Published in final edited form as:

J Mol Cell Cardiol. 2008 January ; 44(1): 188–200.

CARDIAC SULFONYLUREA RECEPTOR SHORT FORM-BASED CHANNELS CONFER A GLIBENCLAMIDE-INSENSITIVE K_{ATP} ACTIVITY

Jie-Lin, Pu^{1,ψ,¶}, Bin Ye^{1,¶}, Stacie L. Kroboth¹, Elizabeth M. McNally², Jonathan C. Makielski¹, and Nian-Qing Shi^{1,*}

¹Department of Medicine, 1300 University Ave., University of Wisconsin, Madison, WI 53706

²Department of Medicine, 5841 S. Maryland Ave., MC6088, The University of Chicago, Chicago, IL 60637

Abstract

The cardiac sarcolemmal ATP-sensitive potassium channel (K_{ATP}) consists of a Kir6.2 pore and a SUR2 regulatory subunit, which is an ATP-binding cassette (ABC) transporter. K_{ATP} channels have been proposed to play protective roles during ischemic preconditioning. A SUR2 mutant mouse was previously generated by disrupting the first nucleotide-binding domain (NBD1), where a glibenclamide action site was located. In the mutant ventricular myocytes, a non-conventional glibenclamide-insensitive (10 μM), ATP-sensitive current (*I*_{KATPn}) was detected in 33% of single-channel recordings with an average amplitude of 12.3±5.4 pA per patch, an IC₅₀ to ATP inhibition at 10 μM, and a mean burst duration at 20.6±1.8 ms. Newly designed SUR2-isoform or variant-specific antibodies identified novel SUR2 short forms in the sizes of 28 and 68 kDa in addition to a 150-kDa long form in the sarcolemmal membrane of wild-type (WT) heart. We hypothesized that channels constituted by these short forms that lack NBD1, confer *I*_{KATPn}. The absence of the long form in the mutant corresponded to loss of the conventional glibenclamide-sensitive K_{ATP} currents (*I*_{KATP}) in isolated cardiomyocytes and vascular smooth muscle cells but the SUR2 short forms remained intact. Nested exonic RT-PCR in the mutant indicated that the short forms lacked NBD1 but contained NBD2. The SUR2 short forms co-immunoprecipitated with Kir6.1 or Kir6.2 suggesting that the short forms may function as hemi-transporters reported in other eukaryotic ABC transporter subgroups. Our results indicate that different K_{ATP} compositions may co-exist in cardiac sarcolemmal membrane.

Keywords

K_{ATP} channel; SUR2; Ischemic preconditioning; Glibenclamide; NBD1

1. Introduction

K_{ATP} channels are widely distributed in various tissues and play important physiological roles in regulating insulin secretion in pancreatic β-cells [1], providing ischemic protections to the

*Address correspondence: Nian-Qing Shi, Ph.D. Department of Medicine University of Wisconsin 1300, University Ave., Room 24, SMI Building, Madison, WI 53706 Tel: (608) 263–1172 Fax: (608) 263–1144 E-mail: nqs@medicine.wisc.edu.

ψCurrent Address: Chinese Academy of Medical Science & Peking Union Medical College, Beijing, China

¶These authors contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

heart [2] and modulating vascular tone in smooth muscles [3]. It is generally agreed that the sarcolemmal K_{ATP} is a hetero-octamer that consists of a Kir6.0 pore and a sulfonylurea receptor (SUR) [4]. SUR1 (~177 kDa) is a high-affinity SUR that is primarily present in pancreatic β -cells [5] while SUR2 (~174 kDa) is a low-affinity SUR that is mainly detected in the heart [6]. Multiple alternative splice variants have been found in each SUR gene [7]. The SUR in cardiac and vascular smooth muscle is encoded by two splice variants of SUR2 (*ABCC9*), *SUR2A* and *SUR2B*, which differ in the alternative use of the last exon [8]. These SUR isoforms and splice variants have increased the diversity of K_{ATP} .

SURs belong to the ATP-binding cassette (ABC) transporter superfamily. A typical eukaryotic ABC transporter [9] contains two symmetric transmembrane domains (TMD1 and TMD2) and two nucleotide-binding domains (NBD1 and NBD2). However, SURs diverge from other ABC transporters [10] by having an additional TMD0 and two asymmetric NBDs, with NBD1 as the initial ATP-binding site while NBD2 is more likely involved in ATP hydrolyzation [11]. The role of SURs has evolved from other ABC transporters by regulating the K_{ATP} complex instead of transporting a specific substrate itself. ABC transporters also exist as hemi-transporters, either in homodimeric or heterodimeric forms, employing the only TMDs to receive regulatory signals from their single NBDs [12].

In SUR1, glibenclamide acts on an N-end component between NBD1/transmembrane helix (TM) 12 and a C-end component located between TM15–16 [13]. Mutating either component abolishes the drug effect. A previously generated SUR2 mutant mouse has a disruption cassette inserted between exons 10–16 to disrupt NBD1 of SUR2 [14]. The glibenclamide effect is expected to be altered in the mutant. Earlier characterizations at the nucleic acid level suggest that the disruption cassette is present in SUR2, which is confirmed by the loss of the conventional glibenclamide-sensitive K_{ATP} currents (I_{KATP}) in isolated cardiomyocytes [14] and vascular smooth muscle cells [15]. This mouse was thought to be a SUR2 null mouse based on lines of functional data. In the present work, however, a non-conventional glibenclamide-insensitive, ATP-sensitive current (I_{KATPn}) was found in the mutant. Using a panel of newly developed SUR2 isoform- or variant-specific antibodies that were unavailable in earlier study [14], novel SUR2 short forms in the sizes of 28–68 kDa were discovered in the mutant. Evidence at both mRNA and protein levels indicated that the short forms lacked NBD1 but had NBD2, and they could interact with Kir6.1 or Kir6.2, which may account for I_{KATPn} . Our observations suggested a greater diversity for K_{ATP} structure as previously proposed based on pharmacological and physiological studies.

2. Materials and Methods

Detailed protocols and additional supporting information are provided in an on-line Supplement

2.1 Genes encoding the K_{ATP} subunits

The SUR2 (*ABCC9*) cDNA clones were from mouse [14] while the SUR1 (*ABCC8*) cDNA clone was from rat [4]. The mouse *KIR6.2* [16] or *KIR6.1* [17,18] gene was cloned from a mouse heart cDNA library (BD BioSciences, San Jose, CA) by PCR. The *KIR6.2* gene was amplified by using primers, P1a: 5'-GGAGCCA TGCTGTCCCGAAAGGGC-3' and P2a: 5'-ACAAGTGAGTGGGGGCCTGAGG-3' while the mouse *KIR6.1* gene was amplified by primers, P3a: 5'-ATGCTGGCCAGGAAGAGCAT-3' and P4a: 5'-GTCATCGGGACTCAGTGAG-3'. PCR conditions were performed as previously described [19] and the *KIR6.2* or *KIR6.1* PCR products were then subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) as pYB2 or pYB1. Primers used in the nested exonic RT-PCR experiment to amplify possible transcripts encoding the SUR2 short forms were P1: 5'-ATGAATCCCCAGAAAGTGAAGCCT-3'; P2: 5'-

ATGTTTCCAGAGACACTCTCATCAA-3'; P3: 5'-
 ATGATTGTAGGCCAAGTGGGTTGTGG-3'; P4: 5'-
 ATGCAGACCCTGGAAGGAAAAGTTTA-3'; P5: 5'-
 ATGTATTCAAGAGAGGCCAAGGCAC-3'; P6: 5'-
 ATGGGCCTCACAGCCGCAAGAAC-3'. P7: 5'-GGCCATGTCGATGGAAGCAG
 TGGC-3'

2.2 The SUR2 mutant mouse

The SUR2 mutant mouse [14] was previously described and generated in C57BL-6J/129SV/J mixed background (Jackson Laboratories, Bar Harbor, Maine). It was on this mixed background that mice were previously characterized. The SUR2 targeted allele was then bred heterologously through six generations onto the FVB background [15]. Heterologous mice were then interbred and genotyped to obtain homozygous mutants. Homozygotes were maintained for experiments described in this work. Mouse protocols were performed following the guidelines of NIH at the University Wisconsin Animal Core Facility.

2.3 Kir6.0 stable cell lines

Cell culture and transfection were performed as described previously [20]. Single colonies were screened and confirmed by RT-PCR using the SuperScript II Kit (Invitrogen) and Western blot analysis. The RT-PCR primers for *KIR6.2*, P5a: 5'-AGAATATCGTCGGGCTGATG-3' and P6a: 5'-GTTTC TACCACGCCTTCCAA-3' and for *KIR6.1*, P7a: 5'-CTATCATGTGGTGGCTGGTG-3' and P8a: 5'-CGTGGTTTTCTTGACCACCT-3'.

2.4 New SUR2 and other related antibodies

T1 was raised against amino acids 268–287 while BNJ-2 was raised against amino acids 636–649 of SUR2. BNJ-39 was raised against the C-terminus of SUR2A (1525–1546) while BNJ-40 was raised against the C-terminus of SUR2B (1523–1536). Epitopes were synthesized by KLH-conjugation at its N-terminus and affinity-purified (Invitrogen) against the peptide. The isoform or variant specificity of each antibody was tested in the Kir6.2 stable cell line by introducing *SUR2* or *SUR1*. Anti-Kir6.2 (sc-11224) or anti-Kir6.1 (sc-11228) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Na_v1.5 (06–811) was obtained from Upstate (Charlottesville, VA) and Anti-HCN4 (APC-052) was from Alomone Labs (Jerusalem, Israel). Anti-Na/K ATPase (ab-7671), anti-VDAC1 (ab-3434) or anti-COXIV (ab-14744) was obtained from Abcam (Cambridge, MA).

2.5 Isolation of ventricular myocytes and immunocytochemistry

Ventricular myocytes were isolated from mouse hearts by enzymatic treatment as previously described [21]. Immunostaining was performed as previously described [22]. BNJ-39 (1:500) or BNJ-40 (1:250) was the primary antibody while the anti-rabbit Alexa Fluor-488 IgG (1:250) was the secondary antibody (Invitrogen). All confocal recordings were performed at room temperature and the images were analyzed by Confocal Assistant software.

2.6 Protein extractions and Western blotting

Protein isolation [23] was handled on ice or at 4 °C to prevent proteolysis and Westerns were performed as described [24]. Concentration was determined by the Lowry method using a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Secondary antibodies were from Invitrogen and Amersham (Piscataway, NJ) and added at 1:10,000–1:12,500. Blots were scanned by the BioSpectrum Imaging System (UVP, Upland, CA).

2.7 Isolation of cardiac membrane fractions from WT and the SUR2 mutant

Cardiac membrane fractions of WT and SUR2 mutant mouse heart tissues were prepared as previously described [25]. Tissues from 8 mice were pooled for isolation. Hearts (the atria were removed) were rapidly removed and put in ice-cold extraction buffer. The crude extracts were separated by a discontinuous gradient containing 21%, 31%, 40% and 55% sucrose at $141,000 \times g$ for 2 h resulting in three distinct interfaces. The very top fraction at 21% sucrose was used for surface membrane protein isolation. Contamination of mitochondria was determined by Western blots using anti-VDAC1 (1:250), anti-COXIV (1:5000) and anti-Na/K ATPase (1:1000). Protein quality was determined by the detections of Na_v1.5 (1:200) and HCN4 (1:200).

2.8 Co-Immunoprecipitation (Co-IP)

Co-IP experiments were carried out using Dynabeads Protein A or G (Invitrogen) by following manufacturer's recommended procedures. 5 μ g of T1 or control IgG was used to IP ~100 μ g purified membrane proteins isolated from WT hearts in the forward IP experiments followed by a Western blot using BNJ-39 (1:2000). In the reverse IP experiments by BNJ-39, T1 was the Western blot antibody (1:2000). In the experiments to study associations of the SUR2 short forms with Kir6.1 or Kir6.2, 5 μ g of BNJ-39, BNJ-40 or control IgG was used to IP ~100 μ g purified membrane proteins isolated from the SUR2 mutant hearts in the forward IP experiments. Anti-Kir6.1 (1:200) and anti-Kir6.2 (1:200) were used as the Western antibodies. In the reverse IP experiments by anti-Kir6.1, BNJ-39 (1:2000) was the Western antibody.

2.9 Recordings of I_{KATP} in isolated ventricular myocytes

I_{KATP} was recorded by single-channel recordings using inside-out configuration at room temperature as previously described [26]. Ventricular myocytes were isolated from mice that were >12-wks-old. In the glibenclamide experiment, macroscopic currents were recorded from whole cells as described [27]. Experimental data was represented as mean $\pm$ SEM. Data analysis and presentation were carried out as described in [26]. A t-test was used in any comparison of two groups while a χ square test was used in any comparison between two variables. $p < 0.05$ was considered a significant difference.

3. Results

3.1 Kinetics, regulation and pharmacology of the non-conventional I_{KATPn}

In the SUR2 mutant mouse with a disrupted NBD1, the conventional glibenclamide-sensitive I_{KATP} was previously reported to be absent in both ventricular myocytes [14] and vascular smooth muscle cells [15]. Single channel recordings in the ventricular myocytes isolated from the SUR2 mutant hearts, however, recorded a non-conventional K⁺ current (designated as I_{KATPn} , Fig. 1A) that significantly differed from the WT conventional K⁺ current (I_{KATP} , Fig. 1B). I_{KATPn} was sensitive to cytoplasmic ATP and completely inhibited at 1 mM concentration (Fig. 1C). In addition, I_{KATPn} was present at a much lower density with fewer patches showing currents (Fig. 2A), and with smaller amplitude per patch (Fig. 2B). K_{ATPn} was four times more sensitive to ATP than K_{ATP} (Fig. 2C) with a 10.0 μ M half-maximal inhibition value (IC_{50}). Open probability (P_o) for K_{ATPn} in the mutant cells was ~10% higher than that for K_{ATP} in WT cells (Fig. 3A) while single channel conductance (pS) for the mutant cells was quite similar to WT cells at a membrane potential of 0 mV (Fig. 3B). The mean burst duration for K_{ATPn} was half of that for K_{ATP} (Fig. 3C). K_{ATPn} showed a significantly shorter time constant for closed intervals within (τ_1) or between bursts (τ_2) than those recorded in K_{ATP} in the open and closed time histogram (Fig. 3D). Resting potential for the mutant or WT cells was -76 ± 0.58 mV and -75.5 ± 0.56 mV (Fig. 4A), respectively. In contrast to I_{KATP} recorded in WT cells (Fig. 4B), I_{KATPn} in the SUR2 mutant cells was insensitive to 10 μ M glibenclamide (Fig. 4C).

Single channel activity consistent with I_{KATPn} (sensitive to ATP, insensitive to 10 μ M glibenclamide) was also detected in WT cells. These results suggested that I_{KATPn} had distinct ATP sensitivity, kinetics and drug response compared to I_{KATP} .

3.2 Development of SUR2 isoform- and variant-specific antibodies

Several types of K_{ATP} channels may account for I_{KATPn} in the SUR2 mutant cells. A Kir6.2 with a truncated C-terminus (Kir6.2 Δ C35) that is able to travel to cell surface without an SUR subunit [28] could be a candidate. However, such a recombinant channel expressed in COS1 cells (n=6) showed a mean burst duration of 4.6 ± 1.0 ms, a P_o of 0.19 ± 0.03 mV and a single channel conductance value of 25.9 ± 0.9 at a membrane potential of 0 mV ($p < 0.05$ compared to K_{ATPn}) in our study (data not shown) suggesting it did not underlie I_{KATPn} . A Kir6.2/SUR1 (glibenclamide-sensitive) channel mainly functions in β -cells [4-5] but not in cardiac cells and displays distinct kinetics from a SUR2-based channel. These observations led us to develop a new panel of SUR2 antibodies (Fig. 5A) to identify whether any SUR2 forms remained in the mutant, which may confer I_{KATPn} . COS1 cell lines stably expressing *KIR6.2* or *KIR6.1* were generated to facilitate specificity tests for the new SUR2 antibodies. Positive candidates from the stable cell line selection were confirmed by RT-PCR (Fig. 5B) and Western blot analysis (Fig. 5C). One Kir6.2 and two Kir6.1 stable lines were obtained. The specificity of each antibody was then tested by expressing each SUR isoform or splice variant into the Kir6.2 stable cell line. T1 or BNJ-2, recognized a 170-kDa SUR2 band only in the cells expressing *KIR6.2* and SUR2A cDNA suggesting they are SUR2 isoform-specific antibodies (Fig. 5D).

BNJ-39 and BNJ-40 were designed to distinguish the two SUR2 splice variants, with BNJ-39 specific to the C-terminus of SUR2A and BNJ-40 specific to the C-terminus of SUR2B. Because SUR2A differs from SUR2B only in the last exon (contains 42 amino acids) and the 42 amino acids in each variant share ~30% homology, a relatively long epitope was designed to achieve better antigenicity for BNJ-39. It was noted that the last 42 amino acids at the C-terminus of SUR2B shares ~70% homology with that of SUR1. We excluded the last 10 of the 42 amino acids in SUR2B to improve the specificity for BNJ-40. These designs set our antibodies apart from other reported SUR2 antibodies. BNJ-39 detected a 170-kDa SUR2A band only in the cells containing SUR2A and *KIR6.2* (Fig. 5E). A trace amount of the 170-kDa band was found in the untransfected COS1 cells indicating that endogenous SUR2A was present in our COS1 line. On the other hand, two bands in the sizes of 170-kDa and 100-kDa were detected by BNJ-40 in the cells when *KIR6.2* and SUR2B were co-expressed (Fig. 5F). The 100-kDa band was present in the untransfected COS1 cells and the cells containing SUR2A and *KIR6.2* but it was present much more abundantly in those cells containing SUR2B and *KIR6.2*. This suggested that our COS1 line had the endogenous 100-kDa SUR2B, which could be a subtype splice variant for SUR2B. Thus, SUR2A or SUR2B was only recognized by the antibody generated based on the corresponding variant.

3.3 Identification of constituents that confer I_{KATPn}

A cardiac membrane fraction from WT mouse hearts was fractionated as previously described [25] and proteins isolated from the very top fraction showed no detectable mitochondrial contamination in gels based on a Western blot analysis (Fig. 6A, left panel) of two mitochondria markers, VDAC1 (outer membrane) and COXIV (inner membrane), while a plasma membrane marker, Na/K ATPase was abundantly present. To assess whether large molecular-weight ion channel proteins (>150-kDa) were intact in this fraction, the α subunit of the voltage-gated sodium channel, $Na_v1.5$ and the pacemaker channel isoform 4, HCN4, were used as quality controls (Fig. 6A, right panel). Both channels appeared intact in the fraction, suggesting that the protein isolation process did not cause proteolysis in these proteins.

When the T1 or BNJ-2 antibody was used to cross-react with the WT cardiac membrane fraction, a 150-kDa band was found (Fig. 6B, Lanes 1&2). The band detected by T1 appeared to be prominent, which was unexpected. We found later that a theoretical secondary glycosylation site was included in this relatively long epitope. Differentiated glycosylation has been reported in SUR proteins [5], therefore, T1 may detect a group of bands in various sizes as observed in Fig. 6B (Lane 1). BNJ-2, however, was raised against a different region of SUR2, did not detect such a pattern. The C-terminal antibody, BNJ-39, only detected two short forms (Fig. 6B, Lane 5) in the sizes of 68-kDa (designated as 68A) and 28-kDa (designated as 28A) from the membrane fraction. The presence of these short forms was confirmed in purified membrane proteins isolated from individual ventricular myocytes (data not shown). SUR2B is thought to be a major splice variant present in smooth muscle and it is not expected to be found in ventricular tissues. BNJ-40, detected a faint 150-kDa band in a whole heart lysate (Fig. 6B, Lane 4), which could be contaminated by some smooth muscle. In the cardiac membrane fraction, BNJ-40 (Fig. 6B, Lane 6) only detected a 28-kDa band (designated as 28B). These results suggested that the SUR2-specific antibodies detected SUR2 in various sizes in mouse cardiac membrane but they detected full-length SUR2 when each variant was co-expressed with Kir6.2 in COS1 cells (Figs 5D-F).

To further understand why BNJ-39 could not detect a 150-kDa band that was detected by the internal antibodies, T1 and BNJ-2, co-immunoprecipitation experiments were carried out. The cardiac membrane fraction was immunoprecipitated with T1 in the forward IP experiments followed by a Western blot using BNJ-39 (Fig. 6C, Lane 1). Two bands in 150-kDa and 28-kDa were detected by BNJ-39 after IP, but the 68-kDa band was not. These results were confirmed in the reverse IP experiments (Fig. 6C, Lane 3) suggesting 28A had an association with a 150-kDa form in our preparations. In the reverse IP experiments, two additional bands in the sizes of 100-kDa and 55-kDa were found, which could be from the T1 antibody because a faint 55-kDa band was observed in the control (Fig. 6C, Lane 4). Because these two bands were not detected in the forward IP, they were probably not part of the 150/28A complex. Immunostaining was performed by using BNJ-39 and BNJ40 in ventricular myocytes isolated from the WT hearts. Both antibodies detected green fluorescence signals in WT ventricular myocytes (Fig. 6D, Panels II). In WT controls where no primary antibodies were added, green fluorescence was not detected (Fig. 6D, Panels IV). These results confirmed the Western blot results in Fig. 6B.

3.4 SUR2 short forms remain intact in the mutant mouse hearts

The SUR2 mutant mouse was generated with a disruption cassette inserted between exons 10 and 16 of *SUR2* (Fig. 7A) to disrupt NBD1 [14]. The SUR2 antibodies were then used to cross-react with a cardiac membrane fraction isolated from the SUR2 mutant. Neither T1 nor BNJ-2 detected a 150-kDa SUR2 band suggesting that it was disrupted as expected (Fig. 7B, Lanes 1&2). This data was consistent with previous observations that glibenclamide-sensitive I_{KATP} was absent in both mutant ventricular myocytes [14] and vascular smooth muscle cells [15]. However, BNJ-39 and BN-40 detected the SUR2 short forms in the mutant heart tissues (Fig. 7B, Lanes 3&4) as observed in WT. Immunostaining was performed to confirm the Western blot results in the mutant. Both C-terminal antibodies were used to detect SUR2 signals in ventricular myocytes isolated from the mutant hearts. BNJ-39 and BNJ-40 detected green fluorescence signals (Fig. 7C, Panels II) suggesting that the SUR2 short forms were intact. In mutant controls where no primary antibodies were added, no green fluorescence was detected (Fig. 7C, Panels IV).

To study the possible locations of SUR2 transcripts encoding the short forms in the SUR2 mutant, six forward primers (see Fig. 7A) were designed for a nested exonic RT-PCR experiment based on regions before exon 10 (P1, P2), within exons 10–16 (P3, P4) and after

exon 16 (P5, P6). Each forward primer was paired with a reverse P7 primer that was designed based on exon 36 of SUR2 to amplify the expected regions using SUR2A cDNA [26] as the templates. PCR products in expected sizes were amplified successfully by all primer pairs (Fig. 7D, top panel). The six primer pairs were then used to perform nested exonic RT-PCR using mRNA isolated from WT or mutant heart tissues. Expected RT-PCR products were amplified from WT (Fig. 7D, middle panel) but RT-PCR products were only amplified by primer pairs 5/7 and 6/7 from the mutant (Fig. 7D, bottom panel). Cloning and sequencing of each RT-PCR band amplified from the mutant suggested that they encode partial SUR2 sequences that lacked NBD1 but contained NBD2. The 1.7-Kb and 1.3-Kb products were overlapped as expected, which could be the partial transcript encoding 68A while the 0.6-Kb product could be a partial transcript encoding 28 forms. These observations provided initial evidence that transcripts encoding the remaining short forms could be generated by alternative splicing and they were located after exon 16.

A protein level approach was attempted to identify the starting amino acid sequences for the SUR2A-based short forms. BNJ-39 was used to immunoprecipitate a mutant cardiac membrane fraction and the sample was separated by 2-dimensional gels (data not shown). A candidate spot at ~68-kDa (for 68A) was detected and confirmed by Western blot analysis using BNJ-39 in one blot. The 68-kDa spot was excised from the other blot and processed for N-terminal protein sequencing to determine the starting amino acids for this candidate. However, this experiment did not yield any information because the N-terminal end of the 68-kDa protein was acetylated, which is a common modification scheme in certain eukaryotic proteins.

3.5 SUR2 short forms are associated with Kir6.1 or Kir6.2

A previous report in a Kir6.2/SUR1 channel study demonstrated that the C-terminus of SUR1 was essential for channel complex trafficking [29]. Kir6.2 was further shown to interact with either TMD0 (transmembrane domain 0) or TMD2 but not TMD1 [30]. By sequence homology between SUR1 and SUR2, TMD1 may not be as critical as TMD0 or TMD2 for binding of SUR2 to the pore. A more recent study in a Kir6.2/SUR2A channel suggested that Kir6.2 interacted with SUR2A at a region of amino acid position 1294–1358, which is located within NBD2 [31]. To investigate whether each SUR2 short form had an association with Kir6.1 or Kir6.2, Co-IP experiments were performed using the purified mutant cardiac membrane fraction. Anti-Kir6.2 detected a 43-kDa Kir6.2 band in the samples immunoprecipitated by either BNJ-39 or BNJ-40 (Fig. 8A). In the reverse IP experiments, BNJ-39 detected both 68A and 28A while BNJ-40 detected 28B. Thus, each SUR2 short form could interact with Kir6.2. By contrast, anti-Kir6.1 detected a 47-kDa Kir6.1 band only in the sample immunoprecipitated by BNJ-39 (Fig. 8B, Lane 2). Therefore, BNJ-39 was tested in the reverse IP, and it detected 68A and 28A (Fig. 8B, Lane 5). This indicated that the SUR2A-based short forms had an association with Kir6.1 as well. These observations and data from Fig. 6C may lead to K_{ATP} channels with different compositions, regulation and pharmacology.

4. Discussion

A novel glibenclamide-insensitive, ATP-sensitive current ($I_{K_{ATPn}}$) was recorded in a SUR2 mutant mouse. Due to the significantly lower current density, smaller amplitude of the current, and insensitivity to glibenclamide, $I_{K_{ATPn}}$ was relatively difficult to detect in WT cells. However, the mutant provided a background in which the 150-kDa SUR2 long form was disrupted allowing for $I_{K_{ATPn}}$ detection. The kinetics, regulation and drug response of $I_{K_{ATPn}}$ were distinctively different from those reported for the conventional $I_{K_{ATP}}$. The constituents possibly accounting for this current were then further investigated in the SUR2 mutant mice. Because the kinetics of $I_{K_{ATPn}}$ differed significantly from that of a recombinant Kir6.2 Δ 35 channel [28], Kir6.2 Δ 35 was probably not responsible for conferring $I_{K_{ATPn}}$. The

unavailability of commercial SUR2 isoform- or SUR2 splice variant-specific antibodies made characterization at the protein level difficult in earlier studies [14,15]. To search for possible SUR2 forms in the mutant, we designed new antibodies to distinguish SUR2 isoforms and splice variants. These SUR2 antibodies detected novel SUR2 short forms in the sizes of 28 and 68 kDa from WT hearts. Nested exonic RT-PCR results suggested that the short forms could be generated by alternative splicing, therefore, they were “unaffected” by the disruption. Such splicing events have been reported earlier in other eukaryotic genes [32]. The complete sequences for these SUR2 variants, however, remain to be identified.

Two SUR2 isoform-specific antibodies, T1 and BNJ-2, detected a 150-kDa SUR2 band but the C-terminal BNJ-39 antibody was able to detect it only after an IP. SUR2 has an estimated size of 174-kDa but we and others [33,34] detected ~140–150-kDa in WT native cells. These observations let us hypothesize that two 150-kDa forms may exist in WT cardiac membrane, one lacks a N-terminal domain while the other lacks a C-terminal domain in the size of ~25–35 kDa. Both forms are expected to be recognized by T1 but BNJ-39 can only detect the first form in a Western blot. This form is thought to be glibenclamide-sensitive (Fig. 8C, I) while the second form is not due to the truncation in the C-terminus, which is required for glibenclamide-sensitivity [13]. The detection of a 150-kDa band by BNJ-39 after an IP indicates that the first form may be present scarcely in the cell. Moreover, the second form can not be detected by BNJ-39 in the Co-IP experiments unless an SUR2 short form is associated with it. The detection of a 28A band in the Co-IP experiments suggested that it could interact with the second 150-kDa form as a complex (Fig. 8C, II). We further hypothesize that the 150/28A complex is glibenclamide-sensitive and it may underlie I_{KATP} in WT cells. In the SUR2 mutant mice, however, both 150-kDa forms are expected to be disrupted leading to glibenclamide-insensitivity. The exact nature and functional/pharmacological consequences of these 150-kDa forms requires further study.

The Co-IP results indicated that the 68A short form probably had no association with the 150-kDa forms. Because 68A could interact with Kir6.1 or Kir6.2, these short form channels may co-exist with the long-form based channels in cardiac membrane (Fig. 8C, III). The 28B short form also had an association with Kir6.2 suggesting Kir6.2/28B channels may be present as well (Fig. 8C, IV). Previous photo-affinity labeling in β -cells identified three SUR1 proteins in the sizes of 140-kDa, 65-kDa and 33-kDa [35]. [3H]Glibenclamide was predominantly incorporated into the 140-kDa and 33-kDa forms simultaneously but not into the 65-kDa form [36]. Instead, the 65-kDa form was primarily labeled by [3H]glimepiride, a sulfonylurea with different receptor binding kinetics. The [3H]glibenclamide labeling of two other SUR1 forms inhibited the labeling of the 65-kDa form by [3H]glimepiride. These results implied that SUR1 proteins exist in different sizes and configurations (a 140/33 kDa complex and a 65-kDa form). The sizes of the detected SUR1 forms by photo-affinity labeling are in close proximity to the SUR2 forms detected by our antibody approach. The biological significance and regulation of the SUR short forms have not been extensively elucidated. This report represents the initial effort of a long-term study to address the composition and function of the SUR2 short form-based channels.

The intended SUR2 mutant mouse was designed to disrupt NBD1, where action sites for several sulfonylurea drugs are located [37], i. e. glibenclamide [13]. Results from the nested exonic RT-PCR experiment in the mutant suggested that the SUR2 short forms lacked NBD1 but contained NBD2. Channels formed by these short forms were therefore expected to be insensitive to glibenclamide. Pharmacological study of I_{KATPn} showed that it was indeed insensitive to 10 μM glibenclamide. Our study was extended to identify the pore-forming subunit that would interact with these SUR2 short forms. Co-IP results showed that each SUR2 short form had an association with Kir6.2 but only the SUR2A-based short forms could interact with Kir6.1. These results suggested that the SUR2 short forms may act as regulatory subunits

for K_{ATPn} possibly by dimerization as hemi-ABC transporters. They could use NBD2 to receive regulatory signals from a TMD as reported previously for other hemi ABC transporters [9]. Obtaining the complete sequences for SUR2 short forms is expected to allow us to further characterize these channels in recombinant systems. We are aware that we can not completely exclude the involvement of SUR1 short forms [35,36] in underlying $I_{K_{ATPn}}$ because several SUR1 splice variants have been reported earlier [38-40], and SUR1 signals have been detected by antibodies in heart [41] probably from a short splice variant [40].

K_{ATP} also exists in mitochondrial inner membrane [42] and may be important in conferring ischemic protection to the heart [43,44]. A long form of SUR2 in the size of 140-kDa [33] and a 28-kDa short form [34] have been detected by SUR2 antibodies in heart mitochondria. The identities and sequences of these proteins, however, are unclear. Although the exact molecular composition for mitochondrial K_{ATP} remains to be elucidated, the novel SUR2 short forms from this study could be SUR2 candidates that are targeted to heart mitochondria. Future study of the molecular mechanisms that generate the novel SUR2 short forms can expand our knowledge on sarcolemmal and mitochondrial hemi-ABC transporters, and the roles for the two types of K_{ATP} channels in ischemic preconditioning. In conclusion, we have identified an ATP-sensitive current with unique kinetics and pharmacology in a SUR2 mutant mouse that contains novel SUR2 short forms. These short forms may act as hemi-transporters similar to reported cases in other eukaryotic ABC transporter subgroups. More study is required to assess the significance of these findings in WT hearts, but our results suggest that K_{ATP} channels with diverse structures, regulatory function and pharmacology may play roles under normal physiological and stress conditions.

Acknowledgements

This study was supported by The American Heart Association National Center (0630268N to N.Q.S.), (0435030N to B.Y.) and by The National Institute of Health (2R01HL-57414 to J.C.M.).

References

1. Cook D, Hales C. Intracellular ATP directly blocks K^+ channels in pancreatic β -cells. *Nature* 1984;311:271–273. [PubMed: 6090930]
2. Gross G, Auchampach J. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992;70:223–233. [PubMed: 1310443]
3. Standen N, Quayle J, Davies N, Brayden J, Huang Y, Nelson T. Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science* 1989;245:177–180. [PubMed: 2501869]
4. Clement JP, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar-Bryan L, Bryan J. Association and stoichiometry of $K_{(ATP)}$ channel subunits. *Neuron* 1997;18(5):827–838. [PubMed: 9182806]
5. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP, Boyd AE III, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA. Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* 1995;268:423–426. [PubMed: 7716547]
6. Noma A. ATP-regulated K^+ channels in cardiac muscle. *Nature* 1983;305:147–148. [PubMed: 6310409]
7. Shi NQ, Ye B, Makielski JC. Function and distribution of the SUR splice variants. *J Mol Cell Cardiol* 2005;39:51–60. [PubMed: 15978902]
8. Chutkow WA, Simon MC, Le Beau MM, Burant CF. Cloning, tissue expression, and chromosomal localization of SUR2, the putative drug-binding subunit of cardiac, skeletal muscle, and vascular K_{ATP} channels. *Diabetes* 1996;45:1439–1445. [PubMed: 8826984]
9. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* 2001;11:1156–1166. [PubMed: 11435397]

10. Higgins CF, Linton KJ. The ATP switch model for ABC transporters. *Nat Structure Mol Biol* 2004;11:918–926.
11. Bienengraeber M, Alekseev AE, Abraham MR, Carrasco AJ, Moreau C, Vivaudou M, Dzeja PP, Terzic A. ATPase activity of the sulfonylurea receptor: a catalytic function for the KATP channel complex. *FASEB J* 2000;14:1943–1952. [PubMed: 11023978]
12. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE, Higgins CF. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 1990;346:362–365. [PubMed: 1973824]
13. Mikhailov MV, Mikhailova EA, Ashcroft SJ. Molecular structure of the glibenclamide binding site of the beta-cell K(ATP) channel. *FEBS Lett* 2001;499(1–2):154–160. [PubMed: 11418131]
14. Chutkow WA, Samuel V, Hansen PA, Pu J, Valdivia CR, Makielski JC, Burant CF. Disruption of Sur2-containing KATP channels enhances insulin-stimulated glucose uptake in skeletal muscle. *Proc Natl Acad Sci USA* 2001;98:11760–11764. [PubMed: 11562480]
15. Kakkar R, Ye B, Stoller DA, Smelley M, Shi NQ, Galles K, Hadhazy M, Makielski JC, McNally EM. Spontaneous coronary vasospasm in KATP mutant mice arises from a smooth muscle-extrinsic process. *Circ Res* 2006;98:675–689. [PubMed: 16469954]
16. Inagaki N, Gonoi T, Clement J, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J. Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. *Science* 1995;270:1166–1170. [PubMed: 7502040]
17. Sakura H, Ammala C, Smith PA, Gribble FM, Ashcroft FM. Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic beta-cells, brain, heart and skeletal muscle. *FEBS Lett* 1995;377(3):338–344. [PubMed: 8549751]
18. Inagaki N, Tsuura Y, Namba N, Masuda K, Gonoi T, Horie M, Seino Y, Mizuta M, Seino S. Cloning and functional characterization of a novel ATP-sensitive potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. *J Biol Chem* 1995;270:5691–5694. [PubMed: 7890693]
19. Ye B, Valdivia CR, Ackerman MJ, Makielski JC. A common human SCN5A polymorphism modifies expression of an arrhythmia causing mutation. *Physiol. Genomics* 2003;12:187–193.
20. Makielski JC, Ye B, Valdivia CR, Pagel MD, Pu JL, Tester DJ, Ackerman MJ. A ubiquitous splice variant and a common polymorphism affect heterologous expression of recombinant human SCN5A heart sodium channels. *Circ Res* 2003;93:821–828. [PubMed: 14500339]
21. Wolska BM, Solaro RJ. Method for isolation of adult mouse cardiac myocytes for studies of contraction and microfluorimetry. *Am J Physiol* 1996;271:H1250–1255. [PubMed: 8853365]
22. Foell JD, Balijepalli RC, Delisle BP, Yunker AM, Robia SL, Walker JW, McEnery MW, January CT, Kamp TJ. Molecular heterogeneity of calcium channel beta-subunits in canine and human heart: evidence for differential subcellular localization. *Physiol Genomics* 2004;17:183–200. [PubMed: 14762176]
23. Guo W, Li H, Aimond F, Johns DC, Rhodes KJ, Trimmer JS, Nerbonne JM. Role of hetero-multimers in the generation of myocardial transient outward K⁺ currents. *Circ Res* 2002;90:586–593. [PubMed: 11909823]
24. Zhou ZF, Gong QM, January CT. Correction of defective protein trafficking of a mutant HERG potassium channel in human long QT syndrome. Pharmacological and temperature effects. *J Biol Chem* 1999;274:31123–31126. [PubMed: 10531299]
25. Balijepalli RC, Lokuta AJ, Maertz NA, Buck JM, Haworth RA, Valdivia HH, Kamp TJ. Depletion of T-tubules and specific subcellular changes in sarcolemmal proteins in tachycardia-induced heart failure. *Cardiovasc Res* 2003;59:67–77. [PubMed: 12829177]
26. Chutkow WA, Makielski JC, Nelson D, Burant CF, Fan Z. Alternative splicing of sur2 Exon 17 regulates nucleotide sensitivity of the ATP-sensitive potassium channel. *J Biol Chem* 1999;274(19):13656–13665. [PubMed: 10224138]
27. Tokuyama Y, Fan Z, Furuta H, Makielski JC, Polonsky KS, Bell GI, Yano H. Rat inward rectifying potassium channel Kir6.2: cloning, electrophysiological characterization and decreased expression in pancreatic islets of male Zucker diabetic fatty rats. *Biochem Biophys Res Commun* 1996;220:532–538. [PubMed: 8607800]

28. Tucker S, Gribble F, Zhao C, Trapp S, Ashcroft F. Truncation of Kir6.2 produces ATP-sensitive K⁺ channels in the absence of the sulfonylurea receptor. *Nature* 1997;387:179–183. [PubMed: 9144288]
29. Sharma N, Crane A, Clement J, Gonzalez G, Babenko A, Bryan J, Aguilar-Bryan L. The C terminus of SUR1 is required for trafficking of K_{ATP} channels. *J Biol Chem* 1999;274(29):20628–20632. [PubMed: 10400694]
30. Chan KW, Zhang H, Logothetis DE. N-terminal transmembrane domain of the SUR controls trafficking and gating of Kir6 channel subunits. *EMBO J* 2003;22:3833–3843. [PubMed: 12881418]
31. Rainbow RD, James M, Hudman D, Al Johi M, Singh H, Watson PJ, Ashmole I, Davies NW, Lodwick D, Norman RI. Proximal C-terminal domain of sulphonylurea receptor 2A interacts with pore-forming Kir6 subunits in KATP channels. *Biochem J* 2004;379:173–181. [PubMed: 14672537]
32. Saad FA, Vitiello L, Merlini L, Mostacciuolo ML, Oliviero S, Danieli GA. A 3' consensus splice mutation in the human dystrophin gene detected by a screening for intra-exonic deletions. *Hum Mol Gene* 1992;1:345–346.
33. Cuong DV, Kim N, Joo H, Youm JB, Chung JY, Lee Y, Park WS, Kim E, Park YS, Han J. subunit composition of ATP-sensitive potassium channels in mitochondria of rat heart. *Mitochondrion* 2005;5:121–133. [PubMed: 16050978]
34. Lacza Z, Snipes JA, Miller AW, Szabo C, Grover G, Busija DW. Heart mitochondria contain functional ATP-dependent K⁺ channels. *J Mol Cell Cardiol* 2003;35:1339–1347. [PubMed: 14596790]
35. Kramer W, Muller G, Girbig F, Gutjahr U, Kowalewski S, Hartz D, Summ HD. Differential interaction of glimepiride and glibenclamide with the beta-cell sulfonylurea receptor. II. Photoaffinity labeling of a 65 kDa protein by [3H]glimepiride. *Biochim Biophys Acta* 1994;1191(2):278–290. [PubMed: 8172913]
36. Kramer W, Ökonomopulos R, Punter J, Summ HD. Direct photoaffinity labeling of the putative sulfonylurea receptor in rat beta-cell tumor membranes by [3H]glibenclamide. *FEBS Lett* 1988;229:355–359. [PubMed: 2831099]
37. Aguilar-Bryan L, Clement J, Gonzalez G, Kunjilwar K, Babenko A, Bryan J. Toward understanding the assembly and structure of K_{ATP} channels. *Physiol Rev* 1998;78(1):227–245. [PubMed: 9457174]
38. Gros L, Trapp S, Michael D, Ashcroft F, Bataille D, Blache P. Characterization of two novel forms of the rat sulphonylurea receptor SUR1A2 and SUR1BA31. *Br J Pharmacol* 2002;137(1):98–106. [PubMed: 12183335]
39. Sakura H, Trapp S, Liss B, Ashcroft F. Altered functional properties of KATP channel conferred by a novel splice variant of SUR1. *J Physiol* 2002;521(2):337–350. [PubMed: 10581306]
40. Hambrock A, Preisig-Muller R, Russ U, Piehl A, Hanley P, Ray J, Daut J, Quast U, Derst C. Four novel splice variants of sulfonylurea receptor 1. *Am J Physiol Cell Physiol* 2002;283(2):587–598.
41. Morrissey A, Rosner E, Lanning J, Parachuru L, Chowdhury PD, Han S, Gwendolyn L, Tong XY, Yoshida H, Nakamura TY, Artman M, Giblin J-P, Tinker A, Coetzee WA. Immuno-localization of K_{ATP} channel subunits in mouse and rat cardiac myocytes and the coronary vasculature. *BMC Physiol* 2005;5:1–18. [PubMed: 15647111]
42. Inoue I, Nagase H, Kishi K, Higuti T. ATP-sensitive K⁺ channel in the mitochondrial inner membrane. *Nature* 1991;352:244–247. [PubMed: 1857420]
43. Garlid KD, Paucek P, Yarov-Yarovoy V, Sun X, Schindler PA. The mitochondrial K_{ATP} channel as a receptor for potassium channel openers. *J Biol Chem* 1996;271:8796–8799. [PubMed: 8621517]
44. O'Rourke B. Mitochondrial ion channels. *Annu Rev Physiol* 2007;69:19–49. [PubMed: 17059356]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Fig. 1.

Representative single channel recordings of K_{ATP} currents in native cells. Open channel activity is shown as downward deflection. Membrane potential was held at 0 mV. Closed levels of single channels are marked as 0 pA. **A:** Single channel recordings of I_{KATPn} in ventricular myocytes isolated from the SUR2 mutant. **B:** Single channel recordings of conventional I_{KATP} in ventricular myocytes isolated from WT. **C:** Sensitivity of I_{KATPn} to various concentrations of ATP.

Fig. 2. Current density and IC_{50} for K_{ATPn} . **A:** Number of patches that contain K_{ATP} currents in ventricular myocytes isolated from the SUR2 mutant or WT. **B:** Mean amplitude of K_{ATP} currents per patch in the mutant or WT cells ($p < 0.015$). **C:** Determination of the half-maximal ATP inhibition value (IC_{50}) for K_{ATPn} in the SUR2 mutant cells or IC_{50} for K_{ATP} in WT cells ($n = 8$ per concentration, $p < 0.05$).

Fig. 3. Gating kinetics for K_{ATPn} . **A:** Open probability for K_{ATPn} . **B:** Single channel conductance for K_{ATPn} . **C:** Mean burst duration for K_{ATPn} . **D:** Open and closed time histogram for K_{ATPn} in the SUR2 mutant cells or for K_{ATP} in WT cells ($p < 0.05$). τ_1 is the time constant for closed intervals within bursts while τ_2 the time constant for closed intervals between bursts.

A **B****WT** **C****SUR2 mutant** **Fig. 4.**

Resting potential and response to glibenclamide for K_{ATP} . **A:** Resting potential for K_{ATP} . **B:** I_{KATP} recorded from WT cells in the absence or presence of 10 μ M glibenclamide. Note that one channel was glibenclamide-insensitive. **C:** I_{KATP} recorded from the SUR2 mutant cells showing sensitivity to ATP but resistance to 10 μ M glibenclamide. Solution changes were applied as marked by solid lines.

Fig. 5.

Design of new SUR2 antibodies and specificity tests. **A:** The 17-transmembrane helix model for SUR2 topology with positions for SUR2 antibodies shown. **B:** RT-PCR screening in COS1 cells contained a stably expressed *KIR6.2* (top panel) or *KIR6.1* (bottom panel). In each panel, Lane 1: PCR products amplified from a mouse cDNA library; Lane 2: Water control; Lanes 3–6: RT-PCR products amplified from selected candidates. Arrows indicate expected sizes of RT-PCR products. **C:** Western blot analysis to confirm COS1 lines stably expressing either a *KIR6.2* (positive from Fig. 5B top panel, Lane 5) or a *KIR6.1* (positive from Fig. 5B bottom panel, Lane 6). Arrows indicate detected sizes of Kir6.2 and Kir6.1 proteins. **D–F:** Specificity tests for T1, BNJ-2, BNJ-39 and BNJ-40 antibodies using isolated proteins from COS1 cells

stably expressing a Kir6.2 pore and SUR2A, SUR2B or SUR1 cDNA. In all experiments, ~25 µg of isolated protein isolated was loaded in each lane of a 4–12% MOPS NuPAGE gel. T1 (1: 2000), BNJ-2 (1:1000), BNJ-39 (1:2000) and BNJ-40 (1:1000) were used as primary antibodies. Secondary antibodies were added at 1:10000–1:12500. Arrows indicate detected protein sizes under our gel system and testing conditions.

A **B** **C** **D** **Fig. 6.**

Western blot analysis using a membrane fraction isolated from WT mouse heart tissues. In all experiments, ~25 μ g of isolated protein was loaded in each lane of a 4–12% MOPS NuPAGE gel. Arrows indicate detected protein sizes under our gel system and testing conditions. Lanes were re-arranged for the purpose of presentation due to the numbers of different antibodies and samples tested. **A:** Western results by markers and controls. Anti-Na/K ATPase (Lane 1, 1:1000), anti-VDAC1 (Lane 2, 1:250) and anti-COXIV (Lane 3, 1:5000) were used as primary antibodies to cross-react with the cardiac membrane fraction. In separate blots, anti-Na_v1.5 (Lane 4, 1:200) and anti-HCN4 (Lane 5, 1:200) were used as the primary antibodies to cross-react with the same fraction. **B:** Western results by various SUR2-specific antibodies. T1 (Lane

1, 1: 2000), BNJ-2 (Lane 2, 1:1000), BNJ-39 (Lane 5, 1:2000) and BNJ-40 (Lane 6, 1:1000) were used as primary antibodies to cross-react with the cardiac membrane fraction. BNJ-39 and BNJ-40 were also used to cross-react with a whole heart cell lysate (Lanes 3 & 4). In Lanes 1–4: data shown represents a molecular weight range >100-kDa. Each sample was tested >3 times. **C:** Co-IP experiments using the WT cardiac membrane fraction. In the forward IP experiment, 5 μ g T1 (Lane 1) or control IgG (Lane 2) was used to IP ~100 μ g purified membrane proteins followed by a Western using BNJ-39 (1:2000). In the reverse IP experiment, 5 μ g BNJ-39 (Lane 3) or control IgG (Lane 4) was used to IP ~100 μ g purified membrane proteins followed by a Western using T1 (1:2000). Arrows indicate detected protein sizes under our gel system and testing conditions. **D:** Immunocytochemical studies by a confocal microscope in ventricular myocytes isolated from WT hearts. **I & III:** Images taken under transmitted light; **II & IV:** Green fluorescent images taken under an FITC filter. **II:** BNJ-39 (1:500) and BNJ-40 (1:250) were used as primary antibodies. **IV:** Controls where no primary antibody was added. In all panels, the secondary antibody was the anti-rabbit Alexa-Fluor 488 IgG (1:250). Scale bar represents 5 μ m.

Fig. 7.

Western blot analysis using a membrane fraction isolated from the SUR2 mutant heart tissues.

A: A schematic diagram of the targeted disruption in the *SUR2* gene. The SUR2 antibodies used in the experiments are labeled by vertical arrows. The position of NBD1 is marked by a dotted line while NBD2 is marked by a solid line. **B:** Western results by four SUR2 antibodies. In all experiments, ~25 μ g of isolated protein was loaded in each lane of a 4–12% MOPS NuPAGE gel. T1 (Lane 1, 1:2000), BNJ-2 (Lane 2, 1:1000), BNJ-39 (Lane 3, 1:2000) and BNJ-40 (Lane 4, 1:1000) were used as primary antibodies. Secondary antibodies were added at 1:10000–1:12500. Each sample was tested >3 times. Arrows indicate detected protein sizes under our gel system and testing conditions. Lanes were re-arranged for the purpose of

presentation. **C:** Immunocytochemical studies by a confocal microscope in ventricular myocytes isolated from the SUR2 mutant. **I & III:** Images taken under transmitted light; **II & IV:** Green fluorescent images taken under an FITC filter. **II:** BNJ-39 (1:500) and BNJ-40 (1:250) were used as the primary antibodies. **IV:** Controls where no primary antibody was added. In all panels, the secondary antibody was the anti-rabbit Alexa-Fluor 488 IgG (1:250). Scale bar represents 5 μ m. **D:** Nested exonic RT-PCR to locate transcripts encoding the SUR2 short forms. Positions of the six forward primers (P1–6) and the reverse primer (P7) are labeled by short horizontal arrows (see Fig. 7A). Top panel: PCR products amplified by all primer pairs using SUR2A cDNA as templates. Middle panel: RT-PCR products amplified by all primer pairs using mRNA isolated from WT mouse hearts. The lower band in Lane 2 was a non-specific product after sequencing. Bottom panel: RT-PCR products amplified by all primer pairs using mRNA isolated from the SUR2 mutant hearts. Arrows indicate sizes for amplified PCR or RT-PCR products.

Fig. 8.

Co-IP results of the SUR2 short forms with Kir6.1 or Kir6.2. 5 µg of a specific antibody or control IgG was used to IP ~100 µg purified membrane proteins isolated from the SUR2 mutant hearts. **A:** Control IgG, BNJ-39 and BNJ-40 were used in the forward IP experiments followed by a Western using anti-Kir6.2 (1:200). Anti-Kir6.2 was used in the reverse IP experiments followed by Westerns using either BNJ-39 (1:2000) or BNJ-40 (1:1000). **B:** Control IgG, BNJ-39 and BNJ-40 were used in the forward IP experiments followed by a Western using anti-Kir6.1 (1:200). Anti-Kir6.1 was used in the reverse IP experiment followed by a Western using BNJ-39 (1:2000). In all panels, secondary antibodies were added at 1:10000–1:12500.

Arrows refer to detected protein sizes under our gel system and testing conditions. **C**: A schematic diagram of each deduced K_{ATP} complex based on results from this work. **(I)**: 150/Kir6.1 or Kir6.2, **(II)** 150/28A/Kir6.1 or Kir6.2; **(III)**: 68A/Kir6.1 or Kir6.2; **(IV)**: 28B/Kir6.2.