus might directly interact with the μ2 subunit of AP2 complex. To test this, we expressed residues 178–364 of Kir6.2 C-terminus as a fusion protein with glutathione-S-transferase (GST) and tested its ability to bind to *in vitro* translated μ2 protein in a pull-down assay. Figure 4B shows GST-Kir6.2CT (lane 2), but not GST alone (lane 1), was able to bind μ2. We next asked if the C-terminus of Kir6.2 in the intact channel complex is accessible to μ2 using GST-μ2 fusion protein in a pull-down experiment. Figure 4C shows that GST-μ2 fusion protein (lane 2) was indeed able to pull-down Kir6.2-HA from cells expressing Kir6.2-HA and SUR1. Thus, both functional and biochemical data indicate that the endocytic signals are present in the C-terminal domain of Kir6.2 and that SUR1 is unlikely to play a major role in endocytosis of the channel.

Internalisation of K_{ATP} channels is mediated by a YXXØ signal located in the C-terminal tail of Kir6.2

The C-terminus of Kir6.2 contains three putative endocytic signals: a dileucine motif (³⁵⁵LL³⁵⁶) and two tyrosine-based motifs (²⁵⁸YHVI²⁶¹ and ³³⁰YSKF³³³) (Figure 5A); the latter conforms to the YXXØ (X = any amino acid;

Ø = hydrophobic) endocytic signal identified in other proteins (Bonifacino and Traub, 2003). Mutation of dileucine to dialanine (AA) failed to prevent internalisation (Figure 5B), but quantitative analysis showed an increase in surface expression by ~2-fold (*P* < 0.05) relative to the WT (Figure 5C). Substitution of alanine for tyrosine (Y258A) in ²⁵⁸YHVI²⁶¹ failed to prevent internalisation, and had no effect on surface expression. However, when the tyrosine in ³³⁰YSKF³³³ was mutated to alanine (Y330A), no detectable internalisation was observed (Figure 5B), and there was a ~2-fold increase (*P* < 0.05) in surface levels (Figure 5C). Hu *et al* (2003) reported that mutation of the dileucine motif prevents PMA-induced internalisation, raising the possibility that both dileucine and tyrosine motifs may contribute to internalisation. To test this, we substituted the dileucine motif with alanines in a channel with the tyrosine internalisation motifs inactivated (LL/AA-YY/AA) to see if there will be an additive effect; Figure 5C shows that dileucine mutation increased the surface expression of the channel relative to the tyrosine mutant, but the increase is not significant (*P* > 0.05). The increase in surface density of the LL/AA mutant relative to the WT (Figure 5C), however, is consistent with the previous report (Hu *et al*, 2003), but the underlying mechanism could be other than reduced endocytosis (see Discussion). Finally, it is important to exclude the possibility that the tyrosine mutation (Y330A) has somehow compromised the ability of the channel to respond to basal activity of PKC, leading to an apparent impairment of endocytosis. Figure 5D shows that this is not the case: PMA treatment failed to induce internalisation of the tyrosine mutant. Taken together, the tyrosine motif, rather than the LL motif, seems to play a role in the endocytosis of K_{ATP} channels.

Certain mutations that cause PNDM prevent endocytosis

Recent studies reported more than 20 mutations in the *KCNJ11* gene that are linked to PNDM (Gloyn *et al*, 2004; Sagen *et al*, 2004; Vaxillaire *et al*, 2004; Ashcroft, 2005; Tammaro *et al*, 2005). Two of these mutations, Y330C and F333I, are located within the trafficking motif identified in this study, raising the interesting possibility that these mutations might reduce internalisation. To test this, we have co-expressed Kir6.2-HA bearing these mutations with SUR1 to represent homozygosity and examined for internalisation. Figure 6A shows that unlike the WT channel, which showed robust internalisation, neither mutant channel displayed internalisation. The effect is specific to mutations in this motif because a PNDM mutation (Q52R) outside of the tyrosine motif has no effect. Consistent with the confocal data, quantitative analysis (Figure 6B) showed over two-fold (*P* < 0.05) more Y330C and F333I mutant channels at the cell surface compared to the WT channels 30 min after internalisation. To mimic the heterozygous phenotype presented by the patients, we expressed a 1:1 mixture of mutant Kir6.2HA with WT Kir6.2 subunits lacking the HA epitope, together with SUR1. The use of WT Kir6.2 ensures that all immunostained channels would contain at least one mutant subunit. Figure 6A (top right panels) shows some internalisation, indicating that the WT subunit could suppress, at least partially, the endocytic defect caused by both the PNDM mutations.

Figure 5 Identification of internalisation signals. (A) Amino-acid sequence of the C-terminus of Kir6.2 showing canonical internalisation motifs in boxes. (B) Internalisation of mutant channels. tsA-201 cells were transiently co-transfected with the indicated WT or mutant Kir6.2-HA subunits and SUR1. Internalisation of anti-HA antibody-coated surface channels was performed at 37°C for 0 min (top panel) or 30 min (bottom panel), as described in Figure 2A; representative confocal images are shown ($n = 3$); bar = 10 μ m. (C) Quantification of internalisation of mutant channels. Internalisation was performed as in (B). Channels density at the cell surface at 0 and 30 min of internalisation was estimated using chemiluminescence (see Materials and methods). Fraction of surface channels remaining after 30 min of internalisation (density at 30 min/density at 0 min) was calculated for the WT and each mutant channel. The value for the WT channel was normalised to 1.0 and the mutant data expressed relative to the WT value. Data represent means $\pm$ s.e. ($n = 5$); **Significant difference from WT ($P < 0.05$). (D) Internalisation of WT and mutant channels during PKC activation. tsA-201 cells transiently transfected with the WT and indicated mutant channels were analysed for internalisation as described in Figure 1B except that the cells were pretreated with PMA (100 nM) at 4°C for 30 min before internalisation.

However, quantitative analysis of internalisation (Figure 6B, top panel) showed that this suppression is incomplete, surface channel density being still ~ 2 -fold greater ($P < 0.05$) compared to the WT. This increase does not appear to be due to overexpression of the mutant subunits relative to the WT because immunoblotting data (Figure 6B, bottom panel) do not indicate differences large enough to account for the large increase in surface density of channels. We do not know how many subunits of WT are required for complete suppression, but the ~ 2 -fold difference suggests that a single subunit may not be enough. Assuming that there is no subunit preference (bias) for channel assembly, the binomial distribution would predict 6.25 % of channels would be homomers of mutant subunits. Thus, if only mutant homomers contribute to decreased internalisation, only a 1.06-fold increase in surface density would be expected. However, we observe no detectable differences in internalisation between mutant plus WT (hetero) and mutant alone (homo) groups (Figure 6B, top panel). Therefore, the inclusion of the WT subunit does not appear to suppress the ability of the mutations to inhibit internalisation. The inability of the WT subunit to suppress the mutant effect enabled us to investigate the effect of PNDM mutations in the INS-1 cell line. When heterologously expressed, we would expect between one and four mutant Kir6.2-HA subunits to assemble with endogenous Kir6.2/SUR1 present in these cells. Because the presence of a single mutant subunit seems to be sufficient to suppress internalisation, we would expect to see a measurable effect on internalisation. As predicted, compared with the WT Kir6.2-HA, channels containing the PNDM mutations in the tyrosine motif (Y330C, F333I), but not in other regions (Q52R), showed significantly ($P < 0.05$) greater surface

density (Figure 6C); the fold increase for Y330C and F333I heteromeric channels were 1.79 ± 0.07 and 2.44 ± 0.15 respectively.

Discussion

In this paper, we have investigated the mechanisms underlying, and molecular basis for, internalisation of K_{ATP} channels. Our key findings are that: (i) K_{ATP} channels undergo rapid internalisation and that this occurs via $\mu 2$ -dependent CME (Figures 2 and 3), (ii) endocytosis is mediated by a tyrosine-based motif, ³³⁰YSKF³³³; located in the C-terminal cytosolic domain of the Kir6.2 subunit of the channel (Figure 5); and (iii) the SUR1 subunit is not essential for endocytosis (Figure 4). Most remarkably, certain mutations in Kir6.2 (Y330C and F333I), recently reported to cause PNDM with severe hyperglycaemia (Sagen *et al*, 2004; Vaxillaire *et al*, 2004), lie within the tyrosine-based motif and prevent internalisation almost completely (Figure 6). This highlights the potential (patho)-physiological importance of the tyrosine-based signal and endocytosis in the regulation of insulin secretion.

Endocytosis of K_{ATP} channels occurs by CME

Three different types of internalisation mechanisms have been described: CME, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis (Conner and Schmid, 2003). Several lines of evidence demonstrate that K_{ATP} channels use CME for internalisation (Figure 3). First, endocytosis is inhibited by hypertonicity and K^+ depletion, treatments known to suppress endocytosis (Zhu *et al*, 1996) (Figure 3A and B). Second, the dominant-

Figure 6 Defective internalisation of K_{ATP} channels bearing PNDM mutations. tsA-201 cells were transiently co-transfected with the indicated WT Kir6.2-HA or mutant Kir6.2-HA subunits (homo), or 1:1 mixture of WT Kir6.2 (lacking the HA epitope) and mutant Kir6.2-HA subunits (hetero) together with SUR1. Internalisation was examined by confocal microscopy (A) as described in Figure 1B (bar = 10 μ m), or quantified using chemiluminescence (B) as described in Figure 5C. Data represent means $\pm$ s.e. ($n = 12$); **Significant difference from WT ($P < 0.05$). Bottom panel shows Western blots of lysates from cells transfected with a 1:1 mixture of the indicated HA-tagged Kir6.2 subunits and untagged WT Kir6.2, together with SUR1. (C) Internalisation of HA-tagged Kir6.2 containing the indicated mutations transiently expressed in INS-1 cells, determined as in Figure 5C ($n = 9$); **Significant difference from WT ($P < 0.05$).

negative forms of proteins required for CME, viz. the μ 2 subunit of the AP2 adaptor complex and dynamins, inhibited endocytosis (Figure 3C). Third, internalised channels show significant co-localisation with clathrin, a component of clathrin-coated endocytic vesicle (Figure 3D). Finally, *in vitro* experiments showed that an immobilised GST- μ 2 fusion protein was able to pulldown Kir6.2-HA from detergent solubilised cells (Figure 4C).

Endocytosis is mediated by a tyrosine-based motif located in Kir6.2 C-terminus

We found that signals for CME are located in Kir6.2, but not in the SUR1 subunit of K_{ATP} channels (Figure 4A). SUR1 was able to reach the cell surface when the RKR-ER retention signal was inactivated by mutating to AAA, but failed to undergo endocytosis. By contrast, Kir6.2-HA Δ C26 lacking the RKR-ER retention signal could undergo endocytosis in the absence of SUR1. Two pieces of evidence suggest that the signals are located in the C-terminus of Kir6.2: First, transfer

of the C-terminus of Kir6.2 to CD4 was able to confer the ability to endocytose on CD4; second, a GST-fusion protein containing the C-terminus of Kir6.2 was able to interact directly with the *in vitro* translated μ 2 subunit of the AP2 (Figure 4B).

The C-terminus of Kir6.2 has three canonical internalisation motifs (Figure 5A): two tyrosine-based motifs, 258 YHVI 261 and 330 YSKF 333 and a dileucine-based motif, 355 LL 356 . However, alanine mutation of only 330 YSKF 333 prevented internalisation (Figure 5B). In a molecular model of Kir6.2 (Supplementary Figure 1), 258 YHVI 261 is buried and hence is inaccessible to μ 2. This explains why mutation of this sequence has no effect. By contrast, both 330 YSKF 333 and 355 LL 356 are solvent exposed, and potentially available to bind μ 2; however, mutation of only 330 YSKF 333 prevented endocytosis.

Although alanine mutation of 355 LL 356 had no apparent effect on internalisation (Figure 5B) quantitative analysis indicated a ~ 2 -fold increase in the cell surface density of LL/AA mutant channels (Figure 5C). A similar increase was

also reported by Hu *et al* (2003) for the LL/AA mutant channels expressed in COS-7 cells; the authors attributed this effect to a decrease in endocytosis. It therefore seemed that both the tyrosine-based motif and the LL motif may contribute to endocytosis. If this was the case, we would expect to see a cumulative effect of these mutations on endocytosis. However, there was no increase ($P > 0.05$) in surface levels when LL/AA mutations were combined with mutations in the tyrosine-based motif (Figure 5C). Thus, it seems possible that LL/AA mutation increases the surface expression by a different mechanism, for example, by increasing recycling. There are several examples in literature where dileucine residues are not involved in endocytosis, but play a role in recycling, such as mannose-6-phosphate receptor 46 (Tikkanen *et al*, 2000) and memapsin 2 (He *et al*, 2005), where mutation of dileucines increased recycling to the plasma membrane. In these cases, the LL pair is preceded by an aspartate residue (D) at -3 position, forming DXXLL sequence. Unlike the YXXØ motif, the DXXLL motif can not detectably bind the AP2 complex and hence is believed to be not involved in endocytosis (Bonifacino and Traub, 2003). Instead, it binds GGA (Golgi-localised, γ -ear containing, ARF-binding protein) adaptor protein, which mediates TGN-endosome trafficking (Ghosh and Kornfeld, 2004). Kir6.2 contains a similar acidic dileucine motif (³⁵²DSRL³⁵⁶), which may explain why mutation of the two leucines does not impair endocytosis.

Taken together, we conclude that the ³³⁰YSKF³³³ tyrosine-based motif governs the endocytosis of K_{ATP} channels. The effect of the Y330C mutation is expected because Y does not tolerate even minor changes, but the result with the F333I mutation was somewhat unexpected, because it is thought that the last residue in the tyrosine motif needs to be hydrophobic, and the F to I mutation does not significantly affect the hydrophobicity (hydrophobicity values of 2.8 and 4.5 for F and I based on Kyte-Doolittle hydropathy index). However, one report (Gough *et al*, 1999) showed that the tyrosine binding motif with an I at the end position binds the μ 2 subunit of the AP2 complex with 400-fold less affinity than when F is present at this position. Thus, the reduced affinity of F333I mutant for μ 2 may account for the observed inhibition of endocytosis of K_{ATP} channels caused by this mutation.

Physiological significance

There are a growing number of examples where the density of channels and receptors at the cell surface is subject to regulation in response to changes in physiological circumstances and that loss of regulation underlies many genetic disorders (Cobbold *et al*, 2003). K_{ATP} channels represent one excellent example. Some mutations impair their trafficking to the cell surface and thereby cause PHHI in humans (Cartier *et al*, 2001; Partridge *et al*, 2001); the reduced density of K_{ATP} channels, and the consequent membrane depolarisation, is responsible for unregulated insulin secretion and severe hypoglycaemia in these patients. A converse situation, where mutations increase the density of channels at the cell surface thereby reducing the insulin secretion is conceivable, but as yet there is no evidence for this. However, recent studies (Sagen *et al*, 2004; Vaxillaire *et al*, 2004) reported two genetic mutations in Kir6.2, Y330C and F333I, which cause severe forms of PNDM with blood glucose levels approaching as high as 70 mM. Since both mutations are expected to

disrupt the function of ³³⁰YSKF³³³, they provided us with the unexpected opportunity to test the hypothesis. As predicted, both mutations suppressed endocytosis completely leading to a two-fold increase in the surface density of the channel in both homomeric and heteromeric situations (Figure 6). More importantly, the mutant channels were able to associate with native K_{ATP} subunits in INS-1 cells and suppress endocytosis (Figure 6C). Since INS-1 cells resemble native β -cells, our data imply that patients harbouring these mutations may have increased density of these channels at the cell membrane.

Using *Xenopus* oocytes as the expression system, Tammaro *et al* (2005) recently reported that both Y330C and F333I mutations reduced the sensitivity of the channel to ATP inhibition and enhanced the stimulatory effect of Mg-ATP. Taken together, these mutations have two distinct effects: a functional effect which allows the channel to remain open at ATP concentrations that normally close the WT channel, and a cell biological effect, whereby the number of channels at the cell membrane remains high.

What is the predicted relative importance of these two effects? The macroscopic conductance is the product of the single channel conductance, open probability and the number of channels (i.e. surface density). In the Y330C and F333I mutants, the reported increase in channel activity (percentage channels unblocked in 1 mM MgATP) was 5.4- and 2.7-fold, respectively in heterozygous states (Tammaro *et al*, 2005); this compares to a roughly 2.5-fold increase in surface density (Figure 6). So these effects are roughly comparable, and both will be expected to contribute to the development of disease. Indeed, the severity of the disease caused by these mutations suggests that a combination of these defects contributes to the pronounced reduction in insulin secretion. Yorifuji *et al* (2005) recently reported the case of a patient with a novel mutation (C42R) in Kir6.2; this mutation caused a reduction in the IC₅₀ of ATP for the channel by ~15-fold and an increase in the spontaneous open probability by four-fold. Despite the severe functional consequences, the patient expressed only a transient form of neonatal diabetes. This was because the mutation reduced the channel density at the plasma membrane. Further support for a role of channel density in regulation of insulin secretion comes from a study of Goto-Kakizaki (GK) rats, a model of NIDDM. Although the glucose sensitivity of the β -cell K_{ATP} current was similar between GK rats and controls, in the absence of glucose, the channel density was larger in GK rats (Hughes *et al*, 1998). Taken together, it seems that there is a clear functional link between the surface density of K_{ATP} channels and insulin secretion. Since endocytosis can control the surface density of K_{ATP} channels, and its inhibition seems to be associated PNDM, we suggest it may play a role in regulating insulin secretion. Studies with other channels, receptors and transporters indicate that endocytosis is not a passive process, but plays crucial physiological roles by regulating cell surface density (Conner and Schmid, 2003). However, with the exception of the LDL receptor where a mutation in its endocytic motif NPXY causes familial hypercholesterolaemia (Chen *et al*, 1990), and this study, there are not many reports linking such genetic mutations to a disease state.

In conclusion, we showed that a tyrosine-based signal in Kir6.2 mediates rapid endocytosis of K_{ATP} channels, and genetic mutations that disrupt this signal are associated

with severe forms of PNDM in humans. These findings imply that endocytosis of K_{ATP} channels may play a crucial role in the normal physiology of the β -cell and regulate glucose stimulated insulin secretion. Furthermore, our findings suggest that future studies should not be restricted to the regulation of channel activity, but should also consider signals and mechanisms that regulate channel density at the cell surface; this is important if we are to fully appreciate the role of K_{ATP} channels in the physiology of glucose stimulated insulin secretion and the causes of diabetes mellitus.

Materials and methods

Materials

HEK293, tsA-201 and HeLa were obtained from ATCC. INS1/INS-1e cells were a kind gift from Dr CB Wollheim, University Medical Center, Geneva, Switzerland). For all other materials, including the sources of antibodies, see Supplementary data.

DNA constructs and expression

A HA epitope and an additional 11 amino-acid linker was introduced into an extracellular loop of mouse Kir6.2. (accession number D50581) by polymerase chain reactions (PCR) as described by Zerangue *et al* (1999) to produce the Kir6.2-HA construct. A cell line stably expressing HA-tagged K_{ATP} channels was produced by co-transfecting Kir6.2-HA in pcDNA3 and hamster SUR1 (accession number L40624) in pcDNA6 into HEK293 cells as described previously (Partridge *et al*, 2001). Transfections were performed using Eugene6 (Roche) or electroporation (for INS1e). Electroporation of INS1e cells was performed in Optimem[®] using 270 V, 950 capacitance, and infinite resistance in a 4 mm gap cuvette using a Bio-Rad Gene Pulser[®] electroporator. Two copies of c-myc epitope were inserted into the first extracellular loop of SUR1 after the first nucleotide binding fold as described previously (Sharma *et al*, 1999); the resultant construct was cloned into the pcDNA6 vector. Site-directed mutagenesis was performed using the Quik-Change method (Stratagene).

The C-terminus of human CD4 was replaced with the C-terminus of Kir6.2 using the overlap extension PCR method. The resulting construct, CD4-Kir6.2-CT, has the N-terminal and the transmembrane domains (residues 1–426) of CD4 fused to Kir6.2 C-terminal domain lacking the ER retention signal (residues 178–364). The chimaeric construct and the WT CD4 were expressed from pcDNA3.

Detection of channel internalisation by immunocytochemistry

Cells expressing the HA-tagged K_{ATP} channels, grown on polylysine-coated coverslips, were first incubated with 0.2 $\mu\text{g ml}^{-1}$ rat anti-HA antibodies at 4°C for 1 h in the labelling medium (DMEM/5% goat serum/1 mM glucose). After washing with chilled phosphate-buffered saline (PBS) to remove unbound antibodies, the cells were fixed with 3% paraformaldehyde (PFA) or methanol (–20°C) for 10 min. The cells were then treated with FITC or Cy3 conjugated goat anti-rat secondary antibodies in 5% goat serum in PBS (blocking medium) for 1 h at room temperature. After washing, the cells were mounted onto microscope slides using Vectashield (VectorLabs). This procedure allows labelling of surface channels only. To examine for internalisation, after the 4°C and 1 h incubation step, temperature was raised to 37°C to induce internalisation. Internalisation was terminated by chilling on ice at time points as specified in the figure legends. The cells were fixed and permeabilised with acetone/methanol (1:1) (–20°C) for 5 min and labelled with secondary antibodies as above. For some experiments, internalisation was performed at 37°C in the continuous presence of anti-HA antibodies (Figures 1B, 3, 4 and 6)

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Labelled cells were then viewed on a Zeiss 510-META laser scanning confocal microscope under an oil-immersion $\times 63$ objective lens (NA = 1.40). FITC (494 nm excitation: 519 nm emission) was excited using an argon laser fitted with 488 nm filters and Cy3 (550 nm excitation: 570 nm emission) was excited using a helium/neon laser fitted with 543 nm filters.

Quantification of K_{ATP} channel internalisation via chemiluminescence

The method is a modification of that described by Zerangue *et al* (1999). HA-tagged K_{ATP} channels at the cell surface were coated with rat anti-HA antibody for 1 h at 4°C, and after washing, incubated at 37°C permit internalisation. After fixing, the anti-HA-antibody bound channels remaining at the cell surface were estimated using the horse-radish peroxidase-conjugated goat anti-rat IgG and Supersignal West Femto-maximum sensitivity substrate (Pierce). Increase in luminescence was measured using Flouroskan Luminometer and the values normalised to the cell protein content (see Supplementary Materials and methods). Data were obtained from a number of separate transfection experiments (stated as *n* in Figure legends), each carried out in duplicate. The data were expressed as mean $\pm$ s.e. Statistical significance of differences was determined using the paired Student's *t*-test when comparing two sets of data, or one-way ANOVA and Bonferroni test, when data for more than two mutants were compared against the WT channel. *P* < 0.05 was accepted as significant.

Biotinylation experiments

Stable HEK293 cells were biotinylated using the membrane impermeable sulpho-NHS-biotin (Pierce) for 20 min at 4°C and the reaction terminated with 50 mM glycine. After washing, cells were incubated at 37°C for different times to allow internalisation. Remaining surface biotin was removed with glutathione and the cells were lysed. Biotinylated proteins in the lysate were adsorbed onto Neutravidin agarose (Pierce) and subjected to Western blotting using anti-HA antibodies (see Supplementary Materials and methods).

Biochemical assay of $\mu 2$ binding

A GST-fusion protein comprising the GST sequence joined to C-terminal sequences (residues 178–364) of Kir6.2 was allowed to bind glutathione Sepharose 4B and used to pull-down the *in vitro* ³⁵S-methionine labelled $\mu 2$ protein. The radiolabelled protein was detected using Phosphorimaging. In a second approach, a GST-human $\mu 2$ (residues 123–435) fusion protein was purified, bound to the glutathione Sepharose 4B resin and used to pulldown Kir6.2 from lysates of stable cells expressing HA-tagged K_{ATP} channels (see Supplementary Materials and methods).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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