RESEARCH PAPER

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ATP-sensitive potassium currents from channels formed by Kir6 and a modified cardiac mitochondrial SUR2 variant

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Keywords: KATP channel, sulfonylurea receptor, alternative splicing, mitochondria, ATP-binding cassette protein, intra-exonic splice variant, ischemic preconditioning, hemi-transporter, inward rectifier channel

Abbreviations: SUR, sulfonylurea receptors; KATP, ATP sensitive K^+-channels; Kir, inward K^+ rectifier; NBDs, nucleotide binding domains; TMDs, transmembrane domains; KCOs, KATP channel opener; KCBs, KATP channel blockers

Cardiac ATP-sensitive potassium channels (KATP) are found in both the sarcoplasmic reticulum (sarcKATP) and the inner membrane of mitochondria (mitoKATP). SarcKATP are composed of a pore containing subunit Kir6.2 and a regulatory sulfonylurea receptor subunit (SUR2), but the composition of mitoKATP remains unclear. An unusual intra-exonic splice variant of SUR2 (SUR2A-55) was previously identified in mitochondria of mammalian heart and brain, and by analogy with sarcKATP we proposed SUR2A-55 as a candidate regulatory subunit of mitoKATP. Although SUR2A-55 lacks the first nucleotide binding domain (NBD) and 2 transmembrane domains (TMD), it has a hybrid TMD and retains the second NBD. It resembles a hemi-ABC transporter suggesting it could multimerize to function as a regulatory subunit. A putative mitochondrial targeting signal in the N-terminal domain of SUR2A-55 was removed by truncation and when co-expressed with Kir6.1 and Kir6.2 it targeted to the plasma membrane and yielded KATP currents. Single channel conductance, mean open time, and burst open time of SUR2A-55 based KATP was similar to the full-length SUR2A based KATP. However, the SUR2A-55 KATP were 70-fold less sensitive to block by ATP, and twice as resistant to intracellular Ca^{2+} inhibition compared with the SUR2A KATP, and were markedly insensitive to KATP drugs, pinacidil, diazoxide, and glybenclamide. These results suggest that the SUR2A-55 based channels would tend to be open under physiological conditions and in ischemia, and could account for cardiac and mitochondrial phenotypes protective for ischemia.

Introduction

ATP-sensitive potassium current (I_{KATP}) was first described in in the sarcolemma of cardiac ventricular cells[1] and later in pancreas, vascular smooth muscle, and many other tissues. I_{KATP} was implicated as being important for ischemic preconditioning, where brief periods of ischemia protect the hearts from a subsequent prolonged ischemia,[2] when it was shown that blocking I_{KATP} prevented preconditioning.[3] The channels carrying I_{KATP} (KATP) are closed under normal physiological conditions but they are activated in response to ischemia.[4] KATP were found to be composed of 1 of 2 isoforms of the inward rectifier pore-forming subunit[5] Kir6.1 or Kir6.2, encoded by separate genes, and 1 of 2 isoforms of a sulfonylurea receptor (SUR) regulatory subunit SUR1 or SUR2, encoded by separate genes.[6,7] Expression of Kir6.1 or Kir6.2 without SUR in heterologous cells yielded no I_{KATP} implicating SUR as a necessary subunit. I_{KATP} was later described in mitochondrial inner membranes[8] and presumed to be a mitochondrial based KATP (mitoKATP). MitoKATP was also proposed to be important in conferring protection from ischemia.[9] In contrast to the better characterized sarcolemmal forms of KATP (sarcKATP), however, the molecular identity of mitoKATP channels remained less well understood.

Multiple alternative splice variants of SUR2, the major SUR subunit in cardiac ventricular muscle, have been reported.[10] The "full-length" and most studied SUR2 splice variants, SUR2A and SUR2B, differ only by the alternative use of the C-terminal exon.[6] In general, SUR1 is considered the pancreatic form, SUR2A the cardiac form, and SUR2B the vascular form.[11] We reported 2 non-conventional SUR2 splice variants that are approximately 55 kDa in size and named SUR2A-55 and SUR2B-55 for size and alternative C-terminal exon usage. They were smaller than full length SUR2A and SUR2B (1/3 the size), were generated by an unusual process of intra-exonic splicing (IES), and were found predominantly in mitochondrial membrane fractions.[12] They possessed a new hybrid transmembrane domain (TMD) formed by splicing the first 4 transmembrane spanning (TM) regions from TMD0 and TM17 from TMD2 of SUR2, which deleted all of

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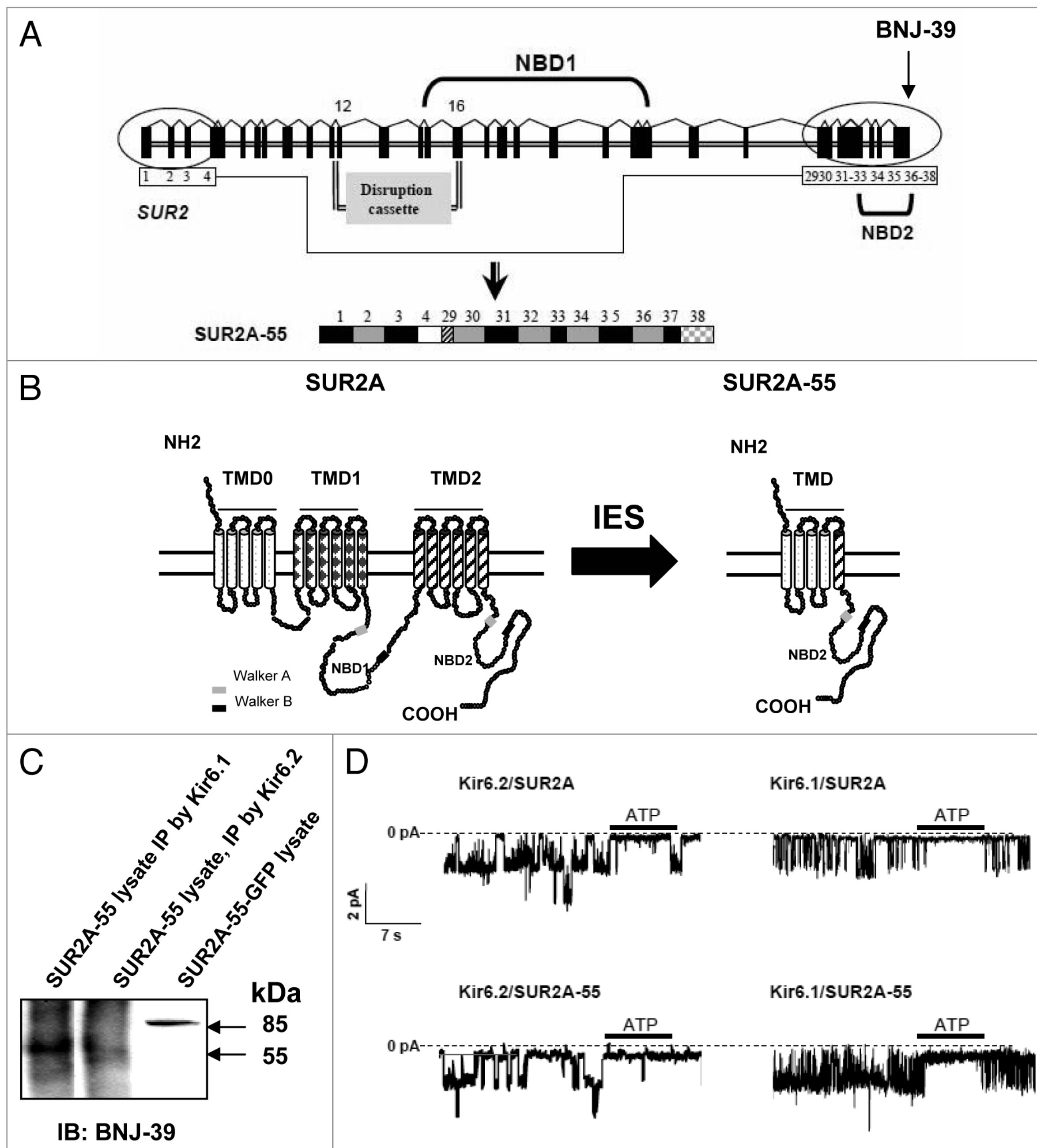

Figure 1. Deduced structure and expression of SUR2–55 based channels. (**A**) Organization of *Sur2*. Horizontal solid double line represents the *Sur2* locus; vertical bars represent the relative exon positions, circled regions represent exons that are present in the intra-exonic splicing (IES) variants; and the location of the targeting cassette for a transgenic mouse[33] and the BNJ-39 antibody is labeled. The exon structure for SUR2A-55 is shown below with black boxes for odd numbered exons, gray boxes for even-numbered exons, hashed box for composite exon made of parts of exon 4 and 29 from the IES, and checked box for last exon of SUR2. (**B**) Exons 4 and 29 undergo IES in the mRNA of SUR2 to generate IES variants, which contain 13 exons. The IES junction is marked by a solid vertical line (**B**) Topology of the full-length SUR2A or SUR2A-55 IES variant. (**C**) Co-immunoprecipitation (Co-IP) results. SUR2A-55-GFP lystate isolated from transfected COS1 cells was immunoprecipitated with anti-Kir6.1 or anti-Kir6.2 antibody. The blot was cross-reacted with an anti-SUR2 antibody (BNJ-39 at 1:1000). Lysates isolated from COS1 cells expressing SUR2A-55-GFP were included as a control. Secondary antibodies were added at 1:10,000. (**D**) Representative I_{KATP} traces from inside out patches for COS-1 cells co-transfected with Kir6.2 (top) or Kir6.1(bottom) and full length SUR2A (top) or IES variant SUR2A-55 (bottom) showing block by 10 mM ATP.

TMD1 and most of TMD2 (**Fig. 1A** and **B**). SUR2–55 retained NBD2 from SUR2, and we hypothesized that 2 or more of these short forms could combine to function as a regulatory subunit in a way analogous to other ABC cassettes that had only a single TM and a single NBD and were called hemi-ABC transporters.[13,14] In our previous study[12] we removed a putative mitochondrial targeting sequence (MTS) by truncation of 30 amino acids from the N-terminus to produce a modified SUR2–55 and found that SUR2A-55 and SUR2B-55 previously targeted to mitochondria

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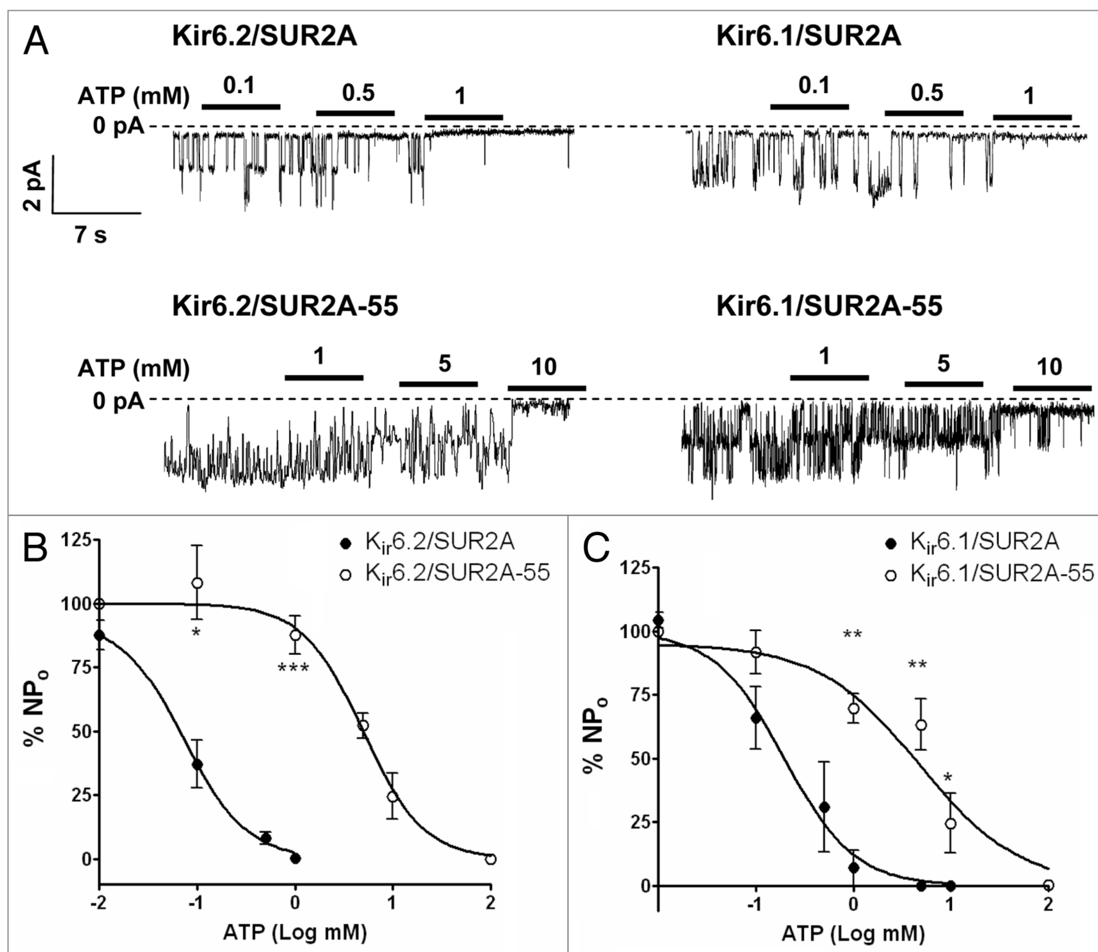

Figure 2. ATP sensitivities recorded from recombinant KATP channels formed by the MTS-less SUR2A-55 or full-length SUR2A and Kir6 pores. (**A**) Representative excised patch current traces from various recombinant KATP channels. (**B**) Summarized P_O values from Kir6.2-based channels against various ATP concentrations. (**C**) Summarized P_O values from Kir6.1-based channels against various ATP concentrations. n = 4, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs full-length SUR2A-based channels.

Table 1. Characterization of intrinsic properties of recombinant KATP channels formed by MTS-less SUR2A-55 or full-length SUR2A and Kir6 pores

Combination	Amplitude (pA)	Conductance (pS)	Mean open time (in ms)	Mean burst duration (in ms)
Kir6.2/SUR2A[c]	-0.77±0.07	29 ± 2	6.9 ± 0.4	58.6 ± 5.2
Kir6.2/SUR2A-55[d]	-0.73±0.09	28 ± 1	8.2 ± 0.4	60.9 ± 4.8
Kir6.1/SUR2A[a]	-0.76±0.02	25 ± 3	7.8 ± 0.5	73.5 ± 10
Kir6.1/SUR2A-55[b]	-0.82±0.08	29 ± 1	6.9 ± 0.3	63.7 ± 12

[a, b, c, d]all recorded values are not significantly different, n = 4, $p > 0.05$.

were re-targeted to the sarcolemma when co-expressed with Kir6 channels, and we recorded I_{KATP} when co-expressed with Kir6 in COS1 cells.[12]

A transgenic mouse with a disrupted SUR2[12] lacked SUR2A and SUR2B, retained SUR2A-55 and SUR2B-55 likely because the IES "jumped" the disruption cassette (**Fig. 1A**), and surprisingly had smaller infarct sizes than wild type as if "autoprotected." These mice also demonstrated a mitochondrial phenotype known to be protective for ischemia which included slightly depolarized membrane potential and increased potassium conductance resistant to ATP and calcium inhibition.[15] We hypothesized that properties of SUR2-55 based KATP in the transgenic mouse as compared with SUR2 based KATP in the wild-type mouse might account for the "autoprotection" from ischemia, and for the altered protective mitochondrial phenotype in the transgenic mouse. Surface targeting of the modified SUR2-55 provided a convenient model to study pharmacology, kinetics, and ATP and calcium sensitivity of I_{KATP} from SUR2-55 based KATP. To

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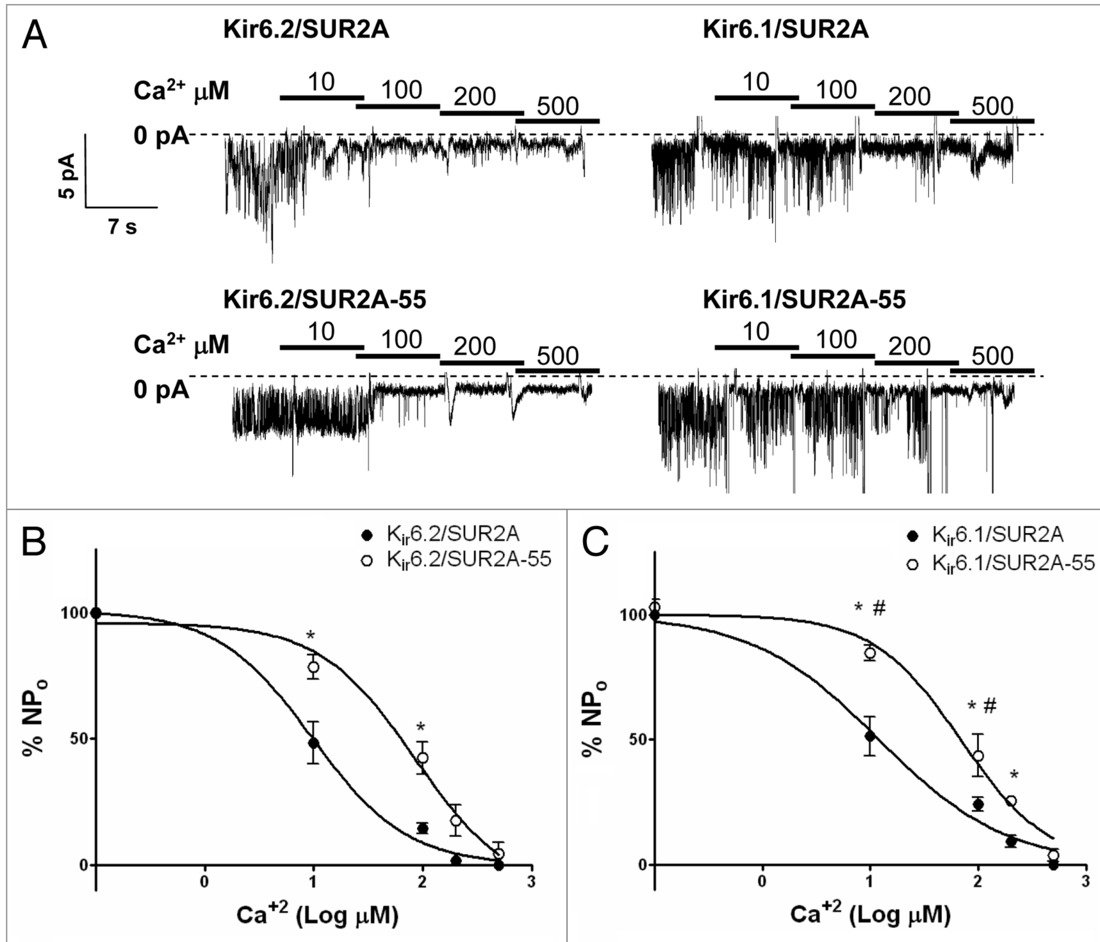

Figure 3. Calcium sensitivities recorded from recombinant KATP channels formed by the MTS-less SUR2A-55 or full-length SUR2A and Kir6.1 or Kir6.2 pores. (**A**) Representative excised patch current traces recorded from various recombinant KATP channels. (**B**) Summarized P_O values from Kir6.2-based channels against various calcium concentrations. (**C**) Summarized P_O values from Kir6.1-based channels against various calcium concentrations. n = 4, * $p < 0.05$, ** $p < 0.01$ vs full-length SUR2A-based channels.

Table 2. Characterization of ATP or Ca^{2+} sensitivity of recombinant KATP channels formed by MTS-less SUR2A-55 or full-length SUR2A and Kir6 pores

Combination		ATP inhibition	Ca^{2+} inhibition	ATP inhibition in Ca^{2+} (10 or 40 μM)
Kir6.2/SUR2A	IC_{50}	0.07 ± 0.02	9.9 ± 1.9	0.07 ± 0.04
	θ	-1.3 ± 0.03	-1.05 ± 0.17	-1.5 ± 0.11
Kir6.2/SUR2A-55	IC_{50}	4.9 ± 1.3**	54.7 ± 2.0***	2.8 ± 0.19***
	θ	-1.2 ± 0.15	-0.92 ± 0.06	-1.09 ± 0.59
Kir6.1/SUR2A	IC_{50}	0.19 ± 0.04	11.9 ± 1.5	0.05 ± 0.09
	θ	-1.2 ± 0.46	-0.88 ± 0.36	-0.89 ± 0.20
Kir6.1/SUR2A-55	IC_{50}	4.7 ± 1.6##	68.9 ± 1.9###	3.7 ± 1.5###
	θ	-0.92 ± 0.27	-1.06 ± 0.20	-1.2 ± 0.23

IC_{50} values (mM for ATP, μM for Ca^{2+}) and the Hill-coefficient (θ) for fits to data from Figures 2, 3, and 4. For ATP inhibition in the presence of Ca^{2+}, an approximately half inhibitory concentration was chosen, 10 μM for SUR2A-55 and 40 μM for SUR2A based channels.. * and #, values compared with Kir6.2/SUR2A and Kir6.1/SUR2A, respectively. *** or ### $p < 0.001$, ** or ## $p < 0.05$.

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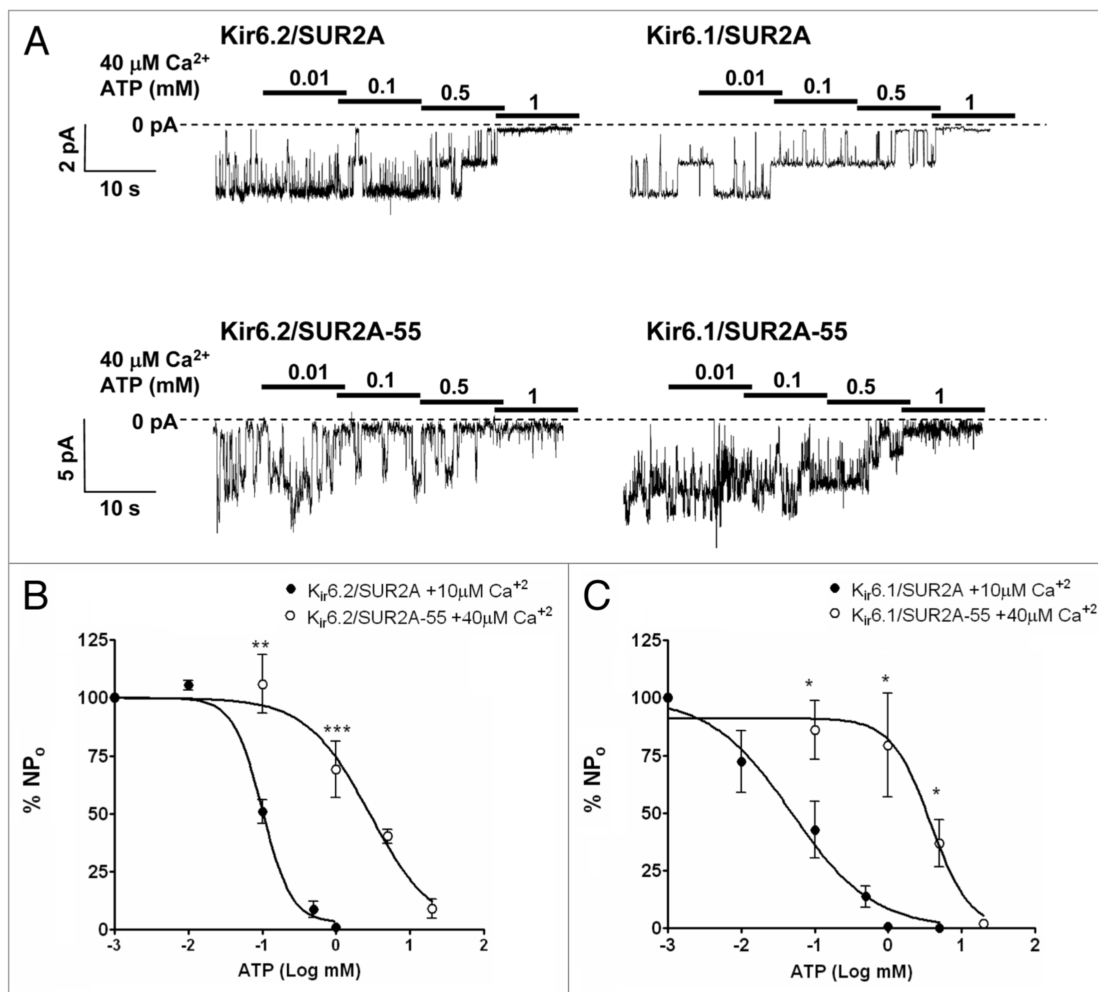

Figure 4. ATP sensitivities in the presence of 40 μM calcium recorded from recombinant KATP channels formed by the MTS-less SUR2A-55 or full-length SUR2A and Kir6.1 or Kir6.2 pores. (**A**) Representative excised patch current traces recorded from the KATP channels. (**B**) Summarized P_O values from Kir6.2-based channels against various calcium concentrations. (**C**) Summarized P_O values from Kir6.1-based channels against various calcium concentrations. n = 4, *p < 0.05, **p < 0.01, ***p < 0.001 vs full-length SUR2A-based channels.

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reduce the experimental scope to a tractable size we focused on SUR2A-55 as this form was more abundant than SUR2B-55 in the autoprotected transgenic mouse.[12]

Results

Removing a putative MTS allowed for expression of I_{KATP}

When unmodified SUR2A-55 was co-expressed in COS1 cells with Kir6.1 or Kir6.2, immunoprecipitation with antibodies for Kir6.1 or Kir6.2 pulled down SUR2A-55 (**Fig. 1C**), giving evidence of association, but no I_{KATP} was detected in these cells. SUR2A-55 was modified by removing the 30 N-terminus amino acids that contained a putative mitochondrial targeting sequence (MTS). For the rest of the paper, we will refer to this truncated form simply as SUR2A-55, and specify "unmodified SUR2A-55" for the non-truncated form. Co-expression of SUR2A-55 with Kir6.1 or Kir6.2 in COS1 cells yielded I_{KATP} that was inhibited by high (10 mM) concentrations of ATP (**Fig. 1D**), whereas SUR2A-55 expressed alone in COS1 cells yielded no detectable I_{KATP} (data not shown). When compared with full-length SUR2A, the I_{KATP} produced by SUR2A-55 with both Kir6.1 and Kir6.2 had the same mean unitary channel amplitude (**Table 1**) with the IV relationship linear over the range of -80 to +20 mv (**Fig. S1**), and the same mean open time (**Table 1**). In the absence of ATP, the average burst open duration of the SUR2A-55 based channels was also not significantly different from full-length SUR2A (**Table 1**).

SUR2A-55 channels were less sensitive to ATP than SUR2A channels

ATP (0.1–10 mM) was added to the bath solutions of the inside-out patches by rapid solution exchange and I_{KATP} was recorded (**Fig. 2A**) and summary data for open probability (P_o) was plotted against ATP concentrations (**Fig. 2B** and **C**). The half-maximal inhibition IC_{50} values for ATP are reported in **Table 2** and range from 70 μM to 4.9 mM. SUR2A-55/Kir6.2 channels were 70-fold less sensitive to ATP than the full length SUR2A channels and SUR2A-55/Kir6.1 channels were 25-fold less sensitive to ATP than the full length SUR2A channels. The

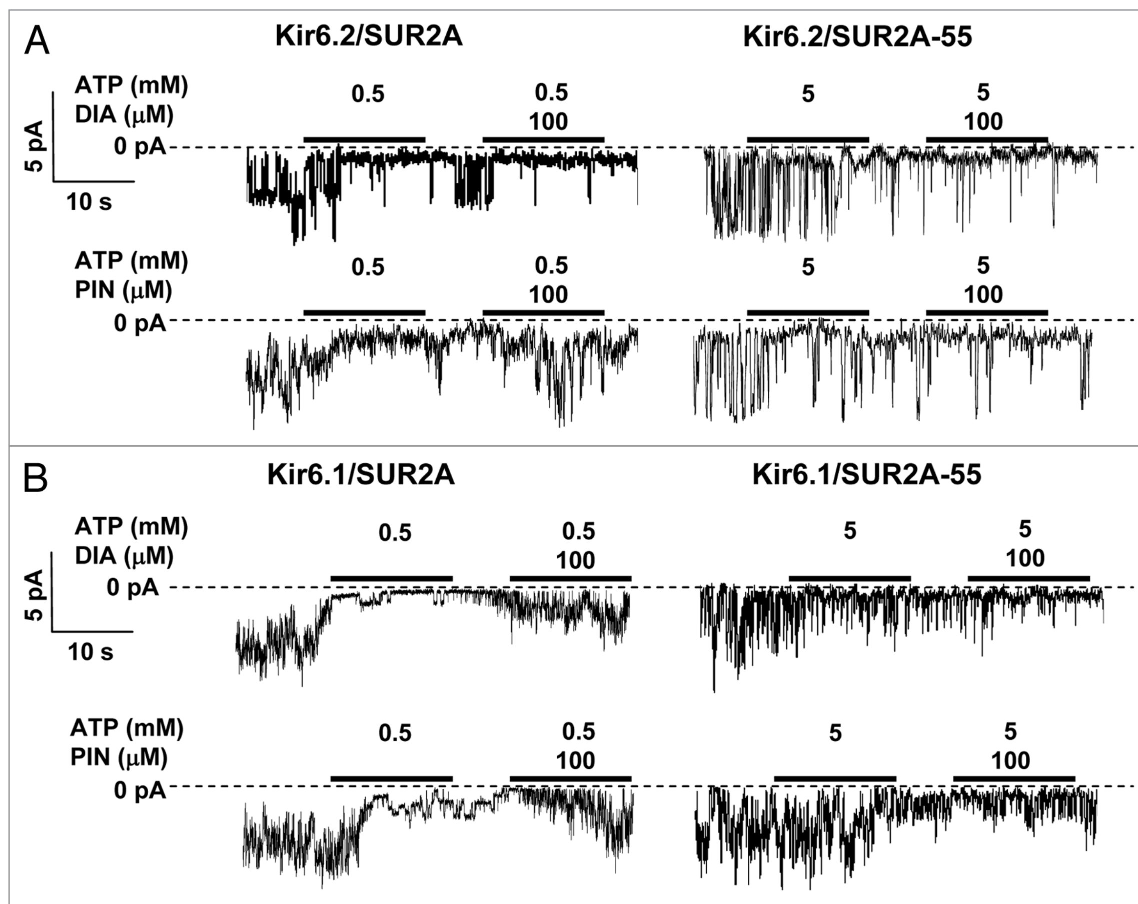

Figure 5. Pharmacology of recombinant KATP channels formed by the MTS-less SUR2A-55 or full-length SUR2A and Kir6.1 or Kir6.2. (**A**) Representative excised patch current traces recorded from Kir6.2-based recombinant KATP channels in the presence of diazoxide (top panels) or pinacidil (bottom panels). (**B**) Representative excised patch current traces recorded from Kir6.1-based recombinant KATP channels in the presence of diazoxide (top panels) or pinacidil (bottom panels).

IC_{50} values for ATP did not differ significantly between the SUR2A-55/Kir6.2 and SUR2A-55/Kir6.1 channels, suggesting that the Kir6 pore did not play a role in altering ATP sensitivity in the short form-based channels. In contrast, the SUR2A/Kir6.1 channels were 3-fold less sensitive to ATP compared with the SUR2A/Kir6.2 channels, indicating that different Kir6 pores may contribute to the ATP sensitivity for the full-length SUR2 based channels.

SUR2A-55 channels were more resistant to inhibition by Ca^{2+}

Intracellular Ca^{2+} levels have been reported[16] to decrease the P_O of native KATP channels. Increasing concentration of Ca^{2+} applied to the bath (cytolasmic side of the channel) decreased P_O for all heterologously expressed channels (**Fig. 3A**). Summary data for % P_O for different Ca^{2+} concentrations are shown (**Fig. 3B** and **C**) and IC_{50} values and Hill coefficients are listed in **Table 2**. The Ca^{2+}-sensitivity of the channels ranged from half inhibition at 9.9–68.9 μM with the order from most sensitive to least sensitive being: Kir6.2/SUR2A > Kir6.1/SUR2A > Kir6.2/SUR2A-55 > Kir6.1/SUR2A-55. The SUR2A-55 based channels were 5.5-fold (Kir6.2) and 5.8-fold (Kir6.1) more resistant to Ca^{2+} than the full-length SUR2A based channels.

Ca^{2+} augmented ATP sensitive closure less in SUR2A-55 based channels

Intracellular Ca^{2+} levels interact with the channel to augment the ATP sensitivity to closure of native KATP channels.[17] We determined % P_O of the SUR2A-55 channels in the presence of ATP (0.01 to 10 mM) when the bath was perfused with 10 or 40 μM Ca^{2+} from the intracellular side (**Fig. 4A**). Based on the previous reports and data in **Figure 3**, the intracellular Ca^{2+} level was tested at 10 μM for SUR2A based channels and 40 μM for SUR2A-55 channels; a concentration at which ~50–60% of the channels are expected to be blocked. The ATP sensitivity of the SUR2A-55 channels was increased in the presence of Ca^{2+} (**Fig. 4B** and **C**). The IC_{50} value for ATP plus 40 μM Ca^{2+} for the SUR2A-55 based channels decreased 4–7 fold relative to the SUR2A based channels (**Table 2**). Overall, the SUR2A-55 channels were more sensitive to ATP in presence of Ca^{2+} but still not as sensitive as the SUR2A channels even at the higher Ca^{2+} concentration.

SUR2A-55 channels had altered pharmacology

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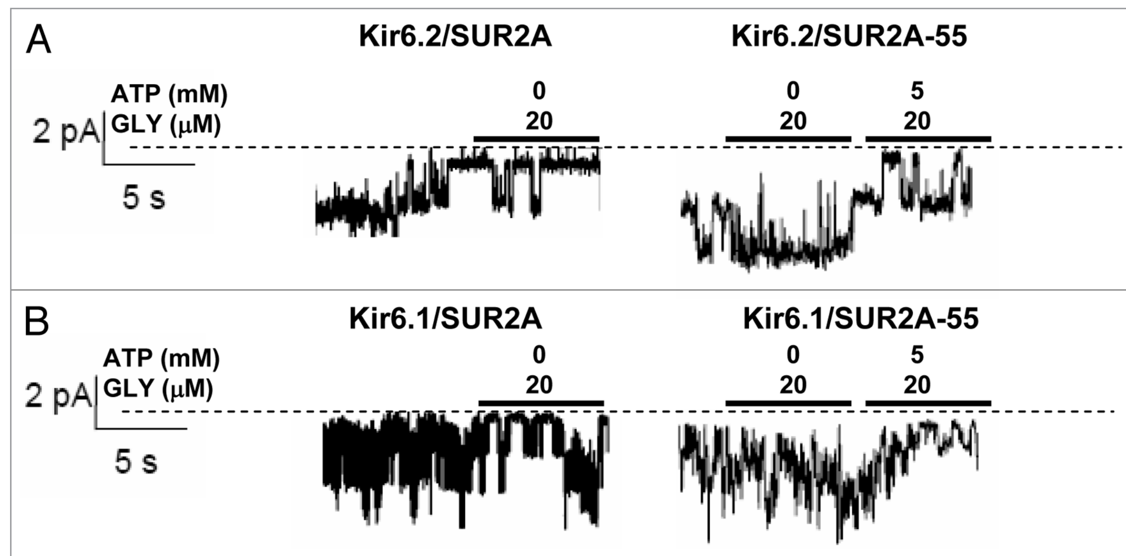

Figure 6. Pharmacology of recombinant KATP channels formed by the MTS-less SUR2A-55 or full-length SUR2A and Kir6 pores. (**A**) Representative current traces recorded from the Kir6.2-based recombinant KATP channels in the presence of glybenclamide or glybenclamide plus ATP. (**B**) Representative excised patch current traces from Kir6.1-based recombinant KATP channels in the presence of glybenclamide or glybenclamide plus ATP.

The SUR2A-55 variant lacks TM5–16, which harbor the action sites for the KATP channel blocker (KCB) sulfonylureas[18] and the KATP channel opener KCOs diazoxide and pinacadil.[19-21] Therefore, SUR2A-55 channels might be expected to be less responsive to both KCOs and KCBs. Pinacidil, a non-selective opener of the full-length SUR2A-based KATP channels, did not open the SUR2A-55 channels that were closed by ATP (10 mM). Diazoxide is a selective opener for mitoKATP channels when it is administered below the concentration of 100 μM.[22] An even higher concentration of pinacidil or diazoxide (1 mM) failed to open the SUR2A-55 channels that were closed by ATP (data not shown). However, diazoxide opened the SUR2A/Kir6.1 channels but not the SUR2A/Kir6.2 channels, suggesting that Kir6 pores contribute to ATP sensitivity in the presence of diazoxide. These observations also support the idea that the binding sites for pinacidil or diazoxide were lost in the SUR2A-55 variant as predicted. Sensitivity to a non-selective inhibitor for KATP channels, glybenclamide, was also tested (**Fig. 6A** and **B**) and did not block the SUR2A-55 based channels, consistent with our previous report for SUR2B-55,[12] however, glybenclamide blocked the SUR2A-based channels as expected.

Discussion

Structure and function of the SUR2–55 based KATP channels

In a prior report[12] we identified SUR2 IES variants in heart and brain mitochondria. A non-conventional intra-exonic splicing event in SUR2 spliced the first 4 transmembrane helixes (TM) of the first TMD (TMD0) with the last TM of the third TMD (TMD2) to form a new hybrid TMD in the SUR2–55 variants (**Fig. 1A** and **B**). These variants retain an intact NBD2 as well as the alternative usage of the C-terminal exon indicated by the SUR2A and SUR2B nomenclature. Recombinant channels with SUR2A-55 and Kir6 pores exhibited signature properties of conventional I_{KATP} such as spontaneous bursting behavior and sensitivity to ATP, albeit at much higher ATP concentrations (**Fig. 1** and **2**). The conductance and kinetic properties were similar to the full-length SUR2A based channels (**Table 1**). It is known that the nucleotide binding domain in the C-terminus of Kir6 pores confer ATP sensitivity to a KATP channel but this sensitivity is significantly increased 100-fold by interacting with a linker region, which is located between TMD0 and TMD1 of a SUR regulatory subunit.[23] This linker is absent in SUR2A-55 due to the IES splicing event, and the ATP sensitivity of SUR2–55 based channels was close to that previously shown for SUR1 based channels with a deleted linker.[24] The SUR2A-55 based channels were inhibited by ATP but the sensitivity was 70-fold lower than the full-length SUR2A channels (**Fig. 3**), suggesting that these SUR2A-55 based channels are relatively insensitive to physiological level of ATP.

The IC_{50} value of ATP for the SUR2A-55 based channels was ~100 μM, which is comparable to the IC_{50} values recorded from a Kir6.2 pore mutant lacking the C-terminal 35 amino acids.[25] An earlier study in SUR1 showed that TMD0 of SUR1 interacts with Kir6.2 in a KATP channel complex.[26] In the SUR2–55 variants, the first 4 of the 5 TMs of TMD0 are present, and could interact with Kir6 pores, and co-IP data (**Fig. 1**) confirmed that SUR2A-55 interacted with Kir6.1 and Kir6.2. SUR2A-55 also yielded I_{KATP} when co-expressed with either Kir6 pore. Although we were able to record I_{KATP} currents from the reconstituted channels, it remains unclear whether Kir6 pores are candidates for the cardiac mitoKATP channels. The literature on whether Kir6.2 is present in cardiac mitochondria is somewhat conflicting, however, Kir6.1 has been proposed to be present.[27] More recently, a RomK2 channel (Kir1.1) was proposed to be the pore for cardiac mitoKATP.[28] Other mitoKATP channels, which do not have a SUR regulatory subunit, may also exist.

Ca^{2+} increases ATP sensitivity of SUR2A-55 based channels

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A characteristic property of I_{KATP} is a phenomenon called "run-down "often attributed to the presence of Ca^{2+} on the intracellular side of the channel. Ca^{2+} causes loss of I_{KATP} within few seconds in an excised patch.[29] The mechanism of this Ca^{2+} effect is not known and it remains controversial whether Ca^{2+} itself binds to SUR to block I_{KATP}. Trypsin treatment of the excised patch that cleaved the C-terminal of the SUR subunits prevented Ca^{2+} induced run-down.[30] Although the exact Ca^{2+} binding site on SUR is unknown, the C-terminus remained intact in the splice variants, and therefore, it is predicted that the SUR2A-55 based channels would be sensitive to Ca^{2+} and would run-down, and indeed the SUR2-55 based channels did run down within a minute (data not shown). I_{KATP} with SUR2A and SUR2A-55 based channels were sensitive to increasing levels of Ca^{2+} on the intracellular side (**Fig. 4**). Interestingly, the SUR2A-55 based channels were 5-fold more resistant to Ca^{2+} than the SUR2A based channels (**Table 2**), suggesting structural components other than the SUR2 C-terminus contribute to Ca^{2+} sensitivity. The extent to which intracellular Ca^{2+} increased the ATP sensitivity of SUR2A or SUR2A-55 based channels is comparable (**Table 2**), indicating that Ca^{2+} may have increased the ATP sensitivity by a common mechanism unrelated to the structure of SUR2. However, the SUR2A-55 based channels were 3-fold less sensitive to ATP than the full-length SUR2A based channels. Although the mechanism for Ca^{2+} increased ATP sensitivity remains unclear, a number of hypotheses have been proposed. Experiments exploring phospholipid actions revealed that molecules with negative charges reduce ATP sensitivity,[31] leading to a hypothesis involving electrostatic binding of the molecule to the Kir6. Accordingly, Ca^{2+}, with its positive charges, may produce a reverse action of potentiating ATP binding affinity to Kir6. In another report, it was hypothesized that Ca^{2+} binds to a site on KATP to enhance ATP block by forming an ATP/Ca^{2+}/ATP complex (at low Ca^{2+} concentrations) or a Ca^{2+}/ATP/Ca^{2+} complex (at high Ca^{2+} concentrations) in the ATP binding site of the channel, which causes increase in ATP block of the channel.[32] The exact mechanism of Ca^{2+} potentiation of ATP block of KATP channels is unclear and implications for cellular physiology remain to be studied.

Altered pharmacology of SUR2A-55 based KATP channels

TMD2 in SUR1 has been shown to be critical for binding of KCOs and KCBs as most of the KCOs bind between TM 12–17 or between TM 6–11[20] while KCBs bind between TM 13–14 and NBD1.[19] Presuming these same homologous structures are important in SUR2, SUR2A-55 lacks these drug action sites. And indeed KCOs and KCBs did not affect the SUR2A-55 based channels (**Figs. 5** and **6**) as predicted by these structural considerations.

Role of SUR2–55 based KATP channels in cardioprotection

In earlier studies, we created a transgenic mouse model SUR2KO that targeted NBD1 of SUR2,[33,34] and characterized the "protective phenotype" in male mutant mice using 2 ischemia models.[12,35] Unexpectedly, these mice were "constitutively" protected from ischemia-reperfusion (I-R) stress without a preconditioning stimulus, suggesting that the SUR2 long forms may not be required to confer cardioprotection in male hearts, or perhaps that their absence may help confer protection. In the SUR2KO hearts, the SUR2–55 variants remained expressed because the IES splicing event "by-passed" the targeting site, allowing the short forms to "escape" the disruption.[12] Overexpression of SUR2A-55 in mice lead to cardioprotection[37] and testosterone induced protection In a cultured myocardial cell model was accompanied by an increase in SUR2B-55, suggesting SUR2–55 may be important in the mechanism for protection.[36] A mitochondrial phenotype study of SUR2KO mice[15] showed that, compared with wild type, the SUR2KO male mitochondria exhibited partial depolarization, a moderately increased level of ROS for I-R stress signaling, a greater K^+ flux (suggesting mitoKATP activity), an insensitivity of mitochondrial membrane potential to ATP and pharmacology, and resistance to Ca^{2+} loading. All of these mitochondrial phenotype differences are consistent with the functional implications of the properties we observe in the I_{KATP} for SUR2A vs. SUR2A-55 based channels. Although the exact and full molecular nature of mitoKATP remains unknown, it suggests SUR2A-55 as a candidate regulatory subunit for mitoKATP.

Under physiological conditions in a resting cell cytosolic ATP concentration is 2–5 mM, Ca^{2+} is 0.25 μM, and mitochondrial Ca^{2+} is approximately 4 μM.[38] Under stressed conditions, depleting ATP causes an increase in mitochondrial Ca^{2+} load to several hundred micromolar.[39] Under stressed conditions, the SUR2A-55 based channels would remain open (and presumably protective) when intracellular Ca^{2+} load is high (**Fig. 3** and **4**). The SUR2KO male mice that are "constitutively" protected lack SUR2A long forms but retain the SUR2–55 forms, suggesting a hypothesis whereby by the remaining SUR2–55 based channels confer protection. Future studies on overexpressing or disrupting SUR2A-55 in vivo and how these forms are altered in ischemic preconditioning may shed new light on the role of short form SUR2–55 based channels in cardioprotection.

Although the I_{KATP} characteristics we observed for SUR2-55 based channels in heterologous expression systems are consistent with causing the protective mitochondrial phenotype we observed in SUR2KO mice, the existence and importance of the SUR2-55 based KATP channels, perhaps with different pores such as Romk2, in vivo in non-transgenic mice and in humans will require further study. If present in vivo, these channels will have important implications. Their altered pharmacology and regulation would require a reinterpretation of previous studies of the role of KATP channels in myocardial protection which prominently used pharmacological tools for the elucidation of mechanism. In addition, these splice variants could potentially form hetero-multimeric ABC complexes with other SUR2 short forms and perhaps other non-SUR2 ABC cassettes such as the mitochondrial ATP binding cassette greatly increasing the possible diversity of I_{KATP} characteristics.

Materials and Methods

Plasmids

A full-length mouse SUR2A cDNA[7] (NM_021041) was subcloned into a pIRES-GFP vector as previously described.

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A mouse SUR2-55A cDNA was cloned and subcloned into a pIRES-GFP.[12] A putative mitochondrial targeting sequence (MTS) from the SUR2A-55 was removed by site-directed mutagenesis as previously described.[12] The MTS-less SUR2A-55 was subcloned into a pIRES-GFP. A mouse Kir6.2 or a rat Kir6.1 cDNA was cloned as reported previously and sub-cloned into pcDNA3 (Life Technologies).[40]

Cell culture and transfection

Approximately 5x10^4 COS1 cells were seeded on 4 coverslips inside a 35-mm plate containing Dulbecco's modified Eagle Medium (MEM), 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acid solution, and 1 mM sodium pyruvate (Life Technologies). Transfection was performed by a TransIT-COS kit (Mirus) according to the manufacturer's recommended protocol. For voltage clamping experiments, 0.7 μg of MTS-less SUR2A-55 or full-length SUR2A cDNA was co-transfected with 0.7 μg of mKir6.1or rKir6.2 into COS1 cells.[40] For MTS-less SUR2A-55 expression, 6 μg DNA was transfected into 1x10^6 COS1 cells in a 100-mm plate for protein isolation.

Co-Immunoprecipitation (Co-IP)

Co-IP experiments were performed using DynalBeads (Life Technologies) following manufacturer's recommended procedures. Twenty-four hours post transfection, total lysates were isolated from transfected cells. Protein concentration was determined using a DC Protein Assay Kit (Bio-Rad). Seven micrograms anti-Kir6.1 or anti-Kir6.2 (Millipore) was then used to immunoprecipitate 250 μg protein that was isolated from transfected COS1 cells. Protein samples were separated on a 4–12% MOPS NuPAGE gel. Anti-SUR2 (BNJ-39) was a previously characterized custom-designed SUR2 antibody[12] and used in the immunoblotting step. Secondary antibody (1:10,000) was obtained from GE Healthcare. Chemiluminescence was detected using an ECL-Plus Detection Kit (GE Healthcare).

Recording I_{KATP} currents

Pinacidil, diaxozide or glybenclamide (Sigma) was dissolved in 100% DMSO. ATP was made freshly before the experiments. I_{KATP} currents were recorded using inside-out excised patches from GFP-positive cells as previously reported.[40] Cells were placed in a recording chamber mounted on an inverted Nikon microscope. The pipette solution contained (in mM); KCl 140, HEPES 5, EGTA 2, $MgCl_2$ 0.2, pH 7.3 and extracellular bath solution contained (in mM); KCl 10, NaCl 130, HEPES 5, $CaCl_2$ 1, $MgCl_2$ 0.2 pH 7.4. A DAD Superfusion System (Scientific Instruments) was employed to allow changing solutions in less than 1 s. K_{ATP} currents were recorded using an inside-out configuration. Patch pipettes were pulled from borosilicate glass capillary tubing with 1.5 mm outside diameter and 0.86 inside diameter (Warner Instrument). The pipettes were fire polished before use with resistances of 4–6 MΩ when filled with pipette solution. The signal was amplified 50 times and recorded using an AXOPATCH 200B patch-clamp amplifier (Axon Instruments) and filtered through a build-in low-pass filter at 1 kHz. The junction potential was adjusted to zero between pipette and bath solutions immediately before making seal. Leak current was subtracted off-line.

Channel kinetics study

For the ATP-sensitivity tests, ATP was added at 0.1, 0.5, or 1 mM for SUR2A based channels and at 1, 5, or 10 mM. ATP was washed out at the end of the experiments to re-gain the current traces. In the Ca^{2+} block tests, $CaCl_2$ was added at 10, 100, 200, or 500 μM. In the tests where both ATP and $CaCl_2$ were added, $CaCl_2$ was added at 40 μM while ATP was added at 0.01, 0.1, 0.5, or 1 mM to the cells containing SUR2A-55. In the cells expressing SUR2A channels, $CaCl_2$ was added at 40 μM while ATP was added at 0.5, 1, 5, and 10 mM. In the tests of the effects of KATP channel drugs, cells expressing SUR2A-55/Kir6.0 or SUR2A/Kir6.0 channels were treated with 0.5 mM ATP or 0.5 mM ATP plus diaxozide (100 or 200 μM), or ATP plus pinacidil (100 or 200 μM). KCOs were mixed with the amount of ATP required to close at least 50% of the channels. All the drugs including ATP and Ca^{2+} were applied to the channels using a rapid solution changer .

Statistical analysis

The patch data were acquired by digitizing at 5 kHz using a 16-bit analog-to-digital converter and analyzed off-line by the pClamp10.2 software (Axon Instruments). Experiments were conducted at room temperature (22–25 °C). A Student t-test was performed. $p < 0.05$ was considered significant. Data are reported as mean ± SEM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:

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