The ATP-sensitive potassium channel in the heart. Functional, electrophysiological and molecular aspects

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Chapter 7

Structural and functional differences between $K_{\text{ATP}}$ channel subunits Kir6.1 and Kir6.2 isolated from rabbit heart

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Chapter 7

Introduction

Myocardial ATP-sensitive potassium channels (KATP) are closed during physiological conditions but are activated during ischemia when the intracellular ATP-level is sufficiently decreased (Noma 1983). Activation of these channels during ischemia constitutes an intrinsic cardioprotective mechanism, resulting in delayed onset of irreversible damage, decreased infarct size and faster recovery of function (reviewed by Grover and Garlid 2000). Furthermore, these channels also play an important role in ischemic preconditioning (Duncker and Verdouw 2000).

The KATP channel complex consists of two different subunits, an inwardly rectifying potassium channel (Kir6.x) and a sulfonyleurea receptor (SUR) (see Seino 1999). Two Kir6.x isoforms, Kir6.1 (mKATP-1) and Kir6.2 (BIR) and three major SUR isoforms (SUR1, SUR2A and SUR2B) have been cloned so far (Inagaki et al. 1995a, Inagaki et al. 1995b, Sakura et al. 1995, Chutkow et al. 1999). KATP channels assemble as tetramers ([SUR/Kir6.x]₄) and the subunit composition determines the KATP channel subtype and its properties. Kir6.1 and Kir6.2 are generally considered not to be functional in the absence of SUR (Aguilar-Bryan et al. 1998, Seino 1999). The classical sarcolemmal cardiac KATP channel consists of 4 Kir6.2 and 4 SUR2A subunits, whereas pancreatic KATP channels are composed of (Kir6.2/SUR1)₄ (Inagaki et al. 1995b, Sakura et al. 1995, Inagaki et al. 1996, Okuyama et al. 1998). Channels composed of Kir6.2 show weak inward rectification and have a conductance of about 70-80 pS under symmetrical potassium concentrations (Inagaki et al. 1995b, Alekseyev et al. 1997, Babenko et al. 1998, Hu et al. 1999). In contrast, Kir6.1-based channels are active under normal conditions, i.e. at physiological ATP concentration, and have a smaller channel conductance (33-36 pS) compared to channels composed of Kir6.2 (Yamada et al. 1997, Takano et al. 1998, Kono et al. 2000). The only combination resulting in channel properties resembling a known physiological KATP channel consists of Kir6.1/SUR2B, which resembles the KATP channel found in vascular smooth muscle (Yamada et al. 1997). However, Kir6.1 is also present in cardiomyocytes (Mederos y Schnitzler et al. 2000), where its role is not yet known. Recent evidence suggests that Kir6.1 may form part of the mitochondrial KATP channel, but conflicting data have been reported (Suzuki et al. 1997, Seharasevon et al. 2000b). Therefore, to gain more insight into the role of Kir6.1 in the myocardium, we have isolated both Kir6.1 and Kir6.2 from a rabbit heart cDNA library and compared their structural characteristics, regional distribution in the heart and electrophysiological properties. Our results show that Kir6.1 and Kir6.2 share many structural characteristics but differ in functional properties. Although Kir6.1 expression is abundant in both atrial and ventricular tissue, its functional role remains to be elucidated.
Structure and function of Kir6.1 and Kir6.2

Methods

cDNA library construction and cDNA cloning
A cDNA library was made from rabbit heart tissue (New Zealand White rabbits, 2.0-3.0 kg) in the λ ZAP Express vector (Stratagene), as outlined in the Materials and Methods section. 1 x 10^6 plaques were screened by hybridisation with a mix of ^32P-labeled partial rat Kir6.1 cDNA fragment (GenBank Accession no. D42145) and rabbit Kir6.2 cDNA (AF006262) used as a probe. Positive colonies were rescreened and the recombinant phage vector was rescued using the Ex-Assist helper phage. Both strands of the positive clones were sequenced using the Big Dye Termination procedure, revealing them to be rabbit heart Kir6.1 and rabbit heart Kir6.2, respectively.

Protein architecture, homology comparison, and structural analysis
After nucleotide sequencing of the isolated clones, the coding sequences encoding the amino acids for both clones were determined using the Open Reading Frame (ORF) Finder programme from NCBI and Kozak sequence analysis (for details, see Chapter 2). Structural and functional features of both Kir6.1 and Kir6.2 proteins, such as transmembrane segments, signal peptides and biologically significant regions or residues were evaluated using the Simple Modular Architecture Research Tool (SMART) and the PROSITE database (ScanProsite). Homology comparison between Kir6.1 and Kir6.2 and multiple sequence alignment between various inward rectifier (Kir) channels was performed using the BLAST (Basic Local Alignment Search Tool) and CLUSTALW multiple alignment programmes, respectively.

Northern blot analysis
For RNA isolation, rabbit heart tissue was separated in atrial, left and right ventricular tissue, frozen in liquid nitrogen and stored at −80°C. RNA was isolated through a CsCl-cushion during overnight centrifugation (for details, see Chapter 2). For Northern blot analysis, 10 μg of total RNA was blotted on a nylon membrane (Hybond-N, Amersham) and hybridised under stringent conditions (65°C) with ^32P-labelled, full-length rabbit heart Kir6.1 or Kir6.2.

Expression and electrophysiological analysis
1-2 μg of pBK-CMV containing full-length cDNAs of rabbit heart Kir6.2 or Kir6.1 (isolated clones A and C respectively) was transfected together with 1 μg pEGFP-N1 (as a marker for successful transfection) in HEK293 and CHO cells using Lipofectamine Reagent (Gibco BRL, Life Technologies 18324-012), according to manufacturer's instructions. In part of the experiments, both rabbit Kir6.1 and Kir6.2 were transfected
together in equal amount (1-2 μg). Unless mentioned otherwise, human SUR1 (1-2 μg) was co-transfected. In addition, a subset of both HEK and CHO cells were either not transfected at all or transfected with 1 μg pEGFP-N1 only, to serve as a negative control. Transfected HEK293 cells were cultured in Minimum Essential Medium containing Earle's salts and L-glutamine (Gibco BRL 11095) supplemented with non-essential amino acid solution, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a 5% CO₂ incubator at 37°C for 1-2 days. Similarly, CHO cells were cultured in F-12 Nutrient Mixture (Ham) containing L-alanyl-L-glutamine (Gibco BRL 31765) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. 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). 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. The pipette solution contained 145 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ and 10 mM HEPES (pH adjusted to 7.4 with KOH). The 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. To test channel inhibition by ATP after patch excision, the bath was perfused with bath solution containing 1 mM ATP. Single-channel currents were measured at various holding potentials between -60 mV and +80 mV, and unitary current amplitudes 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 performed using 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.

**Results**

**Sequence analysis and structural features**

After rabbit heart cDNA library screening, three positive plaques (A, B and C) were isolated and rescreened. Clones A and B were found to be identical. Sequence analysis of clone A (2785 bp=basepairs) revealed an 1170-bp open reading frame preceded by three in-frame termination codons, which encoded a protein of 390 amino acids, and overall
Figure 1. Structure of rabbit Kir6.1 and Kir6.2 proteins and location of phosphorylation, myristoylation and/or glycosylation sites (Φ).
<table>
<thead>
<tr>
<th>Species</th>
<th>Protein Family</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Kir6.1</td>
<td>RKRK</td>
</tr>
<tr>
<td></td>
<td>Kir6.2</td>
<td>RKRK</td>
</tr>
<tr>
<td>Human</td>
<td>Kir1.1</td>
<td>RKRK</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>Kir2.1</td>
<td>RKRK</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>Kir3.1</td>
<td>RKRK</td>
</tr>
</tbody>
</table>

**Figure 2.** Amino acid sequence homology between rabbit heart Kir6.1 and Kir6.2, human Kir1.1, human Kir5.1, guinea pig Kir2.1 and guinea pig Kir3.1. Identical amino acids among Kir family members are boxed in black and conserved substitutions are boxed in grey. The transmembrane domains M1 and M2 and the pore region H5/P are lined, the putative ATP-binding site in Kir1.1 is denoted by an asterisk and potential glycosylation sites on Kir6.1 are indicated by $.
### Table 1. Amino acid positions of protein kinase C (PKC), casein kinase II, and cAMP/cGMP-dependent (PKA) phosphorylation sites, and N-glycosylation and N-myristoylation sites in Kir6.1 and Kir6.2 of various species

<table>
<thead>
<tr>
<th>Protein kinase C:</th>
<th>Rabbit</th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
<th>Guinea</th>
<th>Pig</th>
</tr>
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<tbody>
<tr>
<td>T10</td>
<td>TEK</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>224</td>
<td>SVR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>345</td>
<td>TMK</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>354</td>
<td>SAR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>379</td>
<td>SLR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>385</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>391</td>
<td>SNT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

| cAMP/cGMP: | 231  | RKTT  | +   | +   | +     | +   |
|           | 329  | SNT    | +   | +   | +     | +   |

| Casein kinase II: | 61  | TLVD  | +   | +   | +     | +   |
|                  | 108 | SGME  | +   | +   | +     | +   |
|                  | 113 | SALE  | +   | +   | +     | +   |
|                  | 234 | TSTE  | +   | +   | +     | +   |
|                  | 329 | TEE   | +   | +   | +     | +   |
|                  | 385 | SNT    | +   | +   | +     | +   |

| N-myristoylation: | 159 | GMIESA | +   | +   | +     | +   |
|                   | 114 | GLESTV | +   | +   | +     | +   |
|                   | 166 | GELMINA | +   | +   | +     | +   |
|                   | 298 | GVVETT | +   | +   | +     | +   |
|                   | 417 | GNQNTS | +   | +   | +     | +   |

| N-glycosylation: | 389 | NNSM  | +   | +   | +     | +   |
|                  | 395 | NNSNI | +   | +   | +     | +   |
|                  | 401 | NNSS  | +   | +   | +     | +   |
|                  | 402 | NSSL  | +   | +   | +     | +   |
|                  | 420 | NTSE  | +   | +   | +     | +   |

| Protein kinase C: | 3    | SRK   | +   | +   | +     | +   |
|                  | 37   | SSK   | +   | +   | +     | +   |
|                  | 190  | TLR   | +   | +   | +     | +   |
|                  | 356  | TVK   | +   | +   | +     | +   |
|                  | 345  | TAR   | +   | +   | +     | +   |
|                  | 363  | SAR   | +   | +   | +     | +   |

| cAMP/cGMP: | 221  | RKTT  | +   | +   | +     | +   |
|           | 369  | RRS   | +   | +   | +     | +   |

| Casein kinase II: | 62  | TLVD  | +   | +   | +     | +   |
|                  | 224 | TSTE  | +   | +   | +     | +   |
|                  | 354 | SLID  | +   | +   | +     | +   |

showed 98% nucleotide homology with rabbit Kir6.2 (AF006262). Clone C (2252 bp) showed 86% nucleotide homology with rat and human Kir6.1 and encoded a 424-amino acid protein. Clone A was designated rabbit heart Kir6.2 and clone C was designated rabbit heart Kir6.1; they share 78% nucleotide homology. Hydropathy analysis revealed that both rabbit heart Kir6.1 and Kir6.2 contain two membrane-spanning domains, M1 and M2, in between which a pore-forming region H5 is located which is present in all potassium channels. Using the SMART and PROSITE databases, both Kir6.1 and Kir6.2 sequences were screened for the presence of amino acids sequences predicted to be of functional relevance. No signal peptides, coiled coil regions or internal repeats were present in either Kir6.1 or Kir6.2 according to the SMART programme. Rabbit Kir6.2 contained five putative protein kinase C (PKC) phosphorylation sites in addition to three putative cAMP/cGMP-dependent protein kinase phosphorylation, three casein kinase II phosphorylation and three N-myristoylation sites (Figure 1). Rabbit Kir6.1
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contained six potential protein kinase C (PKC) phosphorylation, two cAMP/cGMP-dependent protein kinase phosphorylation, six casein kinase phosphorylation and four N-myristoylation sites. In contrast to Kir6.2, the rabbit Kir6.1 sequence also predicted five potential N-glycosylation sites in the distal part of the C-terminal region (Figure 1). Interestingly, not all of the sites in rabbit Kir6.1 and Kir6.2 were found to be conserved in other species such as human, rat, mouse and guinea pig (Table 1). Overall, rabbit Kir6.1 and Kir6.2 showed 89% and 92% nucleotide homology to human, 87% and 93% to rat, 88% and 93% to mouse, and 88% and 92% to guinea pig Kir6.1 and Kir6.2, respectively. The guinea-pig Kir6.2 protein contained one PKC-phosphorylation site less compared to rabbit Kir6.2. For Kir6.1, only the human protein contained the same five N-glycosylation sites as rabbit Kir6.1, whereas in rat, mouse and guinea pig at least one of these sites was absent. Both rat and mouse Kir6.1 lacked two N-myristoylation sites observed in the rabbit gene. Conversely, other phosphorylation and/or myristoylation sites observed in both Kir6.1 and Kir6.2 of other species were absent in rabbit (Table 1). In general, the amino acid composition variation between species was more pronounced for Kir6.1 as compared to Kir6.2.

Figure 2 shows multiple sequence alignment of both rabbit Kir6.1 and Kir6.2 as compared to other members of the inward rectifier potassium (Kir) family. A high degree of homology between Kir family members existed in the transmembrane domains M1 and M2 and the pore-forming region H5/P. In contrast to other potassium channels, both rabbit Kir6.1 and Kir6.2 contain the amino acids Gly-Phe-Gly (GFG) instead of Gly-Tyr-Gly (GYG) within their H5 region, corresponding to the weak inward rectifying properties of the KATP channel (Shyng et al. 1997b). It is of interest to note that neither Kir6.1 nor Kir6.2 contain a putative ATP-binding site such as the Walker A type motif Gly-Xa-Gly-Lys (GXaGK, where X is any amino acid) observed in Kir1.1 (denoted by asterisk in Figure 2). Furthermore, it is clear that the distal C-terminal region of Kir6.1 is unique and a similar sequence containing multiple putative N-glycosylation sites (marked ψ) was not observed in either Kir6.2 or other Kir family members. In addition, the extracellular region between M1 and H5 is considerably longer in Kir6.1 compared to Kir6.2 (see also Figure 1).

Expression of Kir6.1 and Kir6.2 in rabbit heart

Northern blot analysis (Figure 3) showed ubiquitous expression of two transcripts of approximately 2.3 and 1.5 kb for rabbit heart Kir6.1 at similar intensities for both atrial and ventricular tissue. In contrast, Kir6.2 showed only one transcript of approximately 2.7 kb with a high level of expression in atrial tissue, but surprisingly, only moderate expression in both left and right ventricle. The transcript sizes of both Kir6.1 and Kir6.2
Structure and function of Kir6.1 and Kir6.2

Electrophysiological analysis
In cells transfected with pEGFP-N1 alone and in untransfected cells, some endogenous channel activity was observed of about 10-20 pS, which was insensitive to ATP and showed no inward rectification. Transfection with Kir6.2 and SUR1 cDNA in HEK cells resulted in functional K\textsubscript{ATP} channel activity, which was observed upon patch excision in low ATP concentration in 6 out of 8 cells (38.1±17.8 channels/patch, mean±SEM). These channels had a single-channel conductance of 61.4 ±3.2 pS (mean±SEM, n=5), showed weak inward rectification and were inhibited by the application of 1 mM ATP (Figure 4). Co-transfection of Kir6.1 and SUR1 also resulted in channel activity, which was present in the cell-attached patch configuration. In general, very few channels were observed in each patch and the activity pattern was quite non-uniform with respect to the frequency of channel openings (Figure 5). In cell-attached patches, channel activity with a mean conductance of 34.3±5.6 pS was observed in 4 out of 16 HEK/CHO cells (0.9±0.4 channels/patch, mean±SEM). Upon patch excision, channel activity did not increase but rather decreased. Transfection of Kir6.1 alone in either HEK293 (n=16) or CHO (n=12) cells did not result in functional channel activity. Interestingly, in 4 out of 25 HEK/CHO cells transfected with Kir6.2, without SUR1, functional K\textsubscript{ATP} channel...
activity was observed (0.4±0.2 channels/patch, mean±SEM). Figure 6 shows channel activity upon patch excision in a CHO cell transfected with Kir6.2 only; at least 3 channels with short open duration were observed. These channels appeared to be weakly inwardly rectifying, but their conductance was difficult to establish due to the short, spiky channel openings. By selecting the channel openings with the longest duration, mean channel conductance for this cell was calculated to be 55.4 pS (overall 50.2±3.0 pS, mean±SEM, n=3). Strikingly, application of 1 mM ATP did not seem to have much effect, although channel activity had already diminished considerably prior to the addition of ATP to the bath.

We also tested the possibility that Kir6.1 and Kir6.2 join together to form heteromultimeric channels together with SUR. Figure 7 shows functional channel activity in a CHO cell transfected with Kir6.1, Kir6.2 and SUR1. Activity increased dramatically upon patch activity and was reversibly inhibited by 1 mM ATP. In total, similar channel activity was observed in 5 out of 13 CHO cells with 29.6±8.2 channels per patch and a mean channel conductance of 56.4±1.6 pS (p=NS versus Kir6.2+SUR1). Channel characteristics appeared similar to that of Kir6.2 together with SUR1, and no channels with intermediate or low mean channel conductance were observed, suggesting that all channels observed were composed of Kir6.2+SUR1. Although these results suggest that Kir6.1 does not readily co-assemble with Kir6.2 and SUR1, it is possible that some heteromultimeric channels were formed, but that they were too few to be noticed or had no functional impact on channel activity.

Discussion

Since the discovery of the K\textsubscript{ATP} channel in cardiac myocytes by Noma in 1983, much effort has been put into unravelling the molecular identity of this ion channel. First, Kir6.1 was isolated from rat pancreas and showed an ubiquitous mRNA expression pattern in various rat tissues (Inagaki et al. 1995a). Next, screening of a human and mouse library revealed a second clone, Kir6.2, which shared 71-74 % homology with Kir6.1 (Inagaki et al. 1995b, Sakura et al. 1995). Kir6.2 showed high mRNA expression in pancreatic islets, heart, skeletal muscle and brain, and was shown to form functional, ATP-sensitive potassium channels when co-transfected with a sulfonylurea receptor (SUR) (Inagaki et al. 1995b). By comparing electrophysiological and pharmacological characteristics of different combinations of Kir6.2 and SURs, it became apparent that Kir6.2+SUR1 constitutes the pancreatic beta-cell K\textsubscript{ATP} channel type (involved in insulin release), whereas Kir6.2+SUR2A underlies the cardiac K\textsubscript{ATP} channel (involved in
Figure 4. Single-channel characteristics of Kir6.2+SUR1 transfected in HEK293 cells; current at various holding potentials (A), I/V-relationship showing inward rectification (B), and effects of patch excision and application and washout of 1 mM ATP (C).

Figure 5. Current traces at various holding potentials from a cell transfected with Kir6.1+SUR1 (cell-attached patch) (A), I/V-relationship (B) and effect of patch excision (C).
cardioprotection during ischemia and preconditioning) (Inagaki et al. 1996, Okuyama et al. 1998). Co-expression of Kir6.1 with SUR results in functional channel activity that is quite different from the classical K_ATP channel. Kir6.1-based channels form weak inwardly rectifying K\(^+\) channels of ~33-36 pS that are active in cell-attached patch and are strongly activated by Mg\(^{2+}\)-nucleotide diphosphates (UDP) (Åmmälä et al. 1996, Yamada et al. 1997, Takano et al. 2000, Kono et al. 2000). Channels composed of Kir6.1/SUR2B are thought to form the nucleotide diphosphate-dependent K\(^+\) channel (K\(_{\text{NDDP}}\)) observed in vascular smooth muscle cells (involved in vasodilatation) (Yamada et al. 1997). Kir6.1 also forms functional channels together with SUR2A and SUR1 in vitro, but the physiological relevance of these combinations is not yet clarified.

**Structural characteristics of Kir6.1 and Kir6.2.** In the present study, we were interested in the role and function of Kir6.1 in cardiac muscle, and therefore we have isolated both Kir6.1 and Kir6.2 from rabbit heart cDNA library and compared their structural and functional characteristics. Like all other inward rectifier K\(^+\) channels, Kir6.1 and Kir6.2 have two membrane-spanning domains, M1 and M2, in between which is the pore-forming region H5, as depicted in Figure 1. The latter is proposed to enter and exit the lipid bilayer from the extracellular side. The N- and C-termini are both located intracellularly and contain certain biologically active regions, a number of which we have shown to vary between rabbit Kir6.1 and Kir6.2. One of the most striking differences which we observed between the amino acid sequences of Kir6.1 and Kir6.2 is the unique distal C-terminal region of Kir6.1 which is not observed in any other Kir family member. This 20-amino acid stretch contains mostly asparagines, serines and arginines, all hydrophilic amino acids, as well as five potential N-glycosylation sites. In N-linked glycosylation, a carbohydrate is attached to the asparagines (Asn/N) in the sequence Asn-X-Ser/Thr-Y, where X and Y denote any amino acid. Whether or not the presence of this consensus sequence actually leads to protein glycosylation is difficult to predict. It has been shown that non-glycosylated sites tend to be found more frequently towards the C-terminal end, and that the presence of proline (P) residues at positions X and Y in the consensus Asn-X-Ser/Thr-Y reduces the likelihood of N-linked glycosylation (Gavel and von Heijne 1990). All five potential glycosylation sites we observed in rabbit heart Kir6.1 are located in the C-terminal region, but none of them contains a proline within the consensus sequence. Although interspecies variation was observed, three of the five glycosylation sites are conserved among rabbit, human, rat, mouse and guinea-pig Kir6.1 suggesting functional importance. Interestingly, these glycosylation sites are located in the region which is predicted to be intracellular according to the membrane topology of all Kir family members. However, in most cases, N-glycosylation sites are found on extracellular domains, as is the case with SUR and other plasma membrane channels (Khanna et al. 2001). Glycosylation occurs on the luminal side of the endoplasmic
reticulum only (see Bennett and Kanner 1997), after which the membrane proteins are transported to the cell membrane by transport vesicles. After fusion of these vesicles the extracellular side of the cell. Accordingly, the location of the putative glycosylation sites protein compared to the current view. Whether this observation underlies any of the observed differences in functional channel activity between Kir6.1 and Kir6.2 remains
speculative. An important issue to address is whether or not the predicted sites are actually glycosylated in the endoplasmic reticulum.

**Electrophysiological characteristics.** In accordance with others, we observed that co-expression of Kir6.1 with SUR1 resulted in a lower number of functional channels compared to Kir6.2+SUR1 (Takano et al. 1998, Kono et al. 2000). In the absence of SUR1, Kir6.1 did not generate any K\textsubscript{ATP} channel activity. Although Kir6.1 was initially reported to generate functional channels in the absence of SUR (Inagaki et al. 1995, Ammala et al. 1996), our observations are in agreement with more recent reports (Yamada et al. 1997, Kono et al. 2000). However, our results indicate that Kir6.2, in contrast to Kir6.1, is capable of forming functional K\textsubscript{ATP} channels when transfected without SUR1. In fact, these findings confirm the results of John et al., who already in 1998 reported channel activity for Kir6.2 in the absence of SUR1. Nevertheless, it is still generally accepted that only Kir6.2 with the last 26 or 36 amino acids removed from the C-terminal end, is capable of independent functional expression (Tucker et al. 1997, Zerangue et al. 1999). As such, a putative endoplasmic reticulum (ER) retention signal, the amino acid triad RKR (Arg-Lys-Arg) located within the distal C-terminal region, is removed. The interaction of Kir\textsubscript{x} subunits with SUR\textsubscript{x} is thought to conceal the retention signal, thereby allowing for surface channel expression. However, the RKR triad is present in both Kir6.1 and Kir6.2, and thus cannot explain the difference in plasma membrane expression between these subunits observed in the present study. Since our rabbit heart Kir6.2 was active in its untruncated form, these results contradict the previous notion that the last 26 amino acids of Kir6.2 prevent its independent functional expression (Tucker et al. 1997). Nevertheless, we cannot rule out the possibility that both HEK293 and CHO cells contain an endogenous SUR-like protein with interacts with Kir6.2 to form functional channels.

**Distribution in the heart.** Our Northern blot results indicate that Kir6.1 mRNA is highly expressed both in atria and ventricle, and previous studies have shown its presence in cardiomyocytes (Mederos y Schnitzler et al. 2000). Nonetheless, the functional role of Kir6.1 in myocardial cells remains elusive. The pharmacological profile of Kir6.1/SUR1 closely resembles that of the native mitochondrial K\textsubscript{ATP} channel (Liu et al. 2001), and electron-microscopic examination of immunogold staining suggested Kir6.1 labelling on the inner mitochondrial membrane of rat skeletal muscle (Suzuki et al. 1997). On the other hand, Kir6.1 did not co-localise with mitochondria in ventricular myocytes (Seharaseyon et al. 2000). Kir6.1 may also form part of the neuronal K\textsubscript{ATP} channel complex, based on the observation that Kir6.1+SUR1 were shown to underlie the glibenclamide-sensitive K\textsuperscript{+} current observed in glucose-receptive neurones (Lee et al. 1999). Another possibility is that Kir6.1 forms heteromultimeric channels together with Kir6.2 and SUR. Although some studies have shown that functional
Structure and function of Kir6.1 and Kir6.2

Figure 7. Single-channel characteristics of Kir6.1+Kir6.2+SUR1 transfected in a CHO cell; current at various holding potentials (A), I/V-relationship showing inward rectification (B), and effects of patch excision and application and washout of 1 mM ATP (C)

co-assembly of Kir6.1 and Kir6.2 occurs in vitro, it is not known whether these heteromeric channels also exist in vivo and whether they are of functional importance (Kono et al. 2000, Cui et al. 2001, Babenko et al. 2000). In fact, adenoviral dominant-negative Kir6.1 gene transfer into ventricular myocytes did not affect endogenous K\textsubscript{ATP} current, which suggests that Kir6.2 is the sole pore-forming subunit (Seharaseyon et al. 2000a). In accordance with this, in the present study no evidence of heteromultimerisation between Kir6.1 and Kir6.2 was observed. It remains clear that Kir6.1-based channels show a very low level of plasma membrane channel activity, suggesting a preference for intracellular distribution and function. In Chapter 8, we will present data from trafficking studies that support this hypothesis.

Structure-function relation. Cloning and mutational analysis of K\textsubscript{ATP} channels has identified several regions and specific amino acid residues associated with specific biophysical properties (Ashcroft and Gribble 1998). The SUR subunit is now considered to confer sensitivity to sulfonylureas, potassium channel openers and MgADP upon the K\textsubscript{ATP} channel, whereas the Kir6.x subunit forms the actual channel pore and controls channel inhibition by ATP (Bryan and Aguilar-Bryan 1999, Ashcroft 2000). The weak inwardly rectifying properties of K\textsubscript{ATP} channels are determined by the pore (H5) region
of the Kir6x subunit (Shyng et al. 1997b). Single channel conductance is controlled by short amino acid residues in the extracellular regions between the first transmembrane domain (M1) and the pore region (H5) and between the H5 and M2 regions of Kir6.2 (Repunte et al. 1999). These findings are reflected in the structural and functional (dis)similarities between rabbit heart Kir6.1 and Kir6.2 described in this study. Both subunits have identical pore (H5) regions and show weak inward rectification. Also, protein sequence homology is low in the extracellular region between M1 and H5, consistent with the differences in single channel conductance between Kir6.1 and Kir6.2. Our observation that Kir6.2 forms functional ATP-sensitive channels in the absence of SUR1 supports the notion that the ATP-binding site is located on Kir6.2, and confirms previous findings from studies with truncated forms of Kir6.2 (Kir6.2ΔC26/36) and direct photo-affinity labelling of Kir6.2 by \[^{32}P\]-8-azido-ATP (Tucker et al. 1997, Tanabe et al. 1999). Mutations in the N-terminal region, the C-terminus and the second transmembrane region have been associated with ATP-sensitivity of Kir6.2 and it has been proposed that the N- and C-termini of Kir6.2 interact and stabilise the ATP-binding region (Shyng et al. 1997a, Tucker et al. 1998, Tanabe et al. 1999, Reimann et al. 1999b). A distal region in the C-terminus of Kir6.2 (amino acids 333-338) is thought to contain an ATP-binding motif (FX4K or Phe-X4-Lys) and has been proposed to form part of the ATP-binding site (Drain et al. 1998). However, this seems unlikely since both Kir6.1 and Kir6.2 contain this motif, whereas their ATP-sensitivity differs greatly. Although our results showed that Kir6.2 can form functional channels on its own, we also observed that the presence of SUR1 greatly enhanced its surface expression and increased the number of channels per patch by roughly 100-fold. Interestingly, SUR1 not only influenced trafficking efficiency of Kir6.2 but also altered certain channel characteristics. Compared to Kir6.2+SUR1, channels composed of Kir6.2 showed short open channel durations with a spiky appearance. Thus, the assembly of Kir6.2 with SUR1 likely leads to certain conformational changes in or near the channel pore, thereby influencing channel kinetics.

In conclusion, in this study we have compared the structural and functional characteristics of the K\(_{\text{ATP}}\) channel subunits Kir6.1 and Kir6.2. The most striking differences between the two subunits include plasma membrane expression efficiency and ATP-sensitivity. Furthermore, we have shown that, in contrast to Kir6.1, Kir6.2 in its untruncated form can form ATP-sensitive potassium channels independent from SUR1. By comparing their structural characteristics, we attempted to provide a basis for the functional differences between Kir6.1 and Kir6.2. Although Kir6.1 mRNA was shown to be highly expressed throughout atrial and ventricular tissue, further studies are necessary to clarify its functional role in the heart.
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