The ATP-sensitive potassium channel in the heart. Functional, electrophysiological and molecular aspects
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Subcellular trafficking of ATP-sensitive potassium channel subunits Kir6.1 and Kir6.2

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Abstract

Aim. To study functional expression and cellular distribution of the K$_{ATP}$ channel subunits Kir6.1 and Kir6.2 using patch-clamp technique and fluorescence imaging.

Methods and Results: Kir6.1 and Kir6.2 were isolated from a cDNA library constructed from rabbit heart tissue. Fluorescent fusion proteins of Kir6.1 and Kir6.2 were made using the pEYFP-N1 vector such that EYFP was fused at the C-terminal end to either Kir6.1 or Kir6.2. These constructs (110-300 ng) were transfected into HEK293 cells in the presence or absence of SUR1. Confocal imaging showed plasma membrane localisation for both Kir6.1 and Kir6.2 fusion proteins in the presence of SUR1. Without SUR1, Kir6.1 was distributed intracellularly, and showed a high degree of co-localisation with the mitochondrion-selective dye MitoTracker® Red, whereas Kir6.2 was localised at the plasma membrane. Single channel activity was measured in cell-attached patch (CAP) and inside-out (I-O) configuration in HEK293 and CHO cells. EYFP\Kir6.2+SUR1 produced a large number of active channels in I-O patches (mean 32.8 ± SEM 15.4 channels/patch) with burst openings and a single channel conductance $\gamma$ of 58.3±1.4 pS. EYFP\Kir6.1+SUR1 generated only few channels active in the CAP configuration (0.6±0.3 channels/patch; $\gamma$ 30.5±2.8 pS). EYFP\Kir6.2 alone -but not EYFP\Kir6.1- also resulted in channel activity in I-O patches (2.1±0.9 channels/patch), characterised by very brief openings and a $\gamma$ of 62.4±2.9 pS.

Conclusion: Rabbit Kir6.2 can traffic to the plasma membrane and form functional K$_{ATP}$ channels in the absence of SUR. Co-expression with SUR1 increases channel density and alters the kinetic properties of Kir6.2. In contrast, Kir6.1 showed an intracellular distribution and a high degree of co-localisation with a mitochondrial dye, suggesting that Kir6.1 may form part of the mitochondrial K$_{ATP}$ channel complex.
Figure 1. Panel A (top) shows a HEK293 cell containing both wild-type GFP and Mitotracker® Red. GFP (green) is distributed evenly throughout the cell and the mitochondria are labeled in red. Panel B shows two HEK293 cells which contain both Mitotracker® Red and Green. The fluorograms with the red and green colocalisation coefficients for both these cells are depicted in panel C.
Introduction

Activation of ATP-sensitive potassium channels (K\textsubscript{ATP}) protects the myocardium during myocardial ischemia and preconditioning (Grover and Garlid 2000, Duncker and Verdouw 2000). Recent attention has focused on K\textsubscript{ATP} channels in the inner membrane of mitochondria and these mitochondrial K\textsubscript{ATP} channels (mitoK\textsubscript{ATP}) are thought to play an important role in cardioprotection during myocardial ischemia and preconditioning (Garlid et al. 1997, Sato and Marban 2000, Grover and Garlid 2000). On the other hand, other studies suggest that both sarcolemmal and mitochondrial K\textsubscript{ATP} channels are involved and together may contribute to myocardial protection (Tanno et al. 2001, Sasaki et al. 2001).

The K\textsubscript{ATP} channel complex consists of two different subunits, an inwardly rectifying potassium channel (either Kir\textsubscript{6.1} or Kir\textsubscript{6.2}) and a sulfonylurea receptor (SUR\textsubscript{1}, SUR\textsubscript{2A} or SUR\textsubscript{2B}) (see Seino 1999). K\textsubscript{ATP} channels assemble as tetramers ([SUR/Kir\textsubscript{6.x}]\textsubscript{4}) and Kir\textsubscript{6.1} and Kir\textsubscript{6.2} are generally considered not to be functional in the absence of SUR (Aguilar-Bryan et al. 1998, Seino 1999). The subunit combination determines the K\textsubscript{ATP} channel type: classical sarcolemmal cardiac K\textsubscript{ATP} channels are composed of (Kir\textsubscript{6.2}/SUR\textsubscript{2A})\textsubscript{4}, pancreatic K\textsubscript{ATP} channels of (Kir\textsubscript{6.2}/SUR\textsubscript{1})\textsubscript{4} and vascular smooth muscle K\textsubscript{ATP} channels of (Kir\textsubscript{6.1}/SUR\textsubscript{2B})\textsubscript{4} (Inagaki et al. 1995, Sakura et al. 1995, Inagaki et al. 1996, Yamada et al. 1997, Okuyama et al. 1998). The molecular structure of mitoK\textsubscript{ATP} is unknown. Although some studies showed that Kir\textsubscript{6.1} may form part of the mitoK\textsubscript{ATP} channel complex, contradictory results have also been reported (Suzuki et al. 1997\textsubscript{a}, Liu et al. 2001, Seharaseyon et al. 2000\textsubscript{b}). Therefore, the molecular composition of the mitoK\textsubscript{ATP} channel remains an area of interest.

K\textsubscript{ATP} channel assembly takes place in the endoplasmic reticulum (ER), after which the completed polypeptide is transported to the Golgi complex for post-translational modification such as N-linked glycosylation (Griffith 2001, Khanna et al. 2001). Finally, the protein is inserted into transport vesicles and directed to the plasma membrane. An ER retention signal present on both Kir\textsubscript{6.x} and SUR subunits has been proposed to inhibit the effective trafficking from the ER to the Golgi and thus prevent transportation to the plasma membrane of either subunit when expressed in the absence of the other (Zerangue et al. 1999). Also, truncation of Kir\textsubscript{6.2} by deletion of the distal C-terminal 26 or 36 amino acids, would remove the retention signal motif and thus explain the observed functional channel activity of truncated Kir\textsubscript{6.2} in the absence of SUR (Tucker et al. 1997, Zerangue et al. 1999). On the other hand, both independent trafficking and functional channel expression of wild-type untruncated Kir\textsubscript{6.2} in the absence of SUR have been reported (Makhina and Nichols 1998, John et al. 1998).
Subcellular trafficking of Kir6.1 and Kir6.2

In this study we have evaluated subcellular trafficking and functional expression of both Kir6.1 and Kir6.2 in the presence and absence of SUR1. Our results indicate that Kir6.2 can traffic to the plasma membrane and form functional channels independent of the presence of SUR1. In contrast, plasma membrane localisation for Kir6.1 was only observed in the presence of SUR1. When transfected alone, Kir6.1 showed an intracellular distribution with a high degree of co-localisation with mitochondria, suggesting that Kir6.1 may form part of the mitochondrial K<sub>ATP</sub> channel complex.

Methods

**Generation of fluorescent fusion protein constructs**
To study the intracellular localisation of both rabbit heart Kir6.1 and Kir6.2 in the mammalian cell line HEK293 (Human Embryonic Kidney), we constructed fusion proteins of each one using the expression vector pEYFP-N1 (Clontech), as described in the Materials and Methods section. Briefly, Kir6.1 and Kir6.2 were inserted into the multiple cloning site (MCS) in the pEYFP-N1 vector located 5’ to the coding sequence of EYFP as follows. The stop codon at the end of the Kir6.1 and Kir6.2 cDNA was abolished and degenerate primers were designed, containing additional amino acids and introducing specific restriction sites, to facilitate the final ligation into the pEYFP-N1 vector. cDNA encoding Kir6.1 or Kir6.2 was ligated into the multiple cloning site (MCS) of pEYFP-N1 such as to encode a protein consisting of Kir6.1 or Kir6.2 with EYFP fused at the carboxyl end.

**Confocal imaging**
For confocal imaging, 100-300 ng of pEYFP-N1\Kir6.1 or pEYFP-N1\Kir6.2 was transfected with or without 1-2 μg human SUR1 (a kind gift from Dr. M. Nishimura) into HEK293 cells using Lipofectamine Reagent (Gibco BRL, Life Technologies 18324-012), according to manufacturer's instructions. Transfected cells were cultured as described in Chapter 7. After 1-2 days in culture, the transfected cells were trypsinised and added to a 12-well plate containing a small plastic coverslip. After 2-3 hours at 37°C, the majority of cells had attached themselves to the coverslip, which was subsequently placed upside down on a glass slide for confocal analysis. The mitochondrial-selective dye MitoTracker® Red CM-H<sub>2</sub>Xros (Molecular Probes M-7513) was added to the medium for 30-45 minutes at 37°C, prior to removal of the coverslip (final concentration 1 μM). In a subset of untransfected cells, both mitochondrial-selective dyes MitoTracker® Red and MitoTracker® Green were added at a final concentration of 1 μM.
in the medium. Confocal imaging was performed using a confocal laser scanning microscope (BioRad MRC1024) equipped with a 15 mV Krypton/Argon laser (scanning enlargement 20-40x), using the 568 and 488 excitation lines and 605DF32 and 522DF35 emission filters for MitoTracker® Red and EYFP, respectively. For simultaneous visualisation of MitoTracker® Red and EYFP, dual-colour red and green images respectively were recorded. To measure the degree of co-localisation between MitoTracker® Red and EYFP, the degree of spatial overlap between the two channels of the dual-colour image was analysed and both red and green co-localisation coefficients were calculated (Manders et al. 1993). After correction for background fluorescence, a 2D fluorogram was constructed indicating the distribution of all pixels in the 2D intensity space. The green (red) co-localisation coefficient is the ratio of the sum of the co-localised green (red) pixel intensities to the sum of all the green (red) pixel intensities. If no co-localisation is present, this ration is 0, whereas a ration of 1 indicates overlap between all red and green pixels. To evaluate this method, co-localisation coefficients were measured in cells containing both mitochondrial-selective dyes MitoTracker® Red and Green.

**Electrophysiological analysis**

For electrophysiology, 1-2 μg of pEYFP-N1/Kir6.1 or pEYFP-N1/Kir6.2 was transfected in either HEK293 or CHO cells, with or without human SUR1 (1-2 μg). Transfection and cell culture were performed as described earlier. Single-channel currents were recorded at room temperature in the cell-attached or inside-out configuration of the patch clamp technique, using an Axopatch 200B amplifier (Axon Instruments) and the following solutions. Pipette solution: 145 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ and 10 mM HEPES (pH adjusted to 7.4 with KOH). Bath solution contained 145 mM KCl, 1 mM MgCl₂, and 10 mM HEPES (pH adjusted to 7.2 with KOH); 1 μM ATP was added to prevent channel run-down. Patch electrodes were made of borosilicate glass on a home-made one-stage puller. The tips of the electrodes were heat-polished and, after filling with pipette solution, had a tip resistance of 2-3 MΩ. Single-channel currents were measured at various potentials from −60 to +80 mV, and unitary currents were obtained from amplitude histograms. Single-channel data were filtered at 500 Hz (low-pass) and digitised at 2 kHz. Voltage control, data acquisition and analysis were accomplished by use of custom-made software.

**Statistical analysis**

Data are presented as mean±SEM. Differences in mean single-channel conductance and number of channels per patch between groups was analysed using the Student’s t-test. A p-value of <0.05 was considered statistically significant.
Subcellular trafficking of Kir6.1 and Kir6.2

Results

Confocal imaging of MitoTracker® Red and Green
Figure 1A (page 161) shows confocal images of a HEK293 cell transfected with pEGFP-N1 and coloured with the mitochondrion-selective dye MitoTracker® Red. The green fluorescent protein is distributed evenly throughout the cytoplasm of the cell and clearly indicates the outlines of the cell. The mitochondria are distributed throughout the cytoplasm. In Figure 1B, untransfected HEK cells are shown which contain both MitoTracker® Red and MitoTracker® Green. Areas of overlap between the red and green dyes are appear yellow. Green or red areas may indicate absence of co-localisation or local intensity differences in both dyes. Thus, visual evaluation of co-localisation may be hampered by technical as well as biological variations and therefore the degree of spatial overlap between the two channels of the dual-colour image was analysed. Figure 1C shows the 2D fluorogram of the dual-colour image for a cell containing both MitoTracker® Red and Green. Theoretically, non-overlapping pixels are located near the axes and co-localising pixels are clustered around the diagonal line at 45° (y=x). In this case, most pixels are found around a diagonal line and thus a high degree of co-localisation is predicted. Indeed, the co-localisation coefficients for red and green are 0.83-0.98 and 0.95-0.99 respectively, indicating that 83-98% of all red pixels co-localise with green and 95-99% of all green pixels co-localise with red. Thus, as expected, the degree of co-localisation between MitoTracker® Red and Green is very high.

Confocal imaging of YFP-con structs
When the Kir6.2 fusion protein (EYFP-N1/Kir6.2) was co-transfected with SUR1 into HEK cells, fluorescence was clearly observed on the plasma membrane and both red (MitoTracker® Red) and green (EYFP) co-localisation coefficients are low (Figure 2). Similarly, EYFP-N1/Kir6.1 transfected together with SUR1 also showed plasma membrane localisation, although the fluorescent signal appeared less intense compared to EYFP-N1/Kir6.2+SUR1. When the same amount of EYFP-N1/Kir6.2 (200 ng) was transfected in the absence of SUR1, no plasma membrane localisation was observed and the fluorescent signal was located inside the cytoplasm (Figure 3). However, when the amount of transfected DNA for EYFP-N1/Kir6.2 was increased, fluorescence was localised at the plasma membrane, indicating that Kir6.2 is targeted to the cell membrane independently of SUR1. In contrast, no plasma membrane labelling was observed in cells transfected with EYFP-N1/Kir6.1 in the absence of SUR1, irrespective of the amount of DNA used (Figure 3). Instead, EYFP-N1/Kir6.1 showed an intracellular distribution and appeared to co-localise with MitoTracker® Red. Indeed, the fluorogram showed a clustering of points around a diagonal line and the green and red co-localisation...
coefficients were 0.98 and 0.33 respectively, suggesting that nearly all of EYFP-N1\Kir6.1 co-localises with mitochondria, whereas roughly a third of all mitochondria contain the EYFP-N1\Kir6.1 signal. Although this may suggest that Kir6.1 is targeted to the mitochondria, a similar degree of co-localisation was also observed in cells transfected with a low concentration of EYFP-N1\Kir6.2 (Figure 3). However, the degree of co-localisation decreased dramatically for EYFP-N1\Kir6.2 when more DNA was transfected, and this was not observed for EYFP-N1\Kir6.1. The differences in subcellular distribution and membrane targeting between Kir6.2 and Kir6.1 are summarised in Table 1.

Electrophysiological analysis of YFP constructs

In mock transfected and untransfected cells, some endogenous channel activity of about 10-20 pS was observed, which was insensitive to ATP and showed no inward rectification. Co-transfection of EYFP-N1\Kir6.2 and SUR1 resulted in typical K_{ATP} channel activity in 5 of 8 cells with 32.8±15.4 (mean±SEM) channels per patch.

Figure 4. Single channel characteristics of EYFP-N1\Kir6.2+SUR1: current traces at various holding potentials (A), I/V-relationship indicating weak inward rectification (B), and effects of patch excision (IO) and application of 1 mM ATP (C)
Figure 5. Single channel characteristics of EYFP-N1\Kir6.2 alone: effects of patch excision (IO) and application of 1 mM ATP (A), current traces at various holding potentials (B) and I/V-relationship indicating weak inward rectification (C)
Figure 2. Confocal analysis of EYFP-N1 Kir6.2 (top) and EYFP-N1 Kir6.1 (bottom) transfected in HEK293 cells together with SUR1. Fluorograms with colocalisation coefficients are shown as insets.
Figure 3. Confocal analysis of EYFP-N1/Kir6.2 (top) and EYFP-N1/Kir6.1 (bottom) transfected in HEK293 cells in the absence of SUR1. The amount of DNA for both constructs is noted. Fluorograms with colocalisation coefficients are shown as insets. With increasing concentration of transfected DNA, EYFP/Kir6.2 fluorescence was visible at the plasma membrane. In contrast, EYFP/Kir6.1 was never observed at the plasma membrane but remained intracellular, irrespective of the amount of DNA transfected.
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Channel activity increased upon patch excision in low ATP concentration and decreased after application of 1 mM ATP to the bath (Figure 4A). These channels had a mean single channel conductance in the negative voltage range of 58.3±1.4 pS (mean±SEM) and showed weak inward rectification, similar to wild-type Kir6.2+SUR1 described in Chapter 7 (Figure 4B/C). The number of channels observed per patch was also not different from wild-type Kir6.2+SUR1 (Table 1). In HEK293 and/or CHO cells transfected with only EYFP-N1/Kir6.2, channel activity was also observed upon patch excision in 5 out of 12 cells (2.1±0.9 channels/patch) (Figure 5A). These channels showed weak inward rectification and had a single channel conductance of 62.4±2.9 pS, which is similar to EYFP-N1/Kir6.2 in the presence of SUR1 (Figure 5C). However, the channel openings observed for EYFP-N1/Kir6.2 alone were quite different in that they were of short duration and had a spiky appearance (Figure 5B). These characteristics were similar to those of wild-type Kir6.2 without SUR1 described in Chapter 7. Although there was a trend towards a higher number of channels observed per patch compared to wild-type Kir6.2, this difference was not significant (Table 1).

Figure 6. Single channel characteristics of EYFP-N1/Kir6.1+SUR1 in cell-attached mode: current traces at various holding potentials (A) and I/V relationship showing weak inward rectification and a single-channel conductance of 32.8 pS (B)
Subcellular trafficking of Kir6.1 and Kir6.2

No differences in channel characteristics were observed between HEK293 and CHO cells. In contrast to EYFP-N1\Kir6.2, no functional channel activity was observed for EYFP-N1\Kir6.1 when transfected without SUR1 in either HEK293 or CHO cells. When SUR1 was co-transfected, channel activity was observed in cell-attached patch in 4 out of 13 cells, which did not increase upon patch excision (0.6±0.3 channels/patch) (Figure 6A). Mean single channel conductance for EYFP-N1\Kir6.1+SUR1 was 30.5±2.8 pS and weak inward rectification was present (Figure 6B). Again, no differences in channel characteristics between EYFP-N1\Kir6.1+SUR1 and wild-type Kir6.1+SUR1 (see Chapter 7) were observed. In addition, the number of channels observed per patch was similar compared to wild-type Kir6.1+SUR1 (Table 1).

Discussion

In the present study, we evaluated the intracellular distribution of K\textsubscript{ATP} channel subunits Kir6.1 and Kir6.2 by confocal imaging of fluorescent fusion proteins transfected into HEK293 cells. Plasma membrane ion channels are assembled at the endoplasmic reticulum (ER), where transmembrane folding of specific segments across the membrane occurs. The completed polypeptide chains then move to the Golgi complex for posttranslational modification (i.e. N-glycosylation, the addition of N-linked oligosaccharide chains) and are finally incorporated into transport vesicles which fuse with the plasma membrane (see Griffith 2001). In mammalian proteins, an ER retention signal has been described which prevents the effective transport of the protein from the ER to its final destination (Zerangue et al. 2001). This retention signal, RKR (Arg-Lys-Arg), is present in both SUR and Kir\textsubscript{x} subunits of the K\textsubscript{ATP} channel and was proposed to prevent their independent functional expression (Zerangue et al. 1999). Co-expression of SUR with Kir6.x was thought to conceal the retention signal allowing for functional expression of the channel. Similarly, truncation of Kir6.2, thereby removing the RKR motif, also resulted in cell surface expression of Kir6.2 in the absence of SUR (Tucker et al. 1997, Zerangue et al. 1999). Contrary to these findings, our confocal imaging data indicate that untruncated Kir6.2 is capable of independent trafficking to the plasma membrane (i.e. in the absence of SUR1, in accordance with previous results reported by Makhina and Nichols (1998). Similarly, we showed that Kir6.2 also forms functional channels by itself and apparently does not require the presence of SUR1 to “override” the retention signal. Thus, the functional relevance of the RKR motif as an ER retention signal seems questionable, although it may still be involved in the regulation of channel expression efficiency. The functional characteristics, such as single-channel conductance
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<th>number of channels/patch</th>
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<td>WT Kir6.2+SUR1</td>
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<td>50.2 ± 3.0</td>
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<td>2.1 ± 0.9</td>
<td>62.4 ± 2.9</td>
<td>+</td>
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<td>34.3 ± 5.6</td>
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<td>30.5 ± 2.8</td>
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<td>EYFP-N1\Kir6.1 alone</td>
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Table 1. Summary of electrophysiological characteristics and plasma membrane localisation of wild-type (WT) and YFP constructs of Kir6.1 and Kir6.1 in the presence and absence of SUR1 (data presented as mean±SEM)

and inward rectification, of the fusion protein EYFP-N1\Kir6.2 are similar to the wild-type Kir6.2 described in Chapter 7. Therefore, the YFP fused to the C-terminal end of Kir6.2 does not seem to interact with the channel pore, nor can it explain the independent functional expression and membrane trafficking of EYFP-N1\Kir6.2, since we have also observed this for wild-type Kir6.2 (Chapter 7). Co-expression of SUR1 with EYFP-N1\Kir6.2 greatly enhanced the number of channels and the fluorescent signal on the cell surface. Thus, the co-assembly of these subunits somehow results in a more efficient transportation to and/or insertion in the plasma membrane. The C-terminal sequence motif (Phe-X-Tyr-Glu-Asp-Glu-Val) in Kir2.1 reported to be both necessary and sufficient for efficient export from the ER to the cell surface, is not present in either Kir6.2 or Kir6.1 (Stockklausner et al. 2001, Ma et al. 2001) (see Chapter 7, Figure 2).

Originally, the protective effect of K<sub>ATP</sub> channel opening was thought to act through sarcolemmal K<sub>ATP</sub> channel (sarcK<sub>ATP</sub>) activation, leading to action potential shortening, reduced calcium influx into the cell resulting in a more favourable energy homeostasis and metabolic state for the myocyte. With the discovery of a K<sub>ATP</sub> channel in the inner mitochondrial membrane, a potential additional site of action was introduced (Inoue et al. 1991). The mechanism of cardioprotection by mitochondrial K<sub>ATP</sub> (mitoK<sub>ATP</sub>)
channel activation still remains unclear, but matrix swelling, mitochondrial calcium homeostasis and production of reactive oxygen species are thought to play a role (Vanden Hoek \textit{et al.} 1998, Wang \textit{et al.} 2001, Gross 2000). Recent studies have indicated that both sarcK\textsubscript{ATP} and mitoK\textsubscript{ATP} channels are involved in the cardioprotection afforded by potassium channel openers and preconditioning, possibly through interaction between the two channel types (Tanno \textit{et al.} 2001, Sanada \textit{et al.} 2001, Sasaki \textit{et al.} 2001). Two possibilities were proposed: 1) sarcK\textsubscript{ATP} activation may reduce the cytosolic level of endogenous mitoK\textsubscript{ATP} inhibitors and/or 2) trigger a signalling pathway for mitoK\textsubscript{ATP} activation (Paucek \textit{et al.} 1996, Tanno \textit{et al.} 2001, Sasaki \textit{et al.} 2001). For more detailed analysis of the role of either channel in cardioprotection, knowledge of their structural features is essential. However, although the subunit composition underlying the sarcK\textsubscript{ATP} channel is now known, the molecular structure of the mitochondrial channel has not yet been identified. Since sarcK\textsubscript{ATP} and mitoK\textsubscript{ATP} channels share many electrophysiological and pharmacological properties, mitoK\textsubscript{ATP} channels are also thought to consist of SUR and Kir subunits. Two components of mitoK\textsubscript{ATP} have been identified through labelling with bodipy-glyburide, a 55 kD channel protein and a 63 kD SUR (Grover and Garlid 2000). In rat skeletal muscle mitochondrial fractions, a 51 kD band was observed with immunoblot analysis using a Kir6.1 antibody and immunofluorescence staining showed Kir6.1 labelling on the inner mitochondrial membrane (Suzuki \textit{et al.} 1997c). In addition, the subunit combination Kir6.1/SUR1 shares pharmacological similarities with the native mitoK\textsubscript{ATP} channel (Liu \textit{et al.} 2001). On the other hand, immunohistochemistry with an anti-Kir6.1 antibody in ventricular myocytes did not show co-localisation with mitochondria (Scharaseyon \textit{et al.} 2000). Thus, it seems undecided whether or not Kir6.1 forms part of the mitoK\textsubscript{ATP} channel complex.

Proteins destined for the mitochondrial membranes are often recognised through specific signal peptides within their amino acid sequence (Lithgow 2000). Although neither Kir6.1 or Kir6.2 contain such a signal peptide sequence (see Chapter 7), little is known about these signal motifs in ion channels. In the present study, we found that Kir6.1 in the absence of SUR1 is localised within the cytoplasm. Furthermore, a high degree of co-localisation was observed between the fusion protein EYFP-N1\textbackslash Kir6.1 and the mitochondrion-selective dye MitoTracker\textsuperscript{®} Red, suggesting that Kir6.1 is located within the mitochondria. However, a similar degree of co-localisation was also observed in a minority of cells containing EYFP-N1\textbackslash Kir6.2, although only when transfected in low concentrations. A possible explanation for this finding may be that the resolution of the method used is insufficient to discriminate between the various intracellular structures. The pixel size of the confocal images obtained in our experiments was calculated to be about 0.6 \textmu m. Since mammalian mitochondria have a diameter of about
0.5-1.0 μm, it is possible that one single pixel represents two separate adjacent organelles, for example a mitochondrion and part of the ER. Therefore, this method may not be sensitive enough to draw any major conclusions concerning the putative localisation of Kir6.1 within the mitochondrial membrane. Nevertheless, the results from the present study indicate that the K\textsubscript{ATP} channel subunits Kir6.1 and Kir6.2 behave differently with regards to subcellular trafficking and membrane targeting. Furthermore, our results are in accordance with the possibility that Kir6.1 forms part of the mitoK\textsubscript{ATP} channel complex.

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