Assembly Limits the Pharmacological Complexity of ATP-sensitive Potassium Channels*

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ATP-sensitive potassium channels (\(K_{\text{ATP}}\) channels) are formed from an octameric complex of an inwardly rectifying K\(^+\) channel (Kir6.0, Kir6.2) and a sulfonylurea receptor (SUR1, SUR2A, and SUR2B). In this study we have attempted to address the question of whether SUR heteromultimers can form using a combination of biochemical and electrophysiological approaches. We have constructed monoclonal stable lines in HEK293 cells co-expressing Kir6.2 with SUR1 and SUR2A. Using coimmunoprecipitation analysis with SUR isotype-specific antibodies two biochemical populations are distinguished, one containing SUR1 and the other SUR2A. It is not possible to detect immune complexes containing both SUR1 and SUR2A. Functional studies were undertaken and whole cell membrane currents were studied using the patch clamp. Concentrations of sulfonylureas and potassium channel openers were determined that selectively inhibited or activated SUR1/Kir6.2 and SUR2A/Kir6.2. In the cell line expressing SUR1/SUR2A-Kir6.2 we were unable to demonstrate a population of channels with unique pharmacological properties. Thus we conclude from these studies that heteromultimeric channel complexes containing both SUR1 and SUR2A are not formed, suggesting an incompatibility between different SUR subtypes. This incompatibility limits the pharmacological complexity of \(K_{\text{ATP}}\) channels that may be observed in native tissues.

ATP-sensitive potassium channels (\(K_{\text{ATP}}\) channels)\(^1\) are formed from a complex of an inwardly rectifying potassium channel (Kir6.0) and a sulfonylurea receptor (SUR), a member of the ATP-binding cassette superfamily of proteins (1–10). The channel is regulated by intracellular levels of adenine nucleotides, with ATP\(^4-\) inhibiting and MgADP activating channel activity (11–14). \(K_{\text{ATP}}\) channels are thus thought to form a link between the metabolic status of the cell and membrane excitability. Its best characterized physiological role is in the pancreatic \(\beta\)-cell, where \(K_{\text{ATP}}\) channels are important in the regulation of glucose-induced insulin secretion (12, 15). \(K_{\text{ATP}}\) channels also play a role in the regulation of tone in vascular and nonvascular smooth muscle (16) and during ischemia \(K_{\text{ATP}}\) channels in cardiac myocytes, skeletal muscle, and neurons have been proposed to serve a protective function (17, 18). The sulfonylurea class of drugs, which inhibit \(K_{\text{ATP}}\) channel activity, are used in the treatment of Type II diabetes mellitus and these channels are potential therapeutic targets for the treatment of hypertension, asthma, and ischemia reperfusion injury (19).

The channel is an octameric complex of Kir6.0 and sulfonylurea receptor subunits with a 1:1 stoichiometry (20, 21). The Kir6.0 subunits form a tetrameric pore and contain the sites for ATP inhibition (22–25). The sulfonylurea receptor subunits play a role in the modulation of channel activity by MgADP and also confer on the channel responsiveness to a range of pharmacological agents, such as the potassium channel openers diazoxide, pinacidil, and cromakalim and the sulfonylurea class of compounds including glibenclamide and tolbutamide, which inhibit channel activity (1, 4, 6, 22). It has been shown that only fully assembled channel complexes can be expressed at the plasma membrane due to masking of endoplasmic reticulum retention signals exposed in partially assembled complexes (22, 26).

Two Kir6.0 subunits have been cloned to date, Kir6.1 and Kir6.2, which differ in their observed single channel conductances (2, 3, 27). Three major types of sulfonylurea receptor have been cloned, SUR1, SUR2A, and SUR2B (1, 4, 6), although other splice variants also exist (9). The properties of a particular \(K_{\text{ATP}}\) channel are dependent upon its subunit composition (7–10, 28, 29). The co-expression of channel subunits in heterologous systems recapitulates many of the properties of native \(K_{\text{ATP}}\) channels. For example, co-expression of Kir6.2+SUR1, Kir6.2-SUR2A, and Kir6.1-SUR2B produces channels with characteristics similar to \(K_{\text{ATP}}\) channels observed in pancreatic \(\beta\)-cells, cardiac tissue, and smooth muscle, respectively. Pharmacologically, the Kir6.2/SUR1 channel complex is typified by high sensitivity to inhibition by sulfonylureas and activation by diazoxide but is relatively unresponsive to pinacidil or cromakalim (30–33). The Kir6.2/SUR2A channel is less sensitive to inhibition by sulfonylureas and is only weakly responsive to diazoxide but can be activated by pinacidil and cromakalim (30–33). The Kir6.1/SUR2B channel has a similar sensitivity to sulfonylureas as the Kir6.2/SUR2A channel. It is activated by diazoxide, cromakalim, and pinacidil (6, 25).

It is clear that the SUR subunits determine the pharmacological profile of the channel. Given that four SUR subunits are present in the channel complex there is a possibility that more than one type of SUR subunit may be present in some channel complexes. It has been shown that binding of potassium channel openers or sulfonylureas to one SUR subunit of the channel complex is sufficient to induce channel opening or closure (34, 35). Therefore, heteromultimeric channel complexes containing more than one type of SUR subunit would have novel pharmacology, thus contributing to the diversity of ATP-sensitive K\(^+\) channels in cardiac myocytes, skeletal muscle, and neurons.
currents observed in native tissues. SUR1 and SUR2 expression overlaps in several tissues, including rat pituitary gland, ventricular myocytes, some brain regions and some smooth muscles (1, 6, 36–40). In this study we have attempted to address the question of whether SUR heteromultimers can form using a combination of biochemical and electrophysiologcal approaches.

MATERIALS AND METHODS

Production of Triply Transfected HEK293 Monoclonal Stable Lines—HEK293 cells were cultured as previously described (41). A monoclonal stable line was established by antibiotic selection with a reduced concentration of the antibiotic hygromycin B (Invitrogen). Monoclonal stable lines were subsequently established as described previously (41). The presence of SUR1 in the stable line was determined by Western blotting with a SUR1-specific antibody (SUR1NBD1, see later) and electrophysiologcal analysis. We generated a line expressing SUR1/SUR2B/Kir6.2 in an analogous fashion after transfection of SUR1 into a SUR2B/Kir6.2 stable line and triple selection.

Use of Antibodies—Antisera were raised in rabbits to peptides corresponding to sequences in the first nucleotide-binding domains of hamster SUR1 (amino acids 942–955) and rabbit SUR2 (amino acids 651–666). Rabbits were immunized with peptides coupled to keyhole limpet hemocyanin. All immunizations and subsequent bleeds were carried out commercially (Regal Group Limited, Great Bookham, Surrey, UK). The peptides used to raise the SUR1 and SUR2 antibodies had the sequences ETVMERKASEQPSGC and DSYEQARRLPRTEDC, respectively (terminal cysteines added for coupling purposes). Antibodies were affinity purified from the terminal bleeds on a column of the appropriate antigenic peptide coupled to an Affi-Gel 10 gel support (Bio-Rad Laboratories, Hemel Hempstead, UK). The antibody raised to the distal C terminus of Kir6.2 has been described in a previous publication (42).

Immunoprecipitation and Western Blotting—The immunoprecipitation experiments were done as described previously with minor modifications (41, 42). Briefly, cell line homogenates (0.8 mg of total protein) were solubilized in solubilization buffer (1% (v/v) Triton X-100 in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, with protease inhibitors (Complete, Roche Molecular Biochemicals) and sonicated material removed by centrifugation at 20,800 × g for 30 min at 4 °C. Solubilized extracts were subsequently pre-cleared by incubation with 3 mg of Protein A-Sepharose CL-4B (Amersham Bioscience) for 2 h at 4 °C. Immune complexes were allowed to form by addition of 1 μl (0.15 μg of protein) affinity purified antibody followed by incubation for 12–16 h at 4 °C with gentle rotation. The immune complexes were precipitated by addition of Protein A-Sepharose followed by further incubation for 2 h at 4 °C with gentle rotation. The Sepharose was pelleted by centrifugation and washed four times with solubilization buffer followed by a final wash in solubilization buffer without Triton X-100. Immune complexes were eluted with 75 μl of 6 × loading buffer (350 mM Tris-HCl, pH 6.8, 10% (v/v) SDS, 5% (v/v) glycerol, 200 mM dithiothreitol, 0.012% (v/v) bromphenol blue) and denatured (100 °C for 3 min) prior to analysis by SDS-PAGE. SDS-PAGE and Western blotting was done as described previously (41). All antibodies were used to probe Western blots at a 1:2000 dilution.

Immunofluorescence Microscopy—Cells were stained and imaged using a previously published method (42).

Electrophysiology—Whole cell recording were performed using an Axopatch 200B amplifier (Axon Instruments). The current signals were filtered at 1 kHz and digitized at 5 kHz (voltage step) or 2 kHz (voltage ramp) using Digidata 1200 interface and analyzed using pClamp software (v6.0, Axon Instruments). Patch pipettes, made from 1.7-mm outer diameter borosilicate capillaries (Clark Electromedical), were pulled and fire polished using a DMZ-universal puller (Zietz Instruments). The pipettes had resistance of 3–7 MΩ for whole cell recording when filled with the intracellular solution. The capacitance of pipettes was reduced by coating pipettes with a paraffin/mineral oil suspension and compensated for electronically. Series resistance was corrected using amplifier circuitry to at least 75%. A solution containing (in mM), 107 KCl, 1.2 MgCl2, 1 CaCl2, 10 EGTA, 5 HEPES (KOH to pH 7.2–7.4). M K+ was used as the pipette solution and another that contained 140 KCl, 2.6 CaCl2, 1.2 MgCl2, 5 HEPES (pH 7.4) was used as the bath solution. The pipette solution was supplemented with nucleotides as indicated in the figure legends and the pH adjusted to 7.2 again if necessary.

Statistical Analysis—Statistical analysis was carried out using one-way ANOVA and a Bonferroni post-hoc test (Prism v3). Statistical significance is as indicated (*, *p < 0.05; **, *p < 0.01; ***, *p < 0.001). Data are presented as mean ± S.E.

RESULTS

Characterization of SUR Subtype-Specific Antibodies—Antibodies raised to the first nucleotide-binding domains of SUR1 and SUR2 were characterized by Western blotting, immunoprecipitation, and immunofluorescence microscopy. The characterization of the SUR2 antibody (designated SUR2NBD1) by Western blotting is shown in Fig. 1A. Two reactive bands that have estimated molecular masses of between 160,000 and 190,000 Daltons are observed on a Western blot of lysates from stable lines expressing Kir6.2 + SUR2A and Kir6.1 + SUR2B but not from a stable line expressing Kir6.2 + SUR1. The two reactive bands are likely to correspond to differentially glycosylated forms of the receptor (43). Immunoprecipitation experiments done on the Kir6.2 + SUR2A and Kir6.2 + SUR1 stable cell lines showed that the SUR2NBD1 antibody can immunoprecipitate SUR2A but not SUR1 (Fig. 1A, ii + iii). In parallel experiments, the SUR1NBD1 antibody was shown to immunoprecipitate SUR1 but not SUR2A (Fig. 1A, ii + iii). The SUR2NBD1 antibody was also characterized by immunofluorescence microscopy (Fig. 1B). In cells where SUR2B is expressed, detectable fluorescent signal can be observed unlike in non-transfected and SUR1-expressing cells. The characterization of the SUR1NBD1 antibody by Western blotting and immunofluorescence microscopy is detailed elsewhere.3 It shows selectivity for SUR1 over SUR2 in these experimental assays (not shown). The characterization studies demonstrate that the SUR1NBD1 and SUR2NBD1 antibodies are subtype-specific and can be used in biochemical studies examining the potential for SUR subunit heteromultimerization.

Production of a Kir6.2 + SUR2A + SUR1 Monoclonal HEK293 Stable Line—Monoclonal stable lines expressing SUR1 + SUR2A + Kir6.2 and SUR1 + SUR2B + Kir6.2 were established as described under “Materials and Methods.” In the studies reported here we largely use the SUR1 + SUR2A + Kir6.2 line because the Kir6.2 and SUR1 stable lines expressing SUR1 + SUR2A and SUR1 + SUR2B + Kir6.2 are activated by similar concentrations of diazoxide. The presence of each component in the SUR1 + SUR2A + Kir6.2 line was checked by immunofluorescence microscopy as shown in Fig. 1C and essentially every cell in this line expresses all three components. This was also confirmed by electrophysiological studies using selective pharmacological tools (see below).

Immunoprecipitation Experiments—The SUR subtype-specific antibodies were used in an attempt to immunoprecipitate channel complexes containing SUR1 and SUR2A from the Kir6.2 + SUR1 + SUR2A stable line. The results of these experiments are shown in Fig. 2A. SUR1 is immunoprecipitated using the SUR1NBD1 antibody without concomitant immunoprecipitation of SUR2A. In the reciprocal experiment, the SUR2NBD1 antibody immunoprecipitates SUR2A without co-immunoprecipitation of SUR1. The presence of two separate populations of SUR subunits was confirmed by a sequential immunoprecipitation of the supernatants from the SUR1NBD1 and SUR2NBD1 immunoprecipitation experiments with the SUR2NBD1 and SUR1NBD1 antibodies, respectively. The ab-
sence of any observed decrease in signal on the Western blot between the SUR1 immunoprecipitated directly from the stable line homogenate and the SUR1 immunoprecipitated from the supernatant of the SUR2NBD1 immunoprecipitation (compare lanes labeled \textit{IP SUR1NBD1} with \textit{IP SUR2NBD1}). This observation also applies to the SUR2A immunoprecipitates (compare lane \textit{IP SUR1NBD1} with \textit{IP SUR2NBD1}). The immunoprecipitation experiments appear to indicate that SUR1 and SUR2A are not present in the same channel complexes. The diagram in Fig. 2B explains this concept and provides an interpretation of the immunoprecipitation analysis. The same experiments were done using a Kir6.2+SUR1+SUR2B monoclonal stable line and no co-immunoprecipitation of SUR1 and SUR2B was observed (data not shown).

**Functional Studies**—We next designed experiments to test if functional populations with novel pharmacological properties could be detected that would be compatible with the presence of SUR1 and SUR2 in an octameric complex. In particular, we examined SUR1 and SUR2A as they have a distinct selective and separable pharmacology. Our initial experiments determined the exact concentrations at which to use the sulfonylurea tolbutamide and the K channel openers diazoxide and pinacidil in our system. Membrane currents were studied with the whole cell configuration of the patch clamp in a SUR1+Kir6.2 stable line and a SUR2A+Kir6.2 stable line. 100 \(\mu\)M diazoxide but not 10 \(\mu\)M pinacidil activated SUR1+Kir6.2 and currents were inhibited by 10 \(\mu\)M tolbutamide and 10 \(\mu\)M glibenclamide (Fig. 3). In contrast, SUR2A+Kir6.2 currents were activated by 10 \(\mu\)M pinacidil but not 100 \(\mu\)M diazoxide and inhibited by 10 \(\mu\)M glibenclamide but not 10 \(\mu\)M tolbutamide (Fig. 4). Thus it is possible to use pharmacological tools to...
functionally discriminate these populations.

As described above Schwanstecher and colleagues (34, 35) have shown that a single sulfonylurea receptor can confer activation by openers and inhibition by sulfonylureas and related drugs on the channel complex. A mixed octamer of SUR1+SUR2A+Kir6.2 might thus be expected to have mixed pharmacology. Fig. 5 shows experiments measuring whole cell currents in the SUR1+SUR2A+Kir6.2 stable line to test whether such populations exist. 100 μM diazoxide activates significant tolbutamide-sensitive currents (Fig. 5A, i) confirming the presence of SUR1+Kir6.2. The cell is subsequently washed and currents activated by 10 μM pinacidil (Fig. 5A, ii). If functional SUR1+SUR2A+Kir6.2 octamers existed it would be expected that SUR1 in the complex would confer some sensitivity to 10 μM tolbutamide. The data clearly show that this is not the case. Currents are subsequently completely inhibited by 10 μM glibenclamide.

We sought to test this question in another fashion. A further difference between SUR2A+Kir6.2 and SUR1+Kir6.2 is that current inhibition after the application of glibenclamide is reversible in the former but not in the latter. For example, currents can be selectively activated by 10 μM levcromakalim in the SUR2A+Kir6.2 cell line or by 100 μM diazoxide in the SUR1+Kir6.2 cell line and then inhibited by co-application of 10 μM glibenclamide. The cells are washed for a 5-min period and the potassium channel opener reapplied (Fig. 6, A and B). This leads to current activation that is comparable in magnitude with SUR2A+Kir6.2 but no activation in the case of SUR1+Kir6.2. 10 μM levcromakalim is selective for SUR2A+Kir6.2 over SUR1+Kir6.2 in a similar manner to 10 μM pinacidil (not shown). We then examined the behavior of the SUR1+SUR2A+Kir6.2 stable line (Fig. 6, C and D). Prior application of glibenclamide and subsequent washing did not lead to a change in the current elicited by a second application of levcromakalim. This data and that in Fig. 5 suggests that there is not a functionally significant heteromeric population containing SUR1 and SUR2A with Kir6.2.

**DISCUSSION**

The major novel finding presented here is that $K_{\text{ATP}}$ channel complexes containing SUR1 and SUR2 with Kir6.2 cannot be formed even after overexpression in HEK293 cells. This pro-
posal is supported by biochemical and functional data. In particular we have focused on SUR1 and SUR2A as it is possible to perform less ambiguous functional experiments based on selective pharmacology. We have performed analogous biochemical studies with SUR1 and SUR2B with similar results. The latter combination is likely to be more important in native tissues as both SUR1 and SUR2B are more widely distributed than SUR2A (see below). However, SUR2A and SUR2B are C-terminal splice variants and differ by under 40 amino acids. SUR2A and SUR2B do display some pharmacological differences (44). However, given the large part of the protein that is identical between the splice variants our results are likely to be generally applicable to interactions between SUR1 and SUR2. Our previous studies have demonstrated that the pore forming subunits, Kir6.1 and Kir6.2 (\textsubscript{H9251}subunits), are able to readily heteromultimerize (42). In contrast, it seems the \textsubscript{H9252}subunits for Kir6.0, i.e. SUR, do not enter into mixed populations and the latter process will limit the potential pharmacological complexity of \textsubscript{K\textsubscript{ATP}} channels in cells where multiple SUR isoforms are expressed. This has implications in the design of therapeutic agents that are able to specifically target \textsubscript{K\textsubscript{ATP}} channels in different tissues given that channels with intermediate pharmacologies due to SUR heteromultimerization are unlikely to occur \textit{in vivo}. The failure of \beta subunits to heteromultimerize is not a general theme in \textit{K}\textsuperscript{+} channel assembly. For the voltage-gated family of \textit{K}\textsuperscript{+} channel, heteromultimerization of auxiliary or \beta \textit{K}\textsuperscript{+} channel subunits has been shown to have functional importance in refining their modulatory effect. For example, co-assembly of \textit{Kv2} and \textit{Kv1} subunits in a voltage-gated \textit{K}\textsuperscript{+} channel complex modulates the inactivation properties of the channel (45, 46). These data have an important bearing on the likely molecular composition of \textsubscript{K\textsubscript{ATP}} channels in native tissues. It is clear that SURs are widely expressed. For example, SUR2B is present to some level in every tissue examined (6). In some neurons SUR1 and SUR2B are co-expressed and \textsubscript{K\textsubscript{ATP}} channels with intermediate sulfonylurea sensitivity have been observed (36). However, it is clear that the sulfonylurea sensitivity of \textsubscript{K\textsubscript{ATP}} channels is dependent upon several factors other than subunit composition such as cellular stress, adenine nucleotide ratios, cytoskeletal disruption, and the operative condition of the channel (47–51). Our data make one of these explanations more likely. Furthermore, based on antisense experiments, it has also been suggested that a population of heteromeric channels containing SUR1 and SUR2A may exist in neonatal ventricular myocytes (38). However, it is not easy to rule out indirect effects such as a reduction in SUR expression affecting the surface expression of Kir6.2. Our data are supported by other studies, however (35). These authors noted in passing that coexpression of SUR1 and SUR2B did not generate currents with the expected pharmacological properties if the two subunits could freely mix. To study the functional stoichiome-
try of channel activation, they had to use a chimera between SUR1 and SUR2B that was largely formed by SUR1.

What are the potential mechanisms by which these SUR subunits are kept apart? The immunoprecipitation experiments showing a lack of biochemical interaction between SUR1 and SUR2 subunits suggest that it is not related to a trafficking defect whereby channel complexes containing more than one type of SUR subunit are prevented from reaching the plasma membrane. The most likely explanation is that SUR heteromultimers do not form due to an inability of different SUR subtypes to co-assemble and potentially this could be determined by SUR-SUR interactions. If each SUR molecule in the channel complex assembled with the tetramer of Kir6.0 subunits independently, then mixed heteromultimers would be expected. Intermolecular interactions between SUR subunits are not implausible as it has been shown a fusion protein of the first nucleotide-binding domain of SUR1 can form a tetramer pointing to a role of NBD1 in SUR assembly (52). The determinants on SUR molecules responsible for co-assembly and subunit compatibility is still an open question and requires further study. It also interesting that ABC transporters can function as oligomers to provide different pathways for the translocation of substrates across the membrane (53).

An additional source of potential pharmacological complexity of $K_{ATP}$ channels may arise through assembly of the two splice variants of SUR2, SUR2A and SUR2B. There are also splice variants of SUR1 with distinct functional properties (54) and similar considerations apply. We do not possess $K_{ATP}$ channel antibodies that discriminate between SUR2A and SUR2B (and SUR1 splice variants) and as such have not been able to determine whether co-assembly can occur. However, given that SUR2A and SUR2B are identical for all but the last 40 amino acids, it remains a possibility that these two subunits can co-assemble to form a functional channel. The identification of regions responsible for compatibility would also shed light on this problem.
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