

Expression of ATP-Sensitive K⁺ Channel Subunits during Perinatal Maturation in the Mouse Heart

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ABSTRACT

Prevailing data suggest that sarcolemmal ATP-sensitive (K_{ATP}) channels in the adult heart consist of Kir6.2 and SUR2A subunits, but the expression of other K_{ATP} channel subunits (including SUR1, SUR2B, and Kir6.1) is poorly defined. The situation is even less clear for the immature heart, which shows a remarkable resistance to hypoxia and metabolic stress. The hypoxia-induced action potential shortening and opening of sarcolemmal K_{ATP} channels that occurs in adults is less prominent in the immature heart. This might be due in part to the different biophysical and pharmacological properties of K_{ATP} channels of immature and adult K_{ATP} channels. Because these properties are largely conferred by subunit composition, it is important to examine the relative expression levels of the various K_{ATP} channel subunits during maturation. We therefore used RNase protection assays, reverse transcription-PCR approaches, and Western blotting to characterize the mRNA and protein expression profiles of K_{ATP} channel subunits in fetal, neonatal, and

adult mouse heart. Our data indicate that each of the K_{ATP} channel subunits (Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B) is expressed in the mouse heart at all of the developmental time points studied. However, the expression level of each of the subunits is low in the fetal heart and progressively increases with maturation. Each of the subunits seems to be expressed in ventricular myocytes with a subcellular expression pattern matching that found in the adult. Our data suggest that the K_{ATP} channel composition may change during maturation, which has important implications for K_{ATP} channel function in the developing heart. (*Pediatr Res* 58: 185–192, 2005)

Abbreviations

K_{ATP}, ATP-sensitive K⁺
Kir6, inward rectifying K⁺ channel, subfamily 6
SUR, sulfonylurea receptor

ATP-sensitive K⁺ (K_{ATP}) channels are robustly expressed in the immature heart (1,2). However, K_{ATP} channels in the immature heart may differ in several respects to those of adult cardiac tissue. In immature rabbit ventricle, K_{ATP} channel open probability is roughly the same as in the adult, but they exhibit a significantly smaller single-channel conductance (~55 pS) and channel density, as defined by the number of open channels per patch (3). In the immature rat heart, K_{ATP} channel density is also high, but the single-channel conductance and the mean open time are the same as in the adult (2). Reports

describing a smaller unitary conductance in neonatal rat atrium (4) suggested that there may be regional differences. The immature rat heart K_{ATP} channel has a lower open probability, and the channels are much more sensitive to block by intracellular ATP (2,5). In addition to these biophysical changes, there are reports that immature K_{ATP} channels may have a different pharmacological profile. The neonatal atrial K_{ATP} channel shows a unique functional and pharmacological profile resembling the pancreatic β cell channel for its unusually high affinity for glibenclamide and diazoxide (4,5). These characteristics suggest that neonatal K_{ATP} channels may differ in their molecular composition relative to the mature heart.

K_{ATP} channels are believed to be hetero-octameric complexes, consisting of four pore-forming subunits (either Kir6.1 or Kir6.2) and four regulatory subunits (either SUR1 or SUR2); the latter belonging to the ATP-binding cassette superfamily (ABC proteins) (6–9). Initially, two isoforms of SUR2 were described (SUR2A and SUR2B) (10,11), but now

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it is known that at least five alternative spliced variants exist (12). Functional channels (*i.e.* inhibited by cytosolic ATP, blocked by glibenclamide, and opened by K^+ channel openers) are formed only when Kir6 and SUR subunits are coexpressed (13,14). Expression of the same pore-forming subunit (Kir6.2) with either SUR1 or SUR2 leads to channels with identical single-channel conductances but with differences in their gating, ATP sensitivity, and pharmacological properties (15,16). It therefore now is commonly believed that different properties of native K_{ATP} channels in various tissues can be attributed to the molecular architecture of the channel complex, with the adult cardiac K_{ATP} channel consisting of Kir6.2/SUR2A heterooctameric complexes (17).

Given the biophysical and pharmacological differences that exist between K_{ATP} channels in immature and adult heart, it is possible that the molecular composition of K_{ATP} channels in immature heart may be different. As a first step in understanding these possible differences, we performed RNase protection assays, reverse transcription (RT)-PCR analysis, and Western blotting to examine mRNA and protein expression of the various K_{ATP} channel subunits (Kir6.1, Kir6.2, SUR1, and SUR2A/B). Our data show expression of each of the K_{ATP} channel subunits in mouse heart at various developmental time points (fetal to adult). The expression of each of the subunits was generally low in the fetal heart and progressively increased with postnatal maturation.

METHODS

All animals were housed within the New York University Medical Center animal facilities and maintained and killed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by our Institutional Animal Care and Use Committee.

Preparation of RNA. The hearts or brains of adult (1–2 mo of age), neonatal (1–2 d after birth), and fetal (12–14 d postgestation) mice were rapidly removed and flash-frozen in liquid N_2 to avoid RNA degradation. The tissue was homogenized (Polytron homogenizer), and total RNA was prepared using a guanidine thiocyanate method (TriReagent solution; Sigma Chemical Co.-Aldrich, St. Louis, MO). The final RNA pellet was resuspended in diethyl pyrocarbonate-treated water or in RNA storage solution (Ambion, Austin, TX). The RNA concentration was determined spectroscopically (OD measurements were made at a wavelength of 260 nm; Genesys5 spectrophotometer, Milton Roy, Rochester, NY). The integrity and the quantification of the preparations were assessed by the presence of clear and distinct ribosomal RNA (18S and 28S) bands on denaturing agarose gel electrophoresis (data not shown).

Generation of biotinylated riboprobes. RNA was reverse-transcribed using Maloney murine leukemia virus reverse transcriptase enzyme (Promega, Madison, WI), and 2 μ L of each of these reactions was used as a template in the RT-PCR reactions to generate subunit-specific riboprobes. The PCR primers used are shown in Table 1. Cycling conditions were empirically determined (usually <35 cycles), and *pfu* polymerase (Stratagene, La Jolla, CA) was used to minimize PCR errors. PCR products were ligated into expression vectors and sequenced on both strands to confirm their identities and sequence accuracy. The following ligations were performed: the Kir6.1 cDNA fragment was subcloned into PCR-Script (Stratagene), the Kir6.2 cDNA fragment was subcloned into pGEM3Z (Promega), the SUR1 fragment was subcloned into PCR-Blunt (Invitrogen, Carlsbad, CA), and the SUR2 fragment was subcloned into pBS-SKII(+) (Stratagene). The cyclophilin and actin cDNA templates were purchased from Ambion.

Each plasmid was linearized on the 5' end of the cDNA fragment by restriction digestion. Antisense probes were synthesized in a run-off transcription reaction using the Maxiscript T3/T7 kit (Ambion). Biotinylated CTP was incorporated into the probe by using a 1:1 ratio of CTP:Biotin-CTP (Invitrogen). The probes were subsequently purified by denaturing PAGE (PAGE; 8% denaturing TBE-Urea gel), extracted in elution buffer (Ambion), and ethanol-precipitated. The probes were resuspended in Ambion RNA storage solution. The concentration of synthesized probe in RNA storage solution was deter-

Table 1. Primer sequences used for RT-PCR to generate K_{ATP} channel-specific riboprobes and for determining relative SUR2A/B expression

| Species | Size | Primer pair | Primer sequence |
|---------|------------------|------------------|------------------------------|
| Kir6.1 | 297 bp | 1032F | 5'-ACCAGAATTCTCTGCGGAAG-3' |
| | | 1032R | 5'-GCCCTGAAGTGGTGTAGAGT-3' |
| Kir6.2 | 309 bp | 1077F | 5'-TTGGAAGGCGTGGTGTAGAAAC-3' |
| | | 1123R | 5'-GGACAAGGAATCTGGAGAGAT-3' |
| SUR1 | 217 bp | 1253F | 5'-GCCTTCGTGAGAAAGACCAG-3' |
| | | 1253R | 5'-GAAGCTTCTCCGGTTTGTCA-3' |
| SUR2 | 148 bp | 1240F | 5'-CCATCATCAGTGTCAAAGC-3' |
| | | 1240R | 5'-GGCTGCTTCTGTTTATTGG-3' |
| SUR2A/B | SUR2A: 357 bp | 1307F | 5'-ATCATGGATGAAGCCACTGC-3' |
| | | SUR2B: 181 bp | |
| | | | 1463R |

Primers were designed against the following mouse cDNA sequences (Genbank Accession numbers in brackets) of Kir6.1 (D88159), Kir6.2 (NM_010602), SUR1 (NM_011510), SUR2A (D86037) and SUR2B (D86038). The intron/exon organization of SUR2 was verified from genomic mouse sequences (AC108508). The suffix 'F' and 'R' of the primer names refer to forward and reverse primers respectively.

mined by OD readings at 260 nm. We used ~200–500 pg of probe per reaction. Probes were tested in a ribonuclease protection assay against mRNA that was extracted from cells that were transfected with the appropriate full-length cDNA or against an unlabeled "probe" sequence generated in the sense orientation.

RNase protection assays. The Hybspeed RPA Kit (Ambion) was used to hybridize the biotinylated riboprobes to RNA (10 min at 68°C) from adult, neonatal, and fetal mouse heart (the reaction contained yeast RNA to keep the total RNA concentration constant at 50 μ g). Two control reactions were always included in each experiment. As a control of the riboprobe quality, the probe was incubated with yeast tRNA and RNases to demonstrate the lack of self-self protection within the probe. This reaction also serves as a check to verify digestion by RNases. In another reaction, the probe was incubated with yeast tRNA but without RNases to verify the probe size and integrity. After hybridization, unhybridized probe and other single-stranded RNA were digested with RNase A/T1, and the product (which contains undigested double-stranded protected fragments) was subjected to denaturing PAGE (6–8% TBE-Urea; Invitrogen) to determine the size and the amount of the protected fragment. Ribonucleotides were transferred by electroblotting (Owl Scientific, Portsmouth, NH) onto positively charged nylon membrane (Bright Star-Plus; Ambion) and cross-linked by controlled ultraviolet exposure (Stratalinker; Stratagene). Each blot was subjected to a nonisotopic detection protocol, using streptavidin alkaline phosphatase (BrightStar BioDetect; Ambion). Several different exposures of the same blot were made to ensure that measurements were within the linear range of the film and that no saturation occurred. Experiments were performed such that each gel contained lanes for every age group under study, and these were compared internally (comparisons were independent of variables such as film exposure time etc.). For each blot, data were normalized relative to the band intensity in the adult lane.

RT-PCR of SUR2 splice variants. A primer pair (1307F and 1463R; Table 1) was designed such that both the forward and the reverse primers were within regions common to SUR2A and SUR2B but on either side of the splice site. In this manner, we could differentiate between the expression of the SUR2A (357 bp) and SUR2B (181 bp) splice variants. RT reactions were carried out using random primers as described above. PCR (Geneamp 9700; Perkin Elmer, Wellesley, MA) was performed (HotStart *Taq* polymerase; Qiagen, Studio City, CA) using 2 μ L of the RT reaction as the template. The cycling conditions consisted of an initial denaturing time of 15 min (at 95°C) followed by 33 cycles (denaturing at 95°C for 30s, annealing at 60°C for 30s, and extension at 72°C for 45s) and a final extension at 72°C for 5 min. PCR products (10 μ L) were separated by agarose gel (1.5%) electrophoresis and visualized by staining with ethidium bromide or Sybr-gold (Molecular Probes, Eugene, PR).

Antibodies to K_{ATP} channel subunits used for Western blotting. We developed a polyclonal rabbit anti-Kir6.1 antibody (NAF1) against a peptide corresponding to the N-terminal sequence ENLRKPRIRDRLP of mouse Kir6.1. The polyclonal goat anti-Kir6.2 antibody (G16) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). We used a commercial poly-

clonal goat anti-SUR1 antibody (C-16; Santa Cruz) targeted to a C-terminal region of the protein to detect SUR1. We also developed a polyclonal rabbit antibody (R2B) specific to a C-terminal peptide (CVMKRGNILEYDTPES) of human SUR2B. Both antibodies that we developed were affinity-purified using the peptide against which they were raised.

Transfection of cells. For characterizing the specificity of the primary antibodies, we performed Western blotting using lysates of cells that were transfected with K_{ATP} channel subunit cDNAs. For some experiments, we used cell lysates that were obtained from HEK-293 cells that were stably transfected with Kir6.1 and either SUR2A or SUR2B subunits (18). For others, we grew COS7L or HEK-293 cells to ~70% confluence before transfection (Fugene6; Roche Applied Science, Indianapolis, IN) with hamster SUR1 cDNA (gift from Dr. Joseph Bryan, Baylor College of Medicine, Houston, TX) and the rat Kir6.1 cDNA (gift from Dr. Susumu Seino, Kobe University Graduate School of Medicine, Kobe, Japan).

Preparation of membrane fractions. Mouse heart tissue (adult or 1- to 4-d neonates) were excised, rinsed in Dulbecco's PBS at 4°C (Invitrogen), and placed in homogenization buffer [in mM: 250 sucrose, 1 EGTA, 1 MgCl₂, and 10 HEPES (pH 7.4)] supplemented with protease inhibitor cocktail (1:100; Sigma Chemical Co.-Aldrich, St. Louis) and 1 mM PMSF (Sigma Chemical Co.-Aldrich). Tissue was snap-frozen in liquid nitrogen and ground into a powder with a mortar and pestle on dry ice. Tissues were resuspended in homogenization buffer (10 mL/g tissue) and homogenized using a Polytron homogenizer (setting 2 for two 20-s bursts). The tissue was further homogenized using a motorized Dounce tissue homogenizer (five strokes). This was followed by centrifugation (1000 × g for 10 min) to remove cellular debris and nuclear material. The supernatant was kept, and the pellet was resuspended in 5 mL/g homogenization buffer. The pellet mixture was rehomogenized (Dounce tissue homogenizer) and recentrifuged at 1000 × g for 10 min. The supernatant of the two steps was combined and centrifuged at 20,000 × g for 1 h. The resulting pellet that contained membrane proteins was resuspended in 0.5 mL TBS/g tissue [supplemented with protease inhibitor cocktail (1:100) and PMSF (1 mM)]. Protein concentrations were measured (BioRad, Hercules, CA), and samples were diluted to 1–5 mg/mL protein with TBS, aliquotted, and stored at –80°C.

Western blotting. Cell lysates were fractionated by SDS-PAGE and subjected to Western blotting for identification of K⁺ channel subunits. Briefly, membrane proteins (30 μg) were fractionated on 10% SDS-polyacrylamide gels and transferred to immunoblot PVDF membranes (BioRad). Membranes were incubated overnight at 4°C in blocking solution TBS-Tween (in mM: 100 Tris-HCl, 150 NaCl, and 0.1% Tween-20) with 5% nonfat milk. The membrane strips were incubated for 1 h with primary antibodies at room temperature (TBS-Tween + 1° Ab). After washing with TBS-Tween (three times), the strips were incubated for 1 h at room temperature in peroxidase-linked donkey anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ) diluted 1:3000 or in peroxidase-linked mouse anti-goat IgG (Sigma Chemical Co.-Aldrich) diluted 1:50,000 in TBS-Tween. After washing (three times with TBS-Tween), bound antibodies were detected using the SuperSignal West Pico (Pierce Biotechnology, Rockford, IL) chemiluminescent alkaline phosphatase substrate, and luminescence was recorded on Kodak x-ray film.

Immunocytochemistry. Single ventricular myocytes were isolated from the hearts of neonatal (1–2 d after birth) mice of either sex. The hearts were pooled in an oxygenated HEPES-buffered saline solution (with heparin, 10 U/mL) that contained (in mM) 20 NaCl, 5.4 KCl, 0.5 MgSO₄, 5 sodium pyruvate, 20 glucose, 20 Taurine, 30 BDM, 10 HEPES (pH 7.0). The atria were removed, and the ventricles were sliced into small pieces (~0.5 mm³). Cells were isolated in a three-step enzymatic digestion procedure (at 35°C). Tissue was transferred into a 50-mL beaker, kept in an incubator that was prewarmed to 35°C, and stirred with a small magnetic bar at 3–5 rpm with different enzymes mixed in HEPES-buffered saline solution. The first enzyme used was protease Type XXIV (Sigma Chemical Co.) for 3–5 min. In the second step, supernatant was discarded and 5 mL of hyaluronidase (80 U/mL) was added for 5 min or until a few rod-shaped cells were seen under the microscope. In the third step, residual tissue was incubated in collagenase (110 U/mL). The progress of the tissue digestion was followed by examining the enzyme solution for increases in turbidity and the presence of single rod-shaped myocytes. The supernatant that was obtained after collagenase was centrifuged (100 × g for 2 min), and the pellet was suspended in KB solution.

Isolated myocytes were allowed to settle on and attach to glass coverslips and were fixed in paraformaldehyde (4%) for 15 min at room temperature, followed by a fixation/permeabilization step by incubation with ice-cold 100% methanol for 5 min at –20°C (19). Myocytes then were washed with Ca²⁺ and Mg²⁺-free PBS (Invitrogen) and incubated with 0.1% Triton X-100 (in PBS) for 15 min at room temperature. After washing (2 × 5 min) and blocking (5% donkey serum in PBS; 2 × 10 min), the cells were incubated with primary antibodies (1 h at room temperature), washed (3 × 10 min in PBS serum), and incubated with secondary antibodies (45 min at room temperature). After four

washes (with PBS; 10 min each), coverslips were mounted and fluorescence was recorded using a confocal microscope (Leica TCS SP2, HCX PL APO CS ×63 oil objective) while excited with a HeNe 543-nm laser.

Antibodies used (all polyclonal) were the rabbit anti-Kir6.1 NAF-1 antibody (see above), the rabbit anti-Kir6.2 76A antibody (a gift from Dr. Tinker, University College, London, UK), goat anti-SUR1 C-16 antibody (see above), and a goat anti-SUR2 C-15 C-terminal antibody (sc-5793; Santa Cruz Biotechnology). Each of these antibodies has been characterized extensively for their specificity to subunits against which they have been raised and for their utility in immunocytochemistry (20–22). Secondary antibodies used were Cy3-conjugated donkey anti-rabbit IgG or Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

RESULTS

RNAse protection assays: determination of probe concentration. For an RNAse protection assay to be semiquantitative, it is important that the probe be present in excess relative to the amount of target mRNA in the sample. We determined this to be the case for all probes used in this study. An example is shown in Fig. 1 for a probe directed against cyclophilin. Various amounts of mouse brain RNA were hybridized with a fixed amount (230 pg) of biotinylated anti-cyclophilin probe. Increasing amounts of protected fragments were observed as the RNA amount was progressively increased until saturation occurred beyond 50 μg of RNA (Fig. 1). Thus, for an abundantly occurring mRNA species such as cyclophilin, this amount of probe was sufficient for quantitative determination of target mRNA abundance in the sample, as long as the amount of RNA is kept at or below 10–30 μg. We performed similar experiments for the K_{ATP} channel subunit probes that were used in the study and found the probe always to be in excess, even with RNA amounts of >50 μg (data not shown). The reason for this is most likely that the amount of mRNA for K_{ATP} channel subunits is much lower than that of a housekeeping gene such as cyclophilin.

Kir6.1 mRNA expression in fetal, neonatal, and adult mouse heart. RNAse protection assay techniques were used to investigate the developmental expression of Kir6.1 mRNA levels in fetal (12–14 d postconception), neonatal (1–2 d), and adult mouse heart. Fig 2A shows Kir6.1 mRNA expression to be low in fetal heart and to increase progressively with age. Experiments were performed using two amounts of sample RNA (10 and 30 μg) as further validation that measurements were made within the linear range as determined by several factors, including the probe concentration used and the film exposure. Thus, Kir6.1 mRNA expression increases with developmental age from fetal to neonatal to adult states.

Normalization of mRNA expression data. It is customary to normalize the amount of target mRNA on a blot relative to the expression of another gene, such as the housekeeping genes cyclophilin, GAPDH, or β-actin. We attempted to normalize developmental Kir6.1 mRNA expression by incubating samples simultaneously with an anti-Kir6.1 riboprobe and an additional probe directed against cyclophilin (Fig. 2B). Multiple samples (prepared from different animal preparations) were loaded side by side to verify the reproducibility of the assay. The developmental expression profile of Kir6.1 was similar to that observed before (increased expression with age). Within a given age group, the degree of cyclophilin expression was constant relative to the RNA amount used in the reaction.

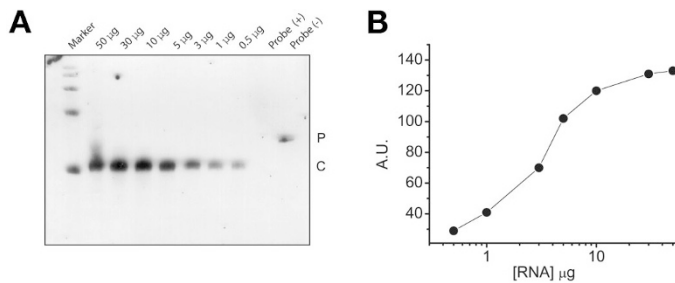


Figure 1. Characterization of the probe concentration used. (A) Different amounts of total mouse brain RNA (50–0.5 μg) were used to determine whether the probe (230 pg) was present in excess amount. In this and subsequent figures, the two control reactions are denoted Probe (+) (probe, yeast RNA with RNAses) and Probe (–) (probe, yeast RNA without RNAses). The two size bands represent the probe (P) and the protected cyclophilin product (C). (B) The signal intensity, measured using densitometry (arbitrary units) and corrected for background, is plotted as a function of the RNA concentration.

However, RNA from fetal hearts contained much more cyclophilin relative to that of the neonatal or adult hearts. As expected, normalizing Kir6.1 expression relative to that of cyclophilin amplified the developmental differences. For the data shown in Fig. 2B, when corrected for cyclophilin expression, Kir6.1 mRNA levels were ~5 and 20% of the adult levels for fetal and neonates, respectively, corresponding to values of 20 and 37% of the adult levels when uncorrected (*i.e.* expressed per amount of RNA in the reaction). Using ethidium bromide denaturing agarose electrophoresis, we determined that developmental alterations occur in the amount of 18S ribosomal proteins relative to the total amount of RNA loaded; therefore, no attempt was made to use the amount of 18S RNA as an internal control to normalize mRNA expression. We found the expression of another housekeeping gene (actin) also to change significantly during this developmental time frame (data not shown), which makes normalization to actin problematic. For these reasons, we consequently decided to convey all subsequent expression data relative to the amount of total RNA used in the RNA protection assay reaction.

Developmental mRNA expression of other K_{ATP} channel subunits. Two representative blots are shown in Fig. 3 to illustrate the developmental expression of Kir6.1, Kir6.2, SUR1, and SUR2 mRNA. In each case, mRNA expression is low in fetal heart and expression is progressively up-regulated with postnatal maturation. We performed multiple similar blots, and the band intensities were analyzed by densitometry. The data are summarized in Fig. 4.

Relative expression of mRNA of the two major SUR2 splice isoforms during development. Functional channels (that can be inhibited by cytosolic ATP, blocked by glibenclamide, and opened by K^+ channel openers) are formed only when Kir6 and SUR subunits are coexpressed (13,14). Initially, two SUR2 isoforms were described (SUR2A and SUR2B) (10,11), but it now is known that several alternatively spliced variants exist (12). However, it is SUR2A and SUR2B that are believed largely to affect the nucleotide sensitivity and pharmacological sensitivity of K_{ATP} channels in the cardiovascular system. The experiments that are shown in the preceding figures could not

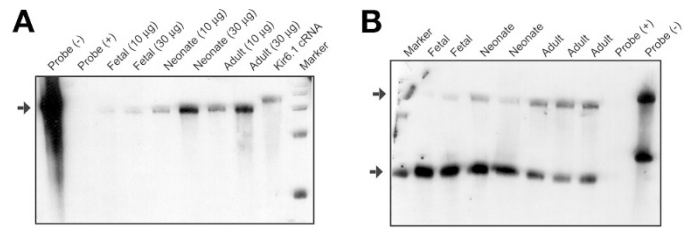


Figure 2. Developmental expression of Kir6.1 mRNA. (A) RNA protection assays (RPAs) were performed using a biotinylated 296-bp anti-Kir6.1 probe (600 pg). Lanes include reactions that were performed using RNA (10 and 30 μg) that was prepared from 13-d fetal, 1-d neonatal, and adult mouse hearts. The Kir6.1-protected fragment is shown by the arrow. To validate the Kir6.1 RNA probe, we additionally included a positive control reaction that contained *in vitro*-transcribed rat Kir6.1 cRNA (prepared from linearized Kir6.1 cDNA as a template). The Kir6.1 probe protected a fragment of slightly larger size in the positive control as a result of additional vector sequences present in the cRNA. (B) RPA reactions with RNA (10 μg each) that was prepared from fetal, neonatal, and adult mouse hearts. The samples were simultaneously hybridized with riboprobes that respectively protected fragments of Kir6.1 mRNA (top arrow) and cyclophilin mRNA (bottom arrow). The different reactions at the same developmental stages refer to different RNA samples prepared on different dates. The two Probe (–) and Probe (+) control lanes contained both of the riboprobes.

distinguish between SUR2 splice variants, because the SUR2 RNA protection assay riboprobe has been designed against a common region that is present in both of the splice variants. To examine the relative expression of SUR2A and SUR2B splice variants, we performed RT-PCR experiments using primers across the splice junction. Using these primers, we were able specifically to detect expression of the SUR2A and SUR2B mRNA species (Fig. 5). Both splice variants were expressed at low levels in the neonate, and their expression was strongly up-regulated during postnatal development. Comparatively, SUR2A seems to be the dominant isoform expressed in the adult heart, whereas SUR2B seems to be expressed at higher levels in the neonate. These results, however, should be interpreted with caution because RT-PCR is not a quantitative technique.

Characterization of K_{ATP} channel antibodies. We developed an anti-Kir6.1 antibody (NAF1) that we previously demonstrated specifically to detect Kir6.1 (and not Kir6.2 subunits) in Western blotting, with an electrophoretic mobility of the Kir6.1 protein corresponding to ~44 kD (20,21). We also previously showed the Santa Cruz G-16 antibody specifically to detect the Kir6.2 protein as a 38 kD band in Western blotting (21).

We characterized the SUR1 antibody using COS7L that have been transfected with hamster SUR1 cDNA. A band with molecular size of 170 kD was observed only in transfected (and not in untransfected) cells, demonstrating that this antibody detects the SUR1 protein (Fig. 6A). Similar experiments were performed to ensure that this antibody does not detect the SUR2 protein (data not shown).

We developed an antibody (R2B) against a peptide that is uniquely present in the SUR2B C-terminal sequence. This peptide has 100% sequence identity in mouse, rat, rabbit, and human but has low homology to the corresponding sequences in SUR1 (67% identity) and SUR2A (50% identity). We tested

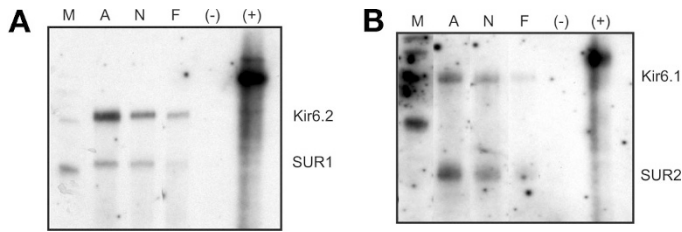


Figure 3. Developmental expression of K_{ATP} channel subunits. Total RNA (30 μ g) isolated from adult, neonatal (1–2 d after birth), and fetal (embryonic d 16) mouse hearts were subjected to RPA using riboprobes for Kir6.2 and SUR1 (A) and Kir6.1 and SUR2 (B). The lanes are as follows: M, biotinylated RNA Century Size Marker (Ambion); A, adult; N, neonate; F, fetal; (–), a control of riboprobe incubated with yeast tRNA and RNases to demonstrate the lack of self-self protection within the probe; and (+), a control of probe incubated with yeast tRNA and without RNases to demonstrate the probe size and integrity. For each of the panels, the lanes shown are from a single blot and have been re-ordered for presentation purposes.

this antibody in cell lysates that were obtained from HEK-293 cells that were stably transfected with Kir6.1 and either SUR2A or SUR2B subunits (18). We also used cell lysates of untransfected cells or HEK-293 cells that were transiently transfected with SUR1 cDNA. That the R2B antibody detected a strong band at ~150 kD in SUR2B-transfected cells but failed to recognize SUR2A or SUR1 protein (Fig. 6B) testifies to the subunit specificity of this antibody.

Developmental expression of K_{ATP} channel subunits: Western blot analysis. To examine the relative expression levels of K_{ATP} channel subunits at the protein level, we performed Western blotting using crude membrane preparations. We focused our data on comparing membrane fractions that were obtained from neonatal (1–4 d after birth) and adult hearts. We loaded gels with equal amounts (30 μ g) of total protein. The results were entirely consistent with our data examining mRNA expression (Fig. 7). We found relative expression levels of each of the K_{ATP} channel subunits that we investigated (Kir6.1, Kir6.2, SUR1, and SUR2B) to be higher in the adult membranes compared with that in the neonate.

Subcellular distribution of K_{ATP} channel subunits in neonatal ventricular myocytes. We used immunocytochemistry with well-characterized antibodies to examine the expression and subcellular distribution of K_{ATP} channel subunits in ventricular myocytes that were enzymatically isolated from the hearts of neonatal (1–2 d after birth) mice (Fig. 8). Kir6.1 subunits are expressed in a regular striated pattern throughout the myocyte. Kir6.2 subunits, in contrast, are expressed more prominently at the myocardial surface, and expression seems to be more punctate. Similarly, SUR1 and SUR2 subunits have distinct subcellular localizations. We consistently observed strong surface staining with SUR1 antibodies, whereas SUR2 antibodies diffusely stain throughout the width of the cell with slightly stronger sarcomeric repeating pattern.

DISCUSSION

Our data indicate that each of the K_{ATP} channel subunits (Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B) is expressed at each of the developmental stages that we studied, ranging from fetal to adult mouse heart. The expression of K_{ATP} channel

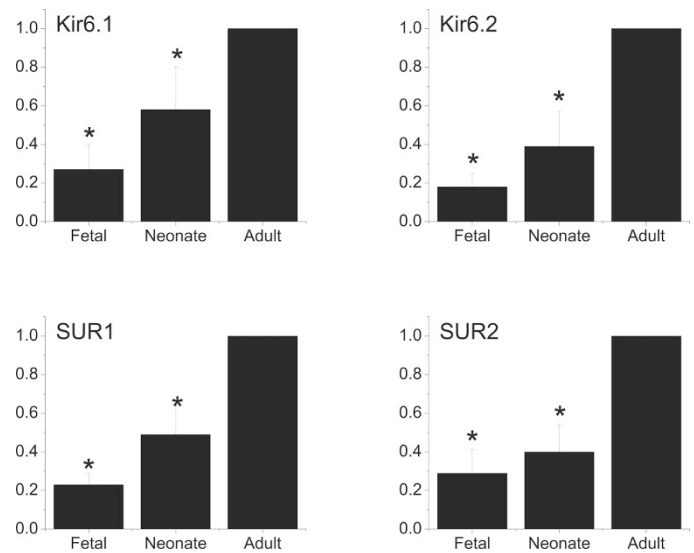


Figure 4. Summary of RPA expression data for K_{ATP} channel subunit mRNA. Data were obtained as in the preceding figure, and blots were subjected to densitometry analysis. For each blot, data were normalized to the density of bands in the adult lane. Pooled expression data are displayed for Kir6.1 (six blots), Kir6.2 (four blots), SUR1 (eight blots), and SUR2 (six blots). **p* < 0.05; one-way ANOVA followed by Dunnett's *t* test vs the adult values.

subunit mRNA was generally low in the fetal heart and was up-regulated during postnatal maturation. We also demonstrated up-regulation of Kir6.1, Kir6.2, SUR1, and SUR2B protein during perinatal maturation.

Expression of Kir6.2 and SUR2A subunits. Cardiac sarcolemmal K_{ATP} channels consist of hetero-octameric complexes of Kir6.2 and SUR2A subunits (23–25). This concept is based on the similarities in the biophysical and pharmacological characteristics when comparing heterologously expressed Kir6.2/SUR2A channels with native cardiac K_{ATP} channels (17) and also because of the known expression of Kir6.2 and SUR2A mRNA and protein in the heart (4,23,26–29). Kir6.2 and SUR2 subunits co-localize in the t-tubules of adult mouse ventricular myocytes (22). We therefore fully anticipated finding Kir6.2 and SUR2A mRNA and protein in the adult mouse heart. However, because functional K_{ATP} channel expression is up-regulated after birth and because there are reports of immature K_{ATP} channels' having biophysical and pharmacological properties that differ from that in the adult, we could not readily predict our results in the immature heart. Our data demonstrate Kir6.2 mRNA and protein as well as SUR2A mRNA to be present (SUR2A protein expression could not be verified because of lack of suitable antibodies). Our finding that both Kir6.2 and SUR2A are expressed in fetal and neonatal heart suggests that (at least some of) the K_{ATP} channels in the immature heart may consist of Kir6.2 and SUR2A subunits. Because Kir6.2 and SUR2B subunits are also expressed in the coronary vascular endothelium (21), increased coronary vascularization during development may also partly account for the increased postnatal expression of these two subunits.

Our immunocytochemistry data suggest that the subcellular distribution of Kir6.2 subunits may change after birth. In adult, Kir6.2 and SUR2 subunits both are expressed at the sarco-

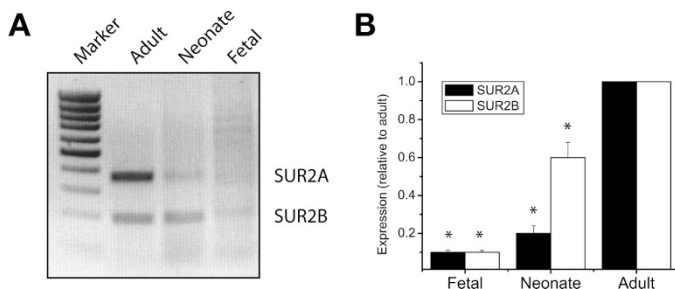


Figure 5. Expression levels of SUR2 splice variants. (A) Agarose gel electrophoresis of RT-PCR reactions from adult, neonatal, and fetal mRNA samples. Primers were designed to amplify both SUR2 splice variants (expected sizes of SUR2A and SUR2B are 357 bp and 181 bp, respectively). (B) RT-PCR data ($n = 4$) were quantified by densitometry and expressed relative to adult values. * $p < 0.05$ determined by Kruskal-Wallis ANOVA on Ranks, followed by a Dunn's t test to test for differences relative to the adult group.

lemma as well as in sarcomeric striated patterns, which we previously attributed to high expression levels of these subunits in t-tubular structures (22). Our observation of predominantly surface expression of Kir6.2 subunits in immature ventricular myocytes is entirely consistent with the fact that neonatal myocytes largely lack t-tubules (30,31). However, we still observe diffuse intracellular staining with the SUR2 antibodies used. A caveat with this observation is that staining was very faint with the SUR2 antibodies, presumably partly because SUR2 protein is expressed at very low levels at this stage of development. Nevertheless, taken at face value, these observations suggest differential expression patterns of the two "cardiac" K_{ATP} channel subunits Kir6.2 and SUR2 and also that SUR2 subunits may well be expressed in intracellular organelles in the immature heart.

Expression of Kir6.1 subunits. We found that Kir6.1 subunits are expressed in the mouse heart. The developmental up-regulation of Kir6.1 mRNA and protein parallels that found for Kir6.2. At least some of the Kir6.1 subunits are likely to be derived from the coronary smooth muscle cells in the vasculature, where they may assemble with SUR2B subunits to form vascular K_{ATP} channels (21,25,32,33). However, Kir6.1 and Kir6.2 subunits both also are expressed in the coronary endothelium, where they may heteromultimerize with SUR2B to form functional K_{ATP} channels (21). The postnatal up-regulation of Kir6.1 mRNA and protein levels observed in this study therefore may be consistent with angiogenesis, which is known to occur during early development. Although there are reports that the Kir6.1 subunit may form part of a mitochondrial K_{ATP} channel (34–36), this view is not necessarily shared by others (22,37,38). However, until independently confirmed, we should consider the possibility that developmental up-regulation of Kir6.1 mRNA may be represented in part by increased production of mitochondria in myocardial cells. Certainly, the subcellular expression pattern of Kir6.1 subunits in immature cells may be consistent with expression in intracellular organelles, such as mitochondria.

Kir6.1 subunits are not thought to be essential components of sarcolemmal K_{ATP} channels in adult ventricular myocytes because these channels are unaffected in mice that are deficient of the Kir6.1 locus (33) or when myocytes are targeted with

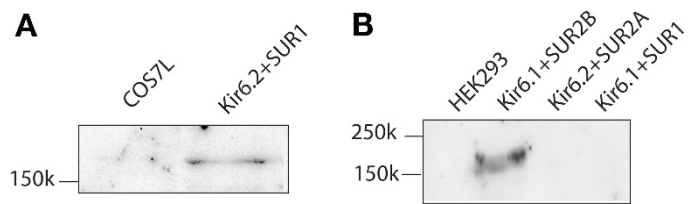


Figure 6. Characterization of anti-SUR1 and anti-SUR2B antibodies. (A) The anti-SUR1 antibody (C-16; Santa Cruz) was used at 1:200 dilution in Western blotting. Lane 1, untransfected COS7L cells; lane 2, COS7L cells transiently transfected with SUR1 cDNA. (B) Western blot performed using an anti-SUR2B antibody (R2B) developed by us (1:500 dilution). HEK-293 were untransfected (lane 1) or transfected with Kir6.1 plus SUR2B (lane 2), Kir6.2 plus SUR2A (lane 3), or Kir6.1 plus SUR1 (lane 4).

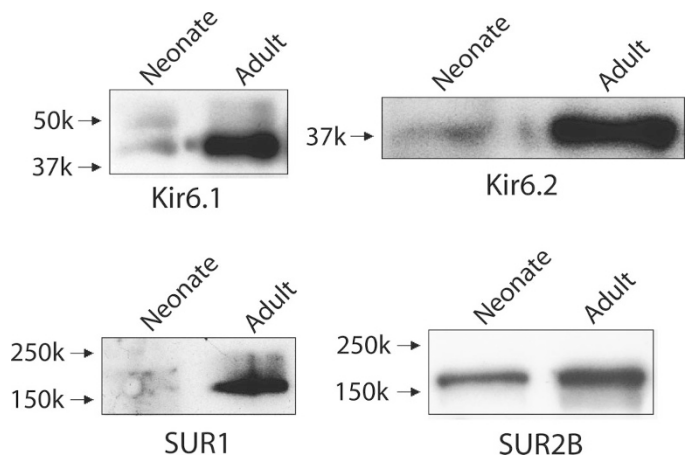


Figure 7. Developmental expression of K_{ATP} channel subunits. We performed Western blotting using membrane fractions that were obtained from the hearts of neonatal or adult mice, using antibodies to Kir6.1 (A), Kir6.2 (B), SUR1 (C), or SUR2B (D). In each case, the expression level was lower in the neonatal heart compared with that in the adult.

dominant negative Kir6.1 subunits (39). Nevertheless, Kir6.1 subunits are expressed in cardiac myocytes (22,40), particularly so in the neonate (4). Our immunocytochemistry data demonstrate a degree of Kir6.1 surface expression in immature myocytes (in addition to strong intracellular expression). Because Kir6.1 and Kir6.2 have the potential to interact with each other to form functional heteromultimers (18,20,21,41–43), it is tempting to consider the possibility that heteromultimeric interaction between Kir6.1 and Kir6.2 subunits may occur to form the immature K_{ATP} channels, similar to the situation in the vascular endothelium (21). This is a particularly tempting thought, considering that immature K_{ATP} channels have a smaller unitary conductance (3), similar to that formed by heteromeric Kir6.1/Kir6.2 channels (41,44–46). Although such heteromeric Kir6.1/Kir6.2 channels have been postulated to exist in the neonate (4), their existence remains to be demonstrated in the neonatal heart.

Expression of SUR2B and SUR1 subunits. Adult ventricular K_{ATP} channels are believed to have SUR2A as the major regulatory subunit. However, we also detected expression of both SUR2B and SUR1 subunits in the mouse heart and found their expression to be up-regulated during postnatal development. The expression of SUR2B can be reconciled easily with

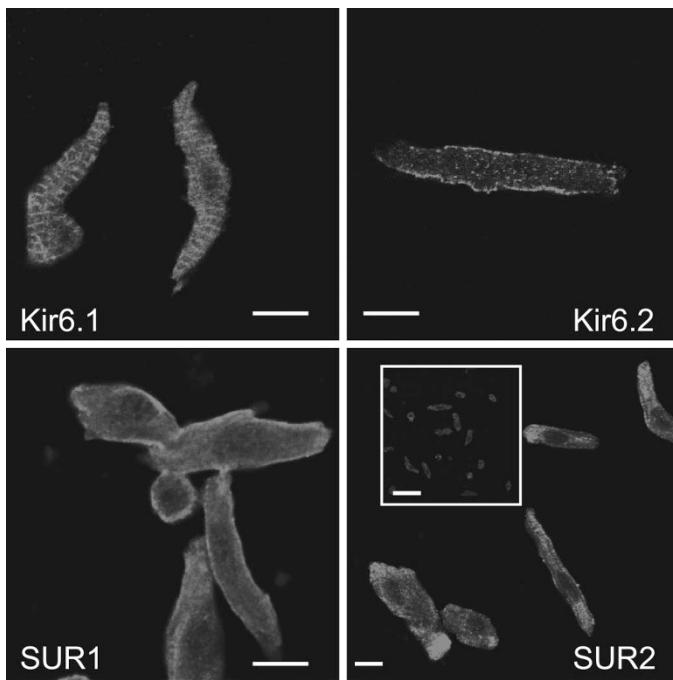


Figure 8. Immunocytochemistry of neonatal (1–2 d after birth) mouse ventricular myocytes. Enzymatically isolated ventricular myocytes were fixed on coverslips and stained with antibodies to Kir6.1, Kir6.2, SUR1, or SUR2 (the secondary antibody was Cy3-conjugated). (Inset) an image with no primary antibody to illustrate the degree of nonspecific staining of the secondary antibody. Bar = 10 μ m (50 μ m in inset).

previous reports describing its expression in the coronary vascular bed, both in the coronary vascular smooth muscle (21,47) and in the coronary endothelium (21,32). These findings are supported by the observations that heterologously expressed Kir6.1/SUR2B channels resemble native vascular smooth muscle K_{ATP} channels (48) and that mice deficient of the SUR2 lack K_{ATP} channels in the vasculature (49). The increased SUR2B expression that occurs during development therefore may reflect increased growth and vascularization of the coronary bed.

We also detected increasing SUR1 mRNA and protein expression in the developing mouse heart. In adult mouse ventricular cells, SUR1 protein is present at the sarcolemmal surface (22). We found an identical sarcolemmal expression pattern of SUR1 subunits in immature heart. The relevance of this observation is not entirely clear, because SUR1 is usually associated with K_{ATP} channels in noncardiac tissues, such as the pancreatic β cell and some neuronal tissues (25). One way to deduce a possible functional role of SUR1 is from its unique pharmacology, given that channels with SUR1 are exquisitely sensitive to tolbutamide and diazoxide (order of potency is SUR1 > SUR2B > SUR2A) (50,51). In this regard, it is interesting to note that the neonatal atrial K_{ATP} channel shows a unique functional and pharmacological profile resembling the pancreatic β cell channel for its unusually high affinity for glibenclamide and diazoxide (4,5). Furthermore, there are reports of SUR1 antisense effectively inhibiting K_{ATP} channel current in rat neonatal ventricular myocytes (5). Thus, the SUR1 subunit may have a role that remains to be fully defined in at least a subpopulation of cardiomyocytes or at a particular

stage of development. This argument may be extended to the coronary vasculature, because there are reports of SUR1 expression in the coronary endothelium (32) [but see (21)]. Thus, SUR1 subunits may well have physiologic roles that are yet to be defined in the developing myocardium.

Role of K_{ATP} channels in the developing heart. Relative to other inward rectifier K⁺ channels, K_{ATP} channels are expressed at high density during early developmental stages and therefore may have an important physiologic role during this period (1–3). However, K_{ATP} channel activity is strongly up-regulated during postnatal development in parallel to that of the classical “inward rectifier current” I_{K1} (52). Our data demonstrating up-regulation of individual K_{ATP} channel subunits during development are consistent with this increased K_{ATP} channel current during perinatal development. However, that the resting potential of the immature heart is relatively insensitive to the external K⁺ concentration as a result of a low K⁺ permeability (53) suggests that K_{ATP} channels are not normally open in the immature heart. The precise function of these channels is yet to be elucidated.

Mouse models deficient of each of the K_{ATP} channel subunits now have been generated (25) and that none of these knockout animals exhibits severe embryonic mortality argues for redundancy in K_{ATP} channel composition and function or alternatively that K_{ATP} channels are physiologically not crucial for normal development. It is very interesting, however, that although Kir6.2 (–/–) mice do not normally have any adverse phenotype (54), these mice fail to adapt (or even die) under conditions of stress (55). In this regard, it is of interest to note that the immature heart is much more resistant to hypoxia and ischemia compared with adult cardiac tissue (56–59). Furthermore, hypoxia induces drastic action potential shortening in isolated ventricular myocytes from adult guinea pig hearts, whereas the action potential of fetal myocytes is largely unchanged (60). Given the role of K_{ATP} channels in action potential shortening during metabolic impairment, it therefore is very likely that the expression levels and/or the inherent properties of K_{ATP} channels in the immature heart (and hence their responsiveness to metabolic stress) may contribute to the resistance of neonates to metabolic impairment.

Reservations. For this type of study, the specificity of antibodies used is always a concern. Although extensively characterized in this and previous studies (18,20–22), the possibility remains that nonspecific interaction might have occurred with other proteins. A definitive study will require the use of tissues that are obtained from knockout animals (*i.e.* the immunostaining should be unequivocally absent in tissues from knockout animals). Viable knockout animals for each of the proteins under consideration have been generated, but we have not been able to obtain these animals (or tissues from these animals) for this purpose. Therefore, although we have taken every step possible to minimize nonspecificity issues, our results should be interpreted within this limitation.

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