Comparison of cloned Kir2 channels with native inward rectifier K⁺ channels from guinea-pig cardiomyocytes


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1. The aim of the study was to compare the properties of cloned Kir2 channels with the properties of native rectifier channels in guinea-pig (gp) cardiac muscle. The cDNAs of gpKir2.1, gpKir2.2, gpKir2.3 and gpKir2.4 were obtained by screening a cDNA library from guinea-pig cardiac ventricle.

2. A partial genomic structure of all gpKir2 genes was deduced by comparison of the cDNAs with the nucleotide sequences derived from a guinea-pig genomic library.

3. The cell-specific expression of Kir2 channel subunits was studied in isolated cardiomyocytes using a multi-cell RT-PCR approach. It was found that gpKir2.1, gpKir2.2 and gpKir2.3, but not gpKir2.4, are expressed in cardiomyocytes.

4. Immunocytochemical analysis with polyclonal antibodies showed that expression of Kir2.4 is restricted to neuronal cells in the heart.

5. After transfection in human embryonic kidney cells (HEK293) the mean single-channel conductance with symmetrical K⁺ was found to be 30.6 pS for gpKir2.1, 40.0 pS for gpKir2.2 and 14.2 pS for Kir2.3.

6. Cell-attached measurements in isolated guinea-pig cardiomyocytes (n = 351) revealed three populations of inwardly rectifying K⁺ channels with mean conductances of 34.0, 23.8 and 10.7 pS.

7. Expression of the gpKir2 subunits in Xenopus oocytes showed inwardly rectifying currents. The Ba²⁺ concentrations required for half-maximum block at −100 mV were 3.24 µM for gpKir2.1, 0.51 µM for gpKir2.2, 10.26 µM for gpKir2.3 and 235 µM for gpKir2.4.

8. Ba²⁺ block of inward rectifier channels of cardiomyocytes was studied in cell-attached recordings. The concentration and voltage dependence of Ba²⁺ block of the large-conductance inward rectifier channels was virtually identical to that of gpKir2.2 expressed in Xenopus oocytes.

9. Our results suggest that the large-conductance inward rectifier channels found in guinea-pig cardiomyocytes (34.0 pS) correspond to gpKir2.2. The intermediate-conductance (23.8 pS) and low-conductance (10.7 pS) channels described here may correspond to gpKir2.1 and gpKir2.3, respectively.

As a consequence of the asymmetrical distribution of potassium ions across the cell membrane, K⁺ channels normally conduct outward current more easily than inward current, i.e. they display outward rectification (Hodgkin & Katz, 1949). The opposite phenomenon, inward rectification, was first discovered as a property of the membrane of skeletal muscle (Katz, 1948) and cardiac muscle (Hall et al. 1963). This strong inward rectification
In the heart, inward rectifier channels are especially important because they largely determine the shape of the cardiac action potential (Nichols & Lopatin, 1997). During diastole the inward rectifier channels provide the dominant membrane conductance of cardiac ventricular muscle and are responsible for setting the resting potential. During the plateau of the action potential the inward rectifier channels are mostly closed, thus reducing the inward current required to maintain depolarization and minimizing the loss of K⁺ ions. The rapid, potential-dependent unblock of inward rectifier channels during the initial phase of repolarization (Ishihara & Ehara, 1998) contributes to the ‘regenerative’ phase of repolarization of the action potential (Shimoni et al. 1992).

Despite their obvious importance, the molecular identity of the channels responsible for inward rectification in cardiac muscle is still far from clear. Even the biophysical properties of cardiac inward rectifier channels are still controversial. The first recordings of single inward rectifier channels have been carried out in cardiac muscle cells isolated from guinea-pig heart by Sakmann & Trube (1984). They reported a single-channel conductance of 27 pS, whereas Shioya et al. (1993) found that the inward rectifier channels in the same guinea-pig cardiomyocytes had a conductance of 33–34 pS. In rat cardiac ventricular muscle, Josephson & Brown (1986) found that the major inward rectifier channel had a conductance of 30–40 pS, whereas Nakamura et al. (1998) reported that inward rectification was largely due to a 21 pS channel which may correspond to Kir2.1.

On the basis of sequence homology inward rectifier channels have been classified into subfamilies Kir1 to Kir7 (Douplnik et al. 1995; Nichols & Lopatin, 1997). The potassium channels with the most pronounced inward rectification belong to the Kir2 subfamily. Using Northern blot and reverse transcriptase (RT)-PCR analysis, expression of Kir2.1 has been demonstrated in mouse, rabbit and human cardiac ventricular muscle (Ishihara & Hiraoka, 1994; Ishii et al. 1994; Raab-Graham et al. 1994), expression of Kir2.1 and Kir2.2 has been demonstrated in human atrium (Wible et al. 1995), and Kir2.3 has been found in human ventricle (Morishige et al. 1994; Perier et al. 1994; Tang & Yang, 1994; Wang et al. 1998). However, in cardiac ventricle only about 30% of the cells are cardiac muscle cells (Rakusan et al. 1980), the remainder consists of endothelial cells, vascular smooth muscle cells, fibroblasts, neurons and other cells. Thus, Kir2 mRNA detected in cardiac ventricular tissue cannot be unequivocally assigned to cardiac muscle cells.

The aim of the present study was to identify the strongly rectifying K⁺ channels of the Kir2 subfamily expressed in isolated cardiomyocytes by molecular biological techniques and to compare the properties of the cloned Kir2 channels with those of the native inward rectifier channels in cardiomyocytes. Preliminary reports of some of our findings have been published (Preisig-Müller et al. 1999a; Liu et al. 2000).

METHODS

All experiments were performed in accordance with the regional animal care committee guidelines (at the Regierungspräsidium Gießen, Germany).

Cloning of guinea-pig Kir2 subunits

Guinea-pigs weighing 200–350 g were killed without prior anaesthesia by decapitation, the heart was quickly excised and RNA was extracted using a modified single-step method (Chomczynski & Sacchi, 1987). Guinea-pig-specific cDNA fragments of Kir2.1, Kir2.2, Kir2.3 and Kir2.4 encoding the region downstream of the second transmembrane domain were amplified using a nested RT-PCR method.

Primers for the first PCR were:

sense, 5' GCGAYATHTTYACNACNTGYGT-3'  
antisense, 5' ACGGYYTCRWNACKRNGCCCA-3'  

Primers for nested PCR were:

sense, 5' TGYYTNTATGGMNGTNGNXAA-3'  
antisense, 5' CATNGCGTNCGCTCNACCATNCC-3'  
(abbreviations as recommended by the IUPAC-IUB). The amplified fragments were subcloned and sequenced (Genetic Analyzer 310, Applied Biosystems). The subcloned gpKir2 fragments were isolated and labelled with digoxigenin-11dUTP (Boehringer Mannheim) for screening. A cardiac ventricle cDNA library was constructed, ligated into the pTriplEx vector (Clontech) and screened as described previously (Mederos y Schnitzler et al. 2000). Genomic clones were isolated from a guinea-pig FIX II genomic library (Stratagene). Total sequence information was obtained by either sequencing the converted pTriplEx and pBluescript SK+ subclones or by direct sequencing of α-DNA.

Cell-specific RT-PCR and immunocytochemistry

A mixed suspension of cardiac cells was obtained by enzymatic digestion of excised guinea-pig hearts as described previously (Preisig-Müller et al. 1999b). For cell-specific detection of gpKir2 transcripts pure fractions of cardiomyocytes and capillary fragments were collected and total RNA was extracted from roughly 1000 cardiomyocytes or 150 capillary fragments (Preisig-Müller et al. 1999b). One-third of the cell RNA eluate were reverse transcribed and amplified (Mederos y Schnitzler et al. 2000). For RT-PCR, the following intron-spanning primers were derived from the 5’ non-coding regions or from the coding region (gpKir2.4) of the cDNAs.

For gpKir2.1 (325 bp fragment):

sense, 5’ OCTCCCAATTCCACTCGCTTTCC-3’  
antisense, 5’ GCCAATCTCATGCGTCCCTTC-3’  

For gpKir2.2 (303 bp fragment):

sense, 5’ GCCACTGACAAAGGTCTGCGGGTG-3’  
antisense, 5’ GCCACCCGACATGGTGACCAAGTGCAG-3’
For gpKir2.3 (290 bp fragment):
sense, 5'-CGTTGAGTGTCGCCAGCAGTG-3';
antisense, 5'-CCACCGAGTGTCGAAATGTC-3'.

For gpKir2.4 (378 bp fragment):
sense 5'-CCGTTGCTGCGGCTGACCCG-3';
antisense 5'-CGTGGGCCCAGAGGGCCTCC-3'.

The purity of the cell fractions was verified by RT-PCR experiments using the cell-specific markers troponin T and endothelin-1 as described previously (Preisig-Müller et al. 1999b). In another series of experiments, total RNA was extracted from intact cardiac ventricular or atrial tissue with a modified single-step method (Chomczynski & Sacchi, 1987) and 2 µg of tissue RNA was reverse transcribed and amplified.

Polyclonal antibodies against the less conserved C-terminus of the Kir2.4 protein (amino acids 393–434; expressed as recombinant protein with pGEX-4T-1 as vector) were raised in rabbits, affinity purified and characterized as detailed for Kv subunits (Veh et al. 1995). Immunocytochemistry was performed on thoracic viscera of five guinea-pigs (decapitated as described above) using a routine indirect immunofluorescence protocol (Kummer et al. 1990) with a FITC-conjugated antiserum.

**Heterologous expression of Kir2 subunits in Xenopus oocytes**

Oocytes were collected under anaesthesia from frogs (*Xenopus laevis*) that were humanely killed after the final collection. For functional expression in *Xenopus* oocytes the coding regions of the gpKir2 cDNAs were subcloned further into the polyadenylation transcription vector pSGEM. Capped run-off poly(A)+ cRNA transcripts from cDNAs were subcloned further into the polyadenylation transcription vector KCl, 2 MgCl2, 0.33 NaH2PO4, 10 glucose, 5 Hepes, pH 7.4 (60 mM K+ solution) or 85 NaCl, 60 KCl, 1 CaCl2, 1 MgCl2, 5 Hepes, 10 glucose; the pH was adjusted to 7.4 with NaOH. The data reported are given as means ± S.D.

**Results**

**Gene structure of guinea-pig Kir2 channels**

To analyse the exon–intron gene structure of Kir2 channels, cDNAs and genes of the four different members of this family were isolated from a cardiac cDNA and a genomic library. Full-length cDNAs of gpKir2.1 (4047 bp, accession number AF183914), gpKir2.2 (2164 bp, accession number AF183916), gpKir2.3 (2388 bp, accession number AF183917) and Kir2.4 (2459 bp, accession number AF187876) were isolated from the cDNA library. The nucleotide sequences of the coding regions of the gpKir2...
cDNAs are 90–94% homologous to the orthologous human or rat Kir2 cDNAs (Perier et al. 1994; Wible et al. 1995; Wood et al. 1995; Krapivinsky et al. 1998; Töpert et al. 1998). They predict open reading frames of 428 amino acids for gpKir2.1, 434 amino acids for gpKir2.2, 444 amino acids for gpKir2.3 and 439 amino acids for gpKir2.4. Comparison of the deduced gpKir2 amino acid sequences to the corresponding human Kir2 amino acid sequences showed 93–99% identity.

Genomic clones of gpKir2.1 (KCNJ2, 11618 bp; GenBank accession number AF187872), gpKir2.2 (KCNJ12, 9642 bp; accession number AF187873), gpKir2.3 (KCNJ4, 2669 bp; accession number AF187874) and gpKir2.4 (KCNJ14, 14865 bp; accession number AF187875) were isolated from a guinea-pig genomic library. A comparison of cDNA and genomic clones revealed that although the coding regions of gpKir2.1, gpKir2.2 and gpKir2.3 are intronless, all genes contain at least one intron in the 5′ non-coding region (Fig. 1). The intronic sequences start 217 bp (gpKir2.1), 56 bp (gpKir2.2) and 39 bp (gpKir2.3) upstream to the start codon with conserved 3′ splice sites of the intron. The identified introns have a length of 4953 bp (gpKir2.1) and 6233 bp (gpKir2.2). However, the genes of the first three gpKir2 members are not complete at the 5′ end, and therefore only a partial genomic structure has been deduced. At least one further intron splits the 5′ region of KCNJ12. Interestingly, the gene of gpKir2.4 showed no intron in the 5′ non-coding region but a 1.6 kb intron splitting the coding region.

**Localization of Kir channels by cell-specific RT-PCR and immunocytochemistry**

The cell-specific expression of Kir2 channel subunits in cardiomyocytes and capillary endothelial cells was studied using multi-cell RT-PCR with intron-spanning primers as described previously (Preisig-Müller et al. 1999b; Mederos y Schnitzler et al. 2000). gpKir2.1, gpKir2.2 and gpKir2.3 mRNA was found in isolated cardiac muscle cells and in capillary endothelium (Fig. 1B). Detection of troponin T was used as a positive control and specific marker for cardiomyocytes; endothelin-1 was used as a specific marker for endothelial cells. The size of the PCR products was as expected from the cloned sequences and the specificity was verified by direct sequencing. As a control, RNA from intact cardiac ventricular and atrial tissue was extracted and the expression of Kir2 channel subunits was analysed by RT-PCR. Surprisingly, all four members of the Kir2 subfamily, including Kir2.4, were found to be expressed in cardiac ventricle and atrium (n = 7; not illustrated). To resolve this apparent contradiction between cell-specific and whole-tissue RT-PCR, and to clarify the localization of Kir2.4 in the heart, immunocytochemical experiments were performed. Kir2.4 immunoreactivity was found exclusively in neuronal elements including perikarya of local parasympathetic ganglia (Fig. 2A) and axons ramifying at the adventitial-medial border of coronary arteries and between cardiomyocytes (Fig. 2B). Neuronal Kir2.4 immunoreactivity was not restricted to the cardiac innervation but was also observed in non-myelinated axon bundles travelling in the phrenic nerve and its pericardiac course.

![Figure 2. Cellular localization of Kir2.4 by immunofluorescence](image-url)

Kir2.4 immunoreactivity was observed in the perikarya and in the neuropil of an epicardial parasympathetic ganglion (A) and in a dense fibre plexus around coronary arteries (B, arrows) as well as in axons between cardiomyocytes (B, arrowheads). The weak fluorescence exhibited in cardiomyocytes (B) represents a non-specific autofluorescence that is also seen in controls. C, bundles of non-myelinated Kir2.4-immunoreactive axons (arrows) in phrenic nerve. Scale bar, 20 µm.
(Fig. 2C) and in adjacent organs, for example the myenteric plexus of the oesophagus.

**Heterologous expression of Kir2 channels in HEK293 cells**

The characteristics of guinea-pig Kir2.1, Kir2.2 and Kir2.3 channels were studied after transient expression in HEK293 cells. Figure 3A shows a typical cell-attached recording of an inward rectifier channel observed after transfection of HEK293 cells with gpKir2.2. The cells were depolarized to approximately 0 mV by elevating the K⁺ concentration in the bathing solution to 140 mM. Inward currents were observed at negative transmembrane potentials; no outward currents were observed at positive potentials. Figure 3B shows typical single-channel recordings of gpKir2.1, gpKir2.2 and gpKir2.3 at a transmembrane potential of −100 mV. The corresponding mean single-channel current–voltage relations are shown in Fig. 6 (filled symbols). The mean single-channel conductance obtained by linear fit of the single-channel amplitude recorded at −80, −100 and −120 mV (the slope conductance at −100 mV) was 30.6 ± 2.5 pS for gpKir2.1 (n = 19), 42.0 ± 2.2 pS for gpKir2.2 (n = 19) and 14.2 ± 1.4 pS for Kir2.3 (n = 12). A histogram of the slope conductances at −100 mV obtained in this way is shown in Fig. 5A.

**Inward rectifier channels in cardiomyocytes**

To compare the properties of the gpKir channels expressed in HEK293 cells with the properties of native inward rectifier channels we carried out cell-attached single-channel recordings in isolated cardiac muscle cells. Only channels showing strong inward rectification, i.e. distinct channel openings in the inward direction and no measurable outward currents, were analysed. Long continuous recordings were performed at different potentials with 150 mM K⁺ in the patch pipette. Figure 4A shows part of a recording from a patch containing two identical inward rectifier channels. The amplitude histogram on the right shows three peaks at approximately equal distance of 3.8 pA. Figure 4B shows a recording in which two different inwardly rectifying channels were observed in the same patch, a large-conductance inward rectifier channel (32 pS) and an intermediate-conductance inward rectifier channel (22 pS). Figure 4C shows a recording of a large- and a small-conductance channel (32.5 and 10.5 pS). These examples illustrate that the channels were really different entities that could sometimes be found even in the same patch. Even small differences in amplitude could be determined reliably from cell-attached recordings of 2–30 min duration.
The single-channel slope conductance at −100 mV was determined as described above for the cloned Kir2 channels. The data from 351 cell-attached recordings of inwardly rectifying channels with symmetrical K⁺ concentration are summarized in Fig. 5B. It can be seen that conductances between 8 and 39 pS were found which can be subdivided into three groups with mean values of 34.0 ± 2.2 pS (n = 325), 23.8 ± 1.2 pS (n = 17) and 10.7 ± 1.2 pS (n = 9), respectively. These results suggest that at least three distinct populations of inwardly rectifying K⁺ channels are expressed in guinea-pig cardiac muscle. The measurements of single-channel amplitude of the three populations were lumped to obtain the single-channel current–voltage relation shown in Fig. 6 (open symbols). Linear regression of the amplitudes measured between −140 and −60 mV gave reversal potentials between −12 and −13 mV. This slight deviation of the extrapolated reversal potential from the expected value of 0 mV, which has also been seen by other groups (Sakmann & Trube, 1984; Shioya et al. 1993), is partly attributable to the liquid junction potential of the patch electrode (+2.8 mV), which was not added. Comparison of the current–voltage relation of the three cloned Kir2 channel subtypes (filled symbols) with the channels found in cardiac inward rectifier channels (open symbols) shows that the three channel populations found in cardiomyocytes had an approximately 25% lower mean conductance than gpKir2.1, gpKir2.2 and gpKir2.3 channels expressed in HEK293 cells (see Discussion).

**Barium block of the inward rectifier channels expressed in Xenopus oocytes**

Looking at Fig. 5A and B it is tempting to correlate the native large-conductance inward rectifier channels with Kir2.2, the intermediate-conductance channels with Kir2.1 and the low-conductance channels with Kir2.3. However, this hypothesis needs to be confirmed by further experimental evidence. It is well known that the different subtypes of guinea-pig inward rectifier channels differ substantially in their sensitivity to Ba²⁺ block. Therefore we first studied Ba²⁺ block of the whole-cell current produced by gpKir2 subunits after expression in Xenopus oocytes. Injection of gpKir2.1, gpKir2.2, gpKir2.3 or gpKir2.4 cRNA in oocytes induced potassium currents showing strong inward rectification. The currents were blocked by barium ions in a time- and

![Figure 4. Single-channel recordings in cardiomyocytes](image-url)

Continuous cell-attached single-channel recordings lasting up to 20 min were performed with 150 mM K⁺ in the pipette solution. The cells were superfused with normal physiological salt solution. The transmembrane potential of the patch (shown above each record) was calculated as the difference between the applied pipette potential and the mean resting potential recorded in the whole-cell configuration. The resting potential was −72 ± 0.19 mV; n = 31. A, typical recording with two identical channels of 33.5 pS in the same patch. The corresponding amplitude histogram shows three narrow peaks. B, recording from another patch containing two channels. The corresponding amplitude histogram shows four peaks, indicating that the two channels had a different conductance (32 and 22 pS). C, recording from a patch containing two channels with conductance 32.5 and 10.5 pS.
voltage-dependent manner. Figure 7A shows a typical recording of the gpKir2.1 current in the presence of 2 µM Ba²⁺. The extracellular K⁺ concentration in this series of experiments was 60 mM.

The concentration dependence of Ba²⁺ block of the inward rectifier current in the steady state was plotted on a semi-logarithmic scale (Fig. 7B). The ordinate shows the fraction of inward rectifier current remaining in the presence of a given concentration of Ba²⁺. The normalized current amplitude was calculated as the ratio between the inward rectifier current measured in the presence of a given concentration of Ba²⁺ ($I_{Ba}$) and the inward rectifier current measured under control conditions ($I_{con}$). As can be seen from Fig. 7, the barium sensitivity of the different gpKir2 subtypes differed considerably, with gpKir2.2 showing the highest and gpKir2.4 showing the lowest Ba²⁺ sensitivity. The Ba²⁺ concentrations required for half-maximum block at −100 mV were 3.24 µM for gpKir2.1, 0.51 µM for gpKir2.2, 10.26 µM for gpKir2.3 and 235 µM for gp2.4.

The voltage dependence of Ba²⁺ block is illustrated in Fig. 8. Assuming a simple one-site model for Ba²⁺ binding (Woodhull, 1973), the relationship between membrane potential ($V_m$) and the equilibrium dissociation constant for Ba²⁺ binding ($K_d$) can be described as:

$$\ln K_d = \ln K_d(0) - \delta (V_m zF/RT),$$

(1)

where $K_d(0)$ is the dissociation constant at 0 mV and $\delta$ is the fraction of the electric field sensed at the barium binding site. The slope of the relation between membrane potential and log $K_d$ is a measure of $\delta$ and the extrapolated intersection with the ordinate gives an estimate of $K_d(0)$. The values of $\delta$ derived from linear fits of log $K_d$ vs. membrane potential were 0.45 for gpKir2.1, 0.62 for gpKir2.2, 0.38 for gpKir2.3 and 0.07 for gpKir2.4.

Barium block of native inward rectifier channels in cardiomyocytes

To get further information on the identity of the cardiac inward rectifier channel subtypes described above we
studied the concentration dependence of Ba\(^{2+}\) block in cell attached single-channel recordings. However, since Ba\(^{2+}\) block also depends on the external K\(^+\) concentration (Shieh et al. 1998; Owen et al. 1999), a meaningful comparison is possible only with identical external K\(^+\) concentrations. Therefore we used a pipette solution with 60 mM K\(^+\) to which various concentrations of Ba\(^{2+}\) were added. The conductance histogram of the channels recorded in this series of experiments (n = 121 channels, Fig. 5C) showed two populations of channels, with conductances in the range 14–17.5 pS and 19–29 pS, respectively. Again only the single-channel amplitudes measured at _80, _100 and _120 mV were used to obtain the slope conductance at _100 mV by linear regression. Comparison of Fig. 5B and C shows that the peaks of the conductance histogram with 60 m M K\(^+\) were shifted to the left, as expected for a lower K\(^+\) external concentration. However, no small-conductance channels were detected

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**Figure 7. Ba\(^{2+}\) block of Kir2 channels expressed in *Xenopus* oocytes**

A, typical record of the dependence of Ba\(^{2+}\) block of Kir2.1 on voltage and time. The extracellular K\(^+\) concentration was 60 mM. Voltage steps to +40, −20, −40, −60, −80 and −100 mV were applied from a holding potential of 0 mV. B, Ba\(^{2+}\) concentration–effect curves for gpKir2.1 ( ), gpKir2.2 ( ), gpKir2.3 ( ) and gpKir2.4 ( ). The data were fitted with the function \( I_{Ba}/I_{con} = 1/(1 + [Ba^{2+}]/K_d) \). \( I_{con} \) was defined as the difference between the inward rectifier current measured under control conditions and the current measured in the presence of a maximal concentration of Ba\(^{2+}\) (5 mM for gpKir2.1 and 1 mM for the other gpKir2 channels, which induced complete block of inward rectifier channels). Analogously, \( I_{Ba} \) was defined as the difference between the current measured in the presence of a given concentration of Ba\(^{2+}\) and the current measured in the presence of a maximal concentration of Ba\(^{2+}\). Fig. 5C) showed two populations of channels, with conductances in the range 14–17.5 pS and 19–29 pS, respectively. Again only the single-channel amplitudes measured at −80, −100 and −120 mV were used to obtain the slope conductance at −100 mV by linear regression. Comparison of Fig. 5B and C shows that the peaks of the conductance histogram with 60 mM K\(^+\) were shifted to the left, as expected for a lower K\(^+\) external concentration. However, no small-conductance channels were detected

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**Figure 8. Comparison of Ba\(^{2+}\) sensitivity in native and cloned inward rectifier channels**

The voltage dependence of the \( K_d \) values for Ba\(^{2+}\) block of cloned Kir2 channels and of native cardiac inward rectifier channels is plotted on a semi-logarithmic scale. Filled symbols: the \( K_d \) for Ba\(^{2+}\) block of gpKir2.1 ( ), gpKir2.2 ( ), and gpKir2.3 ( ) expressed in *Xenopus* oocytes, measured in the presence of 60 mM external K\(^+\). Open circles and dotted line: the \( K_d \) for Ba\(^{2+}\) block of the large-conductance inward rectifier channels in cardiomyocytes (calculated from the data shown in Fig. 9), also measured in the presence of 60 mM external K\(^+\). The lines are linear fits of \( \log K_d \) versus membrane potential.
with 60 mM K+. This may be explained by the fact that such small channels with a high open probability are difficult to detect, especially if superimposed on larger channels.

In agreement with previous reports (Kubo et al. 1993; Makhina et al. 1994), we found that low concentrations of Ba²⁺ (≤1 µM) did not affect the single-channel conductance (n = 58, not illustrated). The effects of Ba²⁺ ions on the kinetics of inward rectifier channels with 60 mM K⁺ in the pipette are illustrated in Fig. 9A. It can be seen that addition of 0.2, 1 or 5 µM Ba²⁺ to the pipette solution caused a concentration-dependent reduction in open probability by inducing long closed states. Figure 9B shows a plot of the mean open probability recorded with different Ba²⁺ concentrations in the pipette solution. The mean Kᵣ for Ba²⁺ block was 1.14 ± 0.13 µM at −80 mV, 0.43 ± 0.05 µM at −100 mV and 0.21 ± 0.03 µM at −120 mV. It should be noted that reliable measurements of the effects of Ba²⁺ on open probability could only be obtained in the range −80 to −120 mV and only for the group of the large-conductance inward rectifier channels (23.5–27.5 pS).

The values for log Kᵣ calculated from these single-channel measurements have been included in Fig. 8 (open circles, dotted line). The measurements in *Xenopus* oocytes and the cell-attached measurements in cardiomyocytes were carried out with the same external K⁺, Ca²⁺ and Mg²⁺ concentrations. Figure 8 shows that the Kᵣ values for Ba²⁺ block of the large-conductance inward rectifier channels in cardiomyocytes coincided with the Kᵣ values obtained for Kir2.2 in oocytes and that the slope of the voltage dependence was nearly identical. These findings suggest that the large-conductance inward rectifier K⁺ channels found in cardiomyocytes may correspond to Kir2.2.

**DISCUSSION**

**Cellular localization of Kir channel subunits in the heart**

We have cloned and sequenced all four members of the guinea-pig Kir2 subfamily. Analysis of the gene structure showed that gpKir2.1, gpKir2.2 and gpKir2.3 have at least one intron in the 5' non-coding region. gpKir2.4 has no intron in the 5' non-coding region, but an intron in the coding region. In trying to elucidate the localization of Kir2 channels in the heart, we found that there are many pitfalls in the cell-specific RT-PCR of Kir channels. To get consistent results we had to exclude the possibility of genomic contamination of the cDNA by using intron-spanning primers throughout. Furthermore, in all experiments control runs were carried out with troponin T and endothelin-1, which have been established as cell-
specific markers of cardiac muscle cells and endothelial cells, respectively, in previous work (Preisig-Müller et al. 1999b). By selecting 800–1000 cardiomyocytes or about 150 capillary fragments (each containing 5–15 endothelial cells) under visual control and by using cell-specific markers we were able to ascertain that the PCR signal was derived exclusively from cardiomyocytes or capillary fragments. We have shown that gpKir2.1, gpKir2.2 and gpKir2.3 subunits are expressed in both cardiac muscle cells and capillary endothelial cells isolated from guinea-pig heart.

Surprisingly, RT experiments with RNA extracted from cardiac ventricle or atrium showed expression of all four Kir2 subunits. This apparent contradiction could be resolved by immunocytochemical experiments showing that expression of gpKir2.4 is restricted to neuronal cells in the heart. This includes the local cholinergic parasympathetic neurons, but the high density of immunoreactive axons in coronary arteries and myocardium suggests that Kir2.4 is also expressed in extrinsic sources of innervation. Kir2.4-immunoreactive nerve fibres bundles run in the phrenic nerve that carries postganglionic sympathetic as well as sensory nerve fibres but no postganglionic parasympathetic axons. Since the brain is an additional site of Kir2.4 gene expression this channel appears to play a general role in the peripheral and central nervous system rather than being restricted to a functionally or topographically defined system.

**Inward rectifier channels in guinea-pig cardiac muscle**

In HEK293 cells we found mean single-channel conductances of 42.0 pS for gpKir2.2, 30.6 pS for gpKir2.1, and 14.2 pS for Kir2.3, in the presence of 150 mM external K⁺, 1 mM Ca²⁺ and 1 mM Mg²⁺. In isolated cardiomyocytes we found three different populations of K⁺ channels showing strong inward rectification (Fig. 5). The single-channel currents had a reversal potential near the calculated K⁺ equilibrium potential and none of the channels showed any measurable current in the outward direction. The mean single-channel conductances measured in cardiomyocytes (with the same pipette solution as in HEK293 cells) were 34.0 pS (large conductance, n = 325), 23.8 pS (intermediate conductance, n = 17) and 10.7 pS (low conductance, n = 9).

Our data are in reasonable agreement with those reported by Noma and co-workers (Shioya et al. 1993) who found a mean conductance of 32.3 ± 0.8 pS in guinea-pig cardiomyocytes, but did not describe intermediate-conductance or low-conductance inward rectifier channels. Our data disagree with the results of Sakmann & Trube (1984) who reported a mean single-channel conductance of 27.5 ± 0.7 pS under very similar experimental conditions. In cardiomyocytes of the rat (Nakamura et al. 1998; n = 15 patches) and in human atrium (Wible et al. 1995; n = 13 patches) the occurrence of multiple conductances of inwardly rectifying channels has been reported. However, the number of measurements in the latter two studies was quite small and channels showing weak inward rectification were not excluded.

**Comparison between cloned and native inward rectifier channels**

The present study is one of the first that rigorously tries to compare the properties of cloned channels with those of native channels in a particular cell type. We have determined the genomic structure of Kir2 channels to be able to design intron-spanning primers. Using multi-cell RT-PCR experiments with appropriate controls we were able to ascertain that the genes for Kir2.1, Kir2.2 and Kir2.3 are transcribed in guinea-pig cardiomyocytes. A large number of cell-attached single-channel measurements yielded information about channel expression at the protein level.

It is tempting to correlate the three populations of inward rectifier K⁺ channels with the three Kir2 subunits expressed in cardiomyocytes. However, the mean conductance of three populations of inward rectifier channels detected in cardiomyocytes (34.0, 23.8 and 10.7 pS) was about 20–25% lower than the mean conductance of the three subtypes of gpKir2 channels expressed in HEK cells (42.0 pS for gpKir2.2, 30.6 pS for gpKir2.1 and 14.2 pS for Kir2.3; see Fig. 5). On the other hand, expression of the mouse and human Kir2 orthologues in *Xenopus* oocytes yielded channels with a conductance of 34–36 pS (Kir2.2), 21 pS (Kir2.1) and 10–17 pS (Kir2.3), with symmetrical K⁺ concentrations (Kubo et al. 1993; Makhina et al. 1994; Takahashi et al. 1994; Wible et al. 1995; Welling, 1997; Zhu et al. 1999). These values are much closer to the values of the three channel populations found in cardiomyocytes. The simplest explanation for these discrepancies is that both oocytes and cardiomyocytes may possess an intracellular ligand or an accessory subunit that reduces the conductance of Kir2 channels, whereas this ligand is absent in HEK293 cells. Interestingly, expression of mouse Kir2.1 in COS-1 cells (a green monkey kidney cell line) also gave a much higher single-channel conductance (34 pS; Omori et al. 1997) than expression of the same clone in oocytes (21 pS, Kubo et al. 1993). In a recent study, Karschin and co-workers have shown that the intracellular binding of PDZ domains to Kir2.3 channels can substantially reduce single-channel conductance (Nehring et al. 2000).

We have used the characteristics of Ba²⁺ block to correlate the cloned gpKir2 channels with the native inward rectifier channels found in isolated cardiomyocytes. Our cell-attached recordings in cardiomyocytes (with 60 mM external K⁺) show that the concentration and voltage dependence of Ba²⁺ block for the large-conductance inward rectifier channels was nearly identical to that found for the Kir2.2 inward rectifier current expressed in *Xenopus* oocytes (with 60 mM external K⁺; Fig. 8). These observations support the idea that the large-conductance inward rectifier channels correspond to Kir2.2.
Previous studies of murine and human Kir2.3 in heterologous expression systems (Makhina et al. 1994; Welling, 1997; Zhu et al. 1999) have shown that it is a small-conductance channel (range, 10–17 pS) with a very high open probability. We have found that gpKir2.3 also has a low conductance (mean, 14.2 pS) and a higher open-state probability than gpKir2.2 and gpKir2.1. Furthermore, we have detected small-conductance (mean, 10.7 pS) inwardly rectifying channels with a high open probability in isolated guinea-pig cardiomyocytes. Since we have also shown robust expression of gpKir2.3 in cardiomyocytes, it is likely that the small-conductance channels correspond to gpKir2.3. Finally, the intermediate-conductance inward rectifier channel found in cardiomyocytes (mean conductance, 23.8 pS) may correspond to gpKir2.1 (Nakamura et al. 1998).

In conclusion, our results suggest that Kir2.1, Kir2.2 and Kir2.3 contribute to the inward rectifier current in guinea-pig cardiac muscle cells and that the largest contribution comes from Kir2.2.


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