Pore loop-mutated rat KIR6.1 and KIR6.2 suppress K\textsubscript{ATP} current in rat cardiomyocytes

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van Bever, Laurianne, Serge Poitry, Cécile Faure, Robert I. Norman, Angela Roatti, and Alex J. Baertschi. Pore loop-mutated rat KIR6.1 and KIR6.2 suppress K\textsubscript{ATP} current in rat cardiomyocytes. Am J Physiol Heart Circ Physiol 287: H850–H859, 2004.First published March 25, 2004; 10.1152/ajpheart.00054.2004.—Cardiomyocytes express mRNA for all major subunits of ATP-sensitive potassium (K\textsubscript{ATP}) channels: KIR6.1, KIR6.2, SUR1A, SUR2A, and SUR2B. It has remained controversial as to whether KIR6.1 may associate with KIR6.2 to form the tetrameric pore of K\textsubscript{ATP} channels in cardiomyocytes. To explore this possibility, cultured rat cardiomyocytes were examined for an inhibition of K\textsubscript{ATP} current by overexpression of pore loop-mutated (inactive) KIR6.x. Bicistronic plasmids were constructed encoding loop-mutated (AFA or SFG for GFG) rat KIR6.x followed by EGFP. In ventricular myocytes, the overexpression of KIR6.1AFA DNA caused, after 72 h, a major decrease of K\textsubscript{ATP} current density of 73.7% and 58.5%, respectively (P < 0.01), relative to corresponding EGFP controls. In atrial myocytes, overexpression of these pore-mutated KIR6.x by 6.0-fold and 10.6-fold, as assessed by quantitative immunohistochemistry, caused a decrease of K\textsubscript{ATP} current density of 73.7% and 58.5%, respectively (P < 0.01). Expression of wild-type rat KIR6.2 increased the ventricular and atrial K\textsubscript{ATP} current density by 58.3% and 42.9%, respectively (P < 0.01), relative to corresponding EGFP controls, indicating a reserve of SUR to accommodate increased KIR6.x trafficking to the sarcolemma. The results favor the view that KIR6.1 may associate with KIR6.2 to form heterotetrameric pores of native K\textsubscript{ATP} channels in cardiomyocytes.

ATP-sensitive potassium channels; atrium; ventricle

ATP-sensitive potassium (K\textsubscript{ATP}) channels are widely distributed in the heart, brain, kidney, pancreas, and blood vessels (1, 4, 33, 42). In the heart, K\textsubscript{ATP} channels are normally closed, and open in metabolically impaired cells (13, 26). They thereby shorten the action potential (25) and limit the calcium transient (9) and thus reduce muscle contraction and energy demand. K\textsubscript{ATP} channels have also been implicated in reducing ischemia-reperfusion damage (37) and in cardiac preconditioning, where a prior mild ischemia protects the heart muscle against subsequent, more severe ischemic insults (10). In pancreatic β-cells, K\textsubscript{ATP} channels are normally open, and they close after a glucose influx-induced increase in ATP, thereby depolarizing the cell and increasing calcium influx and insulin release (3, 22, 24). Thus K\textsubscript{ATP} channels are widely recognized to play vital roles in cardiac and neuronal excitability, vascular tone, ischemic protection, and insulin release.

The assembly of K\textsubscript{ATP} channels varies from tissue to tissue, but the general design is the same. Four subunits, called potassium inward rectifier 6 (KIR6.x), form the channel pore, and four regulatory subunits, called sulfonylurea receptors (SUR), surround this channel pore (1, 4, 33, 42). All major subunits have been cloned. These include KIR6.1 (16) (which is ubiquitously expressed) and KIR6.2 (15), which is expressed mainly in the heart, pancreas, and muscle (1, 6, 33, 42). SUR include SUR1 (15), SUR2A and SUR2B (17), and SUR2C, which may not be expressed in the rat (see citations in Ref. 8). SUR1 has two splice forms, SUR1A (access number L40624) and the shorter SUR1B (AF039595). Reconstitution experiments suggest that the K\textsubscript{ATP} channel is formed from KIR6.2 and SUR2A in the heart (6), KIR6.2 and SUR1 (actually SUR1A) in β-cells (16), and KIR6.1 and SUR2B in vascular smooth muscle (41).

Until recently, it has been generally believed that the channel pore is composed of a KIR6.x homotetramer (1, 4, 33, 42). Suggestive evidence that KIR6.1 can associate with KIR6.2 stems from channel reconstitution in heterologous expression systems. In HEK-293 cells expressing KIR6.1 and KIR6.2, immunoprecipitation revealed KIR6.1/KIR6.2 complexes (12, 28). In COS7 cells, coexpression of the fusion protein KIR6.1-KIR6.2 with SUR2A resulted in functional K\textsubscript{ATP} channels that had a channel conductance of 59 pS, intermediate between KIR6.2 homotetramers (67 pS) and KIR6.1 homotetramers (34 pS) (19). Rat atrial myocytes have a relatively low single channel conductance (58 pS), suggesting the possibility that atrium may express KIR6.1/KIR6.2 heterotetramers (8). However, there is strong evidence against functional heterotetramers of KIR6.1 and KIR6.2 in native K\textsubscript{ATP} channels. Adenoviral transduction of rabbit ventricular myocytes with pore loop-mutated KIR6.1AFA DNA had no effect on whole cell K\textsubscript{ATP} current, suggesting that KIR6.1 does not associate with KIR6.2 (32), whereas transduction with pore-mutated KIR6.2 DNA potentially inhibited the K\textsubscript{ATP} current as expected.

In view of this controversy, we reexamined here whether pore loop-mutated KIR6.1 can functionally associate with native KIR6.2 in cardiomyocytes. Because previous negative results (32) stemmed from the use of heterologous...
loop-mutated Kir6.1 subunits, we designed new loop-mutated rat Kir6.1 and Kir6.2 subunits to be tested in rat cardiomyocytes.

METHODS

Primary Cell Cultures

Atrial and ventricular myocytes were cultured as described previously (8, 9, 18, 27). Briefly, atrial appendage cells from 2- to 4-day-old Sprague-Dawley rats were dispersed enzymatically with 0.2% trypsin (Worthington) and plated on fibronectin-gelatin-coated 12-mm glass slides. Electroporation of DNA was performed after dispersion (7), and cells were plated and cultured in Ham-F10 medium (GIBCO) containing 10% FBS (BioWhittaker), antibiotics, 100 μM vitamin C, 1 μg/ml insulin, 1 μg/ml transferrin, 20 nM selenium, and 3 mM pyruvate. The Ham-F10 medium was adopted as it favored the survival rate after the electroporation.

Ventricular myocytes were dispersed enzymatically from the ventricular apex of 2- to 4-day-old Sprague-Dawley rats with 0.1% collagenase type II (GIBCO) and 0.1% trypsin (Worthington), using six periods of incubation as described by Izumo (details in Ref. 7). The cells were plated and cultured as described above.

For patch-clamp recordings, the cells were plated at a low density to avoid any intercellular contacts. To obtain mRNA extracts used in RT-PCR, 6 million cells were plated in 10-cm-diameter Falcon plates to avoid any intercellular contacts. To obtain mRNA extracts used in RT-PCR, 6 million cells were plated in 10-cm-diameter Falcon plates and cultured as described previously (8). The population of fibroblasts did not exceed 30%, as determined by immunostaining of myocytes for atrial natriuretic peptide (ANP) (9).

Reverse Transcription of K_ATP Subunit mRNA and DNA Amplification (RT-PCR)

To check for potential differences in K_ATP channel expression between atrial and ventricular myocytes, all major subunit mRNAs were tested in RT-PCR. After 48 h, the cultures were rinsed in 4°C PBS (pH 7.3), scraped with a rubber policeman in TRIzol buffer (Life Technologies), harvested, and stored at −80°C pending RT-PCR. For comparison, atrial appendages and ventricular apexes were excised from 8- to 10-wk-old Sprague-Dawley rats, immediately frozen in liquid nitrogen, and stored at −80°C pending tissue homogenization, mRNA extraction, and RT-PCR. RT-PCR was performed as described previously (8).

Plasmid Constructs Encoding Rat Kir6 and Electroporation Protocols

Rat Kir6.x cDNAs were subcloned into the pIRE52-EGFP vector (Clontech). This construction may favor the expression of the first cistron (Kir6.x) over the second (EGFP) (39, 40). All the plasmid constructs were verified by restriction analysis and double-strand DNA sequencing.

Wild-type Rat Kir6.2GFG (Kir6.2 with the normal GFG sequence in the pore loop). Rat Kir6.2GFG was obtained by subcloning the coding sequence of rat Kir6.2GFG cDNA from the pcDNA3-rat Kir6.2GFG (from Cécile Faure’s laboratory) into the pIRE52-EGFP vector. Kir6.2GFG cDNA was excised from pcDNA3-rat Kir6.2GFG using EcoRI and ligated into the EcoRI site of the pIRE52-EGFP vector.

Rat Kir6.2AFA. The pore mutation GFG → AFA was generated by PCR on the pcDNA3-rat Kir6.2GFG vector with the forward primer located in the rat Kir6.2 cDNA sequence 5′-CAGGTGACCATGGCATTCCGAGGGC-3′ (where the restriction sites BstEII and newly created BsmI are underlined and mutated nucleic acids are in bold). The reverse primer was located in the pcDNA3 sequence 5′-GATGGGCTGGCAGCTTACGAGG-3′. The PCR reaction was performed in a solution (50 μl final volume) containing 1.5 units of Taq DNA polymerase (Life Technologies), 250 μM each of dNTP, 20 pM of each primer, 1.5 mM MgCl2, and 5% DMSO with the following protocol: 3 min at 94°C, then 35 cycles composed of 45 s at 94°C, 30 s at 50°C, and 90 s at 72°C. This reaction was terminated by 10 min at 72°C. The PCR product (924 bp) was purified and digested with BstEII and XbaI and ligated into the pcDNA3-rat Kir6.2GFG vector that had been previously digested by BstEII and XbaI. The rat Kir6.2AFA cDNA was then excised from the pcDNA3-rat Kir6.2AFA by EcoRI digestion and ligated into the EcoRI site of the pIRE52-EGFP vector. The presence of the GFG → AFA mutation was confirmed by BsmI digestion.

Rat Kir6.1AFA and rat Kir6.1SFGE. Rat Kir6.1AFA and rat Kir6.1SFGE were obtained by subcloning the coding sequence of the cDNA of the pcDNA3-rat Kir6.1AFA and pcDNA3-rat Kir6.1SFGE vectors (kindly provided by Andrew Tinker) into the pIRE52-EGFP vector. Direct subcloning was not possible (due to incompatible restriction sites and the requirement of using pBS as an intermediate vector). These rather large plasmids (~7 kb) were difficult to electroporate. To minimize these plasmid digestion constraints, the size of these plasmids was reduced. Whole cDNA was excised by SalI/SmaI digestion, and the last 798 bp of the 3′-untranslated region were removed by SpI digestion. The shortened DNA was then reinserted into the pIRE52-EGFP vector that had been linearized by SalI/SmaI.

Electroporation of plasmids was performed with a Gene-Pulser II (Bio-Rad) as described previously for successful imaging of secretory vesicles in atrial myocytes (7). The electroporation procedure yields green fluorescent, healthy looking, beating myocytes over at least 3 days in culture. Fibroblasts did not express EGFP over this time period, contrary to what happens with other transfection methods. The number of EGFP-positive cells was low, however, thus rendering the patch-clamp studies more difficult than with adenoviral transduction protocols. For each electroporation, 400,000 cells were placed in 0.2 ml of calcium- and magnesium-free PBS mixed with 4–8 μg DNA in a cuvette (4 mm electrode gap) and exposed to a 200-V shock. Capacitance was 800 μF, and resistance was either 200 or 600 Ω. The resistances of 200 or 600 Ω were set in separate electroporations to increase the chances of obtaining green fluorescent cells. Cells were placed at various dilutions to obtain isolated myocytes. The cells were then immediately placed in culture medium and returned to the incubator. Electroporation controls were performed with pEGFP-N1 (Clontech), as the empty pIRE52-EGFP vector yields extremely faint fluorescence. Electroporation per se did not change the expression of maximal K_ATP current density. In atrial myocytes, the maximal current density was 64.9 ± 6.6 pA/pF (n = 4) without electroporation and 64.1 ± 4.9 pA/pF (n = 12) with electroporation of EGFP-N1 DNA under otherwise identical recording conditions.

Patch-Clamp Recording

Whole cell recordings of K_ATP current were performed as described previously (8, 27). Initially beating, green fluorescent myocytes without contact to neighboring cells were selected on a Nikon inverted fluorescence microscope (TMD Diaphot) and approached with the recording pipette by means of a Burleigh piezo-micro manipulator. The extracellular medium consisted of (in mM) 5 KCl, 1 CaCl2, 1 MgCl2, 110 NaCl, 10 glucose, and 10 HEPES, 1.1 ATP-Mg, 0.1 ADP-K; pH 7.2. A maximal K_ATP current density of ~60 pA/pF was obtained as reported previously (27). Whole cell K_ATP currents were recorded at room temperature (22–23°C) with an Axon

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200B amplifier using pCLAMP software. Recordings were started every 30 s from a −40-mV holding potential by imposing a 10-s voltage ramp from −80 to +90 mV. The −40-mV holding potential inactivated voltage-dependent channels, and the slow ramp further prevented their activation. To minimize chloride channel interference, the amplitude of the activated KATP current was measured at 0 mV, close to the chloride equilibrium potential. The current was measured in pA and normalized by cell capacitance (pA/pF). Mean capacitance was 17.7 ± 1.5 pF for 40 atrial myocytes and 15.0 ± 1.1 pF for 30 ventricular myocytes. Membrane polarization was measured in the current clamp mode at 0 pA. Inhibition of the activated current by 0.1–1 μM glyburide verified that the recorded current was due to KATP channels (8, 27).

Experimental Protocol

In a series of experiments on 40 atrial and 30 ventricular myocytes, electroporation was used to transfer plasmids encoding EGFP-N1, rat KIR6.1AFA-pIRES2-EGFP, rat KIR6.2AFA-pIRES2-EGFP, rat KIR6.1SFG-pIRES2-EGFP, rat KIR6.2GF-pIRES2-EGFP, or rat KIR6.2GF-pIRES2-EGFP DNA.

Recordings were performed between 72 and 80 h after electroporation. This interval was required for full expression of the pore loop-mutated Kir6.x subunits (32). Upon establishment, whole cell configuration currents were recorded during a 5- to 10-min control period. The KATP current either activated spontaneously or during the subsequent period lasting ~20 min, when up to 10 μM diazoxide and 100 μM pinacidil were applied. Diazoxide was also used to stimulate atrial myocytes as reported previously (8, 27). The protocol was terminated by applying 0.1–1 μM glyburide.

Quantification of Kir6.x Overexpression

Atrial myocytes were electroporated with one of the following plasmids (see constructs): EGFP-N1, rat KIR6.2AFA-pIRES2-EGFP, rat KIR6.1SFG-pIRES2-EGFP, or rat KIR6.2GF-pIRES2-EGFP. A total of 47 live cells was imaged 3 days later by obtaining z-stacks of up to 60 confocal optical sections (0.15 μm apart) of each cell. The cultures were then fixed in 2% parafomaldehyde and stained with primary antiserum directed against rat Kir6.1 or Kir6.2 (dilution 1:1000) (35) followed by 1:300 diluted Texas red-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology). The green fluorescent myocytes were then imaged again by acquiring z-stacks for EGFP and Texas red. Background Texas red staining was determined by omitting the primary antibody. The imaging system consisted of two lasers (488 and 568 nm) with an electronic wavelength selection and timing device, a Nikon Diaphot 300 inverted microscope with a x100 fluorescence oil objective (numerical aperture 1.3), a piezoelectric objective z-drive (Precision Instruments), a QLC-100 spinning wheel confocal head (Visitech), and a CoolSnap-HQ cooled charge-coupled device (CCD) camera (VisiRon Systems). The whole system was driven by Metamorph software (Universal Imaging). Mean fluorescence intensities (CCD units) of optical sections were analyzed, background corrected with MetaMorph, and normalized per 100-μs (EGFP) or 1,000-ms illumination duration (Texas red). The overexpression was quantified by dividing the background-corrected mean fluorescence intensity of Texas red by the green fluorescent myocyte by the background-corrected mean fluorescence intensity of Texas red for the neighboring (not green fluorescent) myocyte. There was no overlap in EGFP and Texas red light emissions.

Statistical Analysis

For each myocyte, we determined the time course of the current (pA), current density (pA/pF), and membrane potential (mV). Their maximal values were averaged over each group, cell type, and DNA expression to yield means ± SE. Because the myocytes were maximally stimulated (27), the maximal currents reflected the product of the number of open channels times their average channel conduction. Current-voltage curves were established for each myocyte. Differences in means were analyzed for statistical significance by ANOVA using SAS software from the SAS Institute (Cary, NC). Statistical analysis (ANOVA) of rank-ordered fluorescence intensities of imaged myocytes was also performed with SAS software.

RESULTS

RT-PCR of KATP Channel Subunits

To test for potential differences between atrial and ventricular channel subunit composition, RT-PCR was performed on RNA extracts from cultures, neonatal tissue, and adult tissue (Fig. 1). β-Actin expression served as a control. Expression of mRNA for ANP was strong in the neonate atrium and ventricle, weaker in the adult atrium, and not detectable in the adult ventricle, as already known. Samples not subjected to RT (−) but to PCR were all negative. There were no remarkable differences between atrial and ventricular tissues, nor between atrial and ventricular neonate cultures. All major PCR products migrated to their expected position (see Ref. 8 for details). Samples from the atrium and ventricle showed expression of mRNA for Kir6.1, Kir6.2, SUR1A, SUR2A, and SUR2B, and none showed SUR2C mRNA, as previously reported for proANP or Kir6.x, these results fairly represent the myocyte cultures used in these experiments.

Effect of Electroporation of Cultured Cardiomyocytes with Pore Loop-Mutated Rat Kir6 Subunit cDNA

In rat atrial myocytes, electroporation of rat Kir6.1AFA-IRE2-EGFP (Fig. 2B), rat Kir6.2AFA-IRE2-EGFP (Fig. 2C), and rat Kir6.1SFG-IRE2-EGFP (Fig. 2D) caused a strong inhibition of the KATP current. KATP current was inhibited maximally by applying up to 10 μM diazoxide plus 100 μM pinacidil. Electroporation of rat Kir6.2GF-IRE2-EGFP DNA, which represents wild-type Kir6.2, or EGFP-N1 DNA resulted in large KATP currents (Fig. 2, A and E). In ventricular myocytes, the pore loop-mutated Kir6.2GF-IRE2-EGFP constructs all strongly reduced the KATP current (Fig. 3, B–D), whereas EGFP-N1 and wild-type Kir6.2GF-IRE2-EGFP resulted in large KATP currents (Fig. 3, A and E).

Figure 4 displays the statistical summary from the experiments on 40 atrial and 30 ventricular myocytes. After 72 h, the electroporation of pore loop-mutated Kir6.x-IRE2-EGFP constructs strongly inhibited whole cell KATP current (Fig. 4, A and B, top) and decreased the current density (Fig. 4, A and B, middle) in atrial myocytes by up to 73.7% (P < 0.01) and in ventricular myocytes by up to 85.8% (P < 0.01) relative to EGFP-N1 controls. The wild-type rat Kir6.2GF-IRE2-EGFP construct significantly boosted KATP current density in both atrial myocytes (+42.9%, P < 0.01) and ventricular myocytes (+58.3%, P < 0.01) (Fig. 4, A and B, middle). The membrane potential was significantly less polarized with the pore loop-mutated Kir6 in the majority of cases (Fig. 4, A and B, bottom).
Estimation of KIR6.x Overexpression in Atrial Myocytes

We next tested whether electroporation caused a massive or modest overexpression of the pore loop-mutated KIR6.x subunit relative to endogenous wild-type KIR6.x. A hundred-fold overexpression, for example, would imply that the dominant negative effect entirely stemmed from competition between wild-type and pore loop-mutated KIR6.x subunits for SUR-mediated trafficking rather than from an association of pore loop-mutated KIR6.x with native KIR6.x. Electroporation with EGFP-N1 DNA caused, 3 days later, a major expression of green fluorescence in living atrial myocytes (Fig. 5A); a laser excitation at 488 nm, on the minimal illumination setting lasting only 50–100 ms, was sufficient for generating hundreds of CCD units. In comparison, electroporation of rat KIR6.2AFA-IRES2-EGFP, rat wild-type KIR6.2GFGR-RES2-EGFP, and rat KIR6.1SFA-IRES2-EGFP resulted in 10 to 20 times less green fluorescence (Fig. 5A). After fixation and immunostaining, EGFP-N1 DNA electroporated myocytes showed a similar staining intensity (of Texas red) as neighboring non-green fluorescent myocytes (Fig. 5B). Staining intensity (of Texas red) for KIR6.2AFA, wild-type KIR6.2, and KIR6.1SFA in green fluorescent myocytes was 10.6, 7.0, and 6.0 times greater, respectively, than in neighboring non-green fluorescent myocytes, and these ratios were roughly proportional to the green fluorescence measured in living cells. An example of a green fluorescent wild-type KIR6.2-IRES2-EGFP electroporated atrial myocyte is shown in Fig. 5C, with the corresponding immunostaining for KIR6.2 in Fig. 5D. The neighboring myocytes were faintly stained, similarly to other non-green fluorescent myocytes of the same culture. Individual variations showed KIR6.x overexpression ranging up to 40-fold, indicating that KIR6.x immunostaining was not saturated within the range shown in Fig. 5B.

DISCUSSION

This study shows for the first time that electroporation of a pore loop-mutated rat KIR6.1 DNA strongly inhibits K$_{\text{ATP}}$ current in both atrial and ventricular rat cardiomyocytes. This
Fig. 2. Effect of electroporation of EGFP (A), rat KIR6.1AFA-pIRES2-EGFP (r6.1A-I2-EGFP; B), rat KIR6.2AFA-pIRES2-EGFP (r6.2A-I2-EGFP; C), rat KIR6.1SFG-pIRES2-EGFP (r6.1S-I2-EGFP; D), and rat KIR6.2GFG-pIRES2-EGFP (r6.2G-I2-EGFP) DNA (E) on activated atrial K\(_{\text{ATP}}\) current. AFA and SFG refer to mutations of wild-type GFG in the pore loop of the KIR6.x subunit. Diazoxide (10 \(\mu\)M; D-5 or D) plus 10–100 \(\mu\)M pinacidil (P-5 or P-4) was added to ensure maximal activation. Left: original traces; middle: potential-current relationships; right: time-dependent activation of K\(_{\text{ATP}}\) current and membrane potential. Note the strong inhibition by glyburide (1 \(\mu\)M; G-6). Typical examples are shown for each type of construct. Mean results from 40 atrial myocytes are shown in Fig. 4.
Fig. 3. Effect of electroporation of EGFP (A), rat KIR6.1AFA-pIRES2-EGFP (B), rat KIR6.2AFA-pIRES2-EGFP (C), rat KIR6.1SFG-pIRES2-EGFP (D), and rat KIR6.2GFG-pIRES2-EGFP DNA (E) on activated ventricular K$_{ATP}$ current. Diazoxide (10 μM) plus 10–100 μM pinacidil was added to ensure maximal activation. Left: original traces; middle: potential-current relationships; right: time-dependent activation of K$_{ATP}$ current and membrane potential. Note the strong inhibition by glyburide (1–10 μM). Typical examples are shown for each type of construct. Mean results from 30 ventricular myocytes are shown in Fig. 4.
new finding is consistent with the possibility that KIR6.1/ KIR6.2 heterooligomers may associate to form sarcolemmal channels. Either an insufficient DNA expression or species differences in molecular sequence could explain previous negative results in rabbit ventricular myocytes (32). Expression of wild-type KIR6.2 enhanced KATP current density; thus competition in membrane trafficking between KIR6.2 and pore loop-mutated KIR6.x for SUR might explain only part of the inhibitory effects on KATP current. Furthermore, this study has succeeded in estimating the overexpression of loop-mutated KIR6.x subunits. The relatively modest overexpression (6- to 10-fold) makes it unlikely that the functional association of KIR6.1/KIR6.2 subunits was coerced. The following subsections address these issues in more detail.

**Evidence for Functional Heteromultimerization of KIR6.1/KIR6.2 in Cardiac KATP Channels**

A comparison of KIR6.x protein sequences (Blast, NCBI) shows that mouse KIR6.1 displays an amino acid homology of 72% with rabbit KIR6.2, 70% with rat KIR6.2, 98% with rabbit KIR6.1, and 98% with rat KIR6.1. Thus, for each KIR6 subtype, differences in the molecular sequence between the rat, rabbit, and mouse are of the order of 2%, but such small species differences at critical sites cannot be excluded to play a role and might explain the differences in results between our study and previous negative findings (32). We constructed expression vectors coding for rat pore loop-mutated KIR6.x followed by an IRES sequence and EGFP. This both eliminated species differences in the KIR6.x sequence in the rat experiments and potentially enhanced the expression of loop-mutated KIR6.1 over EGFP, as expected from other studies (39, 40). We also tested a SFG loop-mutated KIR6.1 version, shown by Tinker’s group (12) to have more powerful dominant negative effects than the AFA mutation. The results (Figs. 2–4) clearly indicated that electroporation of all three loop-mutated KIR6.x vectors strongly diminished activated KATP current in both atrial and ventricular myocytes, with loop-mutated KIR6.1SFG having the most powerful effect on current density. We propose that the negative results in rabbit ventricular myocytes (32) were probably due to insufficient expression of nonhomologous KIR6.1AFA that, in addition, was less efficient in suppressing KATP current than the SFG pore mutant.

**Does Limited Trafficking of Endogenous KIR6.2 Subunits Explain the Suppression of KATP Current?**

It is known that the SUR subunit is required to transfer the KIR6.x subunit from the endoplasmic reticulum to the plasma membrane (43). It may be conjectured that a massive overexpression of pore loop-mutated KIR6.x subunits would sequester the SUR subunits and thus inhibit trafficking of endogenous KIR6.2. To address this question, an expression vector was constructed coding for wild-type rat KIR6.2GFG followed by an IRES sequence and EGFP. If trafficking of KIR6.2 was limited by a limited supply of SUR2A subunits, then activated KATP current density would not be increased by electroporation of this construct. However, activated KATP current density increased significantly in both atrial and ventricular myocytes, by 45% and 58%, respectively, compared with the EGFP control (Figs. 2–4). This suggests that extra SUR subunits were available to accommodate trafficking of pore loop-mutated KIR6.x subunits to the plasma membrane. It must be noted that species and gender differences may exist. In guinea pig ventricular tissue, the mRNA expression for SUR2A, but not for KIR6.1 or KIR6.2, is less in males than in females (31). Moreover, in H9c2 cells, KIR6.2 protein appears to be ex-
pressed in excess over SUR2A (11, 30). Thus, to further address the question as to whether pore loop-mutated KIR6.6 subunits were massively overexpressed in the electroporation experiments, recently developed antisera against KIR6.1 and KIR6.2 (35) were used to immunostain the electroporated myocyte cultures. Quantification of KIR6.x staining in EGFP-positive relative to EGFP-negative myocytes indicated that KIR6.1SF was significantly less overexpressed (6.0-fold) than KIR6.2AFA (10.6-fold; Fig. 5); however, KIR6.1SF was as (or more) effective in suppressing K_{ATP} current density in atrial myocytes (Fig. 4). These results indicate that 1) overexpression of loop-mutated KIR6.x was rather modest in the electroporation experiments; and 2) competition in KIR6.x trafficking, although not excluded, would only explain part of the inhibition of K_{ATP} current. From cell lines, it is known that a DNA ratio of KIR6.2SF to KIR6.2 of 5:1 is required in transfection to inhibit the $^{86}$Rb$^+$ efflux by $\sim80\%$ (23). The degree of overexpression measured in the electroporation experiments herein are thus compatible with an inhibition of K_{ATP} current of 45–80\%.

**Perspectives**

This study favors the view that pore-forming KIR6.1 and KIR6.2 subunits can form heteromultimers in primary cultured rat cardiac myocytes and reconciles previously held differences in opinion on K_{ATP} channel assembly in reconstitution studies and cardiomyocytes (12, 19, 28, 32). Heteromultimerization within potassium channels in vivo is not unprecedented, as it has been demonstrated already in the KIR3.x subfamily (citations in Ref. 38) and within (but not among) the K_{Ca} Shaker, Shab, Shal, and Shaw subfamilies (citations in Ref. 29). Whether KIR6.1/KIR6.2 heteromers exist in cardiac myocytes remains to be established biochemically, but technical difficulties encountered by many laboratories have so far precluded this approach for native cardiac K_{ATP} channels (e.g., Ref. 35). Although all major K_{ATP} channel subunit mRNAs are expressed in both atrial and ventricular myocytes, ventricular myocytes express mainly KIR6.2 and SUR2A in the sarcoplasmic K_{ATP} channels, with a single channel conductance of the reconstituted human channel of 80 pS recorded at room temperature (6). Confirming this view, knockout of the KIR6.2 subunit results in nondetectable K_{ATP} currents in adult mouse ventricular myocytes (36). K_{ATP} Channel composition in atrial myocytes is not yet established, but the single channel conductance, at room temperature, has been shown to range from 58 pS in rats (8) to 73 pS in humans (44). The lower value is close to the single channel conductance of KIR6.1/KIR6.2 heteromers coassembled with SUR2A (see Introduction). Assembly of KIR6.1/KIR6.2 with SUR1 also lowers the single channel conductance (6). Native channels from human atrial and neonatal rat ventricular myocytes display a calculated channel conductance between 49 and 63 pS (5). A relatively low single channel conductance has also been measured in Purkinje cells (20) and rabbit ventricular myocytes (14), raising the possibility of KIR6.1/KIR6.2 heteromers in different regions of the heart.

Could there be situations favoring the assembly of KIR6.1/KIR6.2 heterotramers? Indeed, when subjected to ischemia, the rat heart (2) [and kidney (34)] upregulates the expression of mRNA for KIR6.1, and severe hypoxia decreases the KIR6.2 to KIR6.1 mRNA ratio (21). Thus ischemia and hypoxia would increase the probability of forming KIR6.1/KIR6.2 heterotramers. Although experiments along these lines have not yet been conducted, such a mechanism could serve the purpose of...
reducing maximal $K_{ATP}$ current during ischemia, thus preventing an excessive reduction of the refractory period and reducing the risk of arrhythmia. Because even small $K_{ATP}$ currents are capable of hyperpolarizing the myocyte (Figs. 2 and 3), KIR6.1/KIR6.2-type $K_{ATP}$ channels would still be capable of reducing calcium transients to reduce the energy demand.

In conclusion, this study favors the hypothesis that KIR6.1 and KIR6.2 can form heterotetramers in atrial and ventricular myocytes. Previous results to the contrary are probably explained by an insufficient expression of nonhomologous pore loop-mutated KIR6.x subunits. A heterotetrameric channel pore would explain the lower single channel conductances found in different regions of the heart. The true physiological and pathophysiological significance of this finding remains to be determined but may relate to the delicate choice between protection against hypoxia and prevention of cardiac arrhythmia.

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