The Molecular Assembly of ATP-sensitive Potassium Channels

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ATP-sensitive potassium channels form a link between membrane excitability and cellular metabolism. These channels are important in physiological processes such as insulin release and they are an important site of drug action. They are an octomeric complex comprised of four sulfonylurea receptors, a member of the ATP-binding cassette family of proteins, and four Kir 6.0 subunits from the inward rectifier family of potassium channels. We have investigated the nature of the interaction between SUR1 and Kir 6.2 and the domains on the channel responsible for the biochemical and functional manifestations of coupling. The results point to the proximal C terminus determining biochemical interaction in a region that also largely governs homotypic and heterotypic interaction between different Kir family members. While this domain may be necessary for functional communication between the two proteins, it is not sufficient since relative modifications of either the N or C terminus are able to disrupt many aspects of functional coupling mediated by the sulfonylurea receptor.

EXPERIMENTAL PROCEDURES
Molecular Biology
Standard molecular cloning and mutagenesis techniques were used. For cell transfections all sulfonylurea receptor cDNAs (hamster clone) (SUR2A) and smooth muscle (SUR2B), have now been characterized (3–5). Two members of Kir 6.0 have so far been isolated (Kir 6.1 and 6.2) and topology models predict an intracellular N and C terminus with two transmembrane domains (M1 and M2) and a pore forming hairpin loop (H5). Heterologous expression of the cloned SUR1 and Kir 6.2 in mammalian cells and Xenopus laevis oocytes reconstitutes the properties of the analogous native current seen in pancreatic beta cells (3, 4, 6–11). The channel is thought to be an octomeric complex made up of four SUR1 subunits and four Kir 6.2 subunits (12–14).

Evidence has accumulated indicating that activation by ADP and diazoxide and inhibition by the sulfonylurea class of drug is a property conferred by the sulfonylurea receptor on the channel complex, while the site for ATP inhibition resides on the pore forming Kir 6.2 subunit (9, 15–18). Furthermore, the combined expression of the two proteins is required to generate current. An unresolved and critical question then is how the sulfonylurea receptor “talks” in molecular terms to the ion conducting subunit Kir 6.2. Such information would be invaluable in building a molecular model of how the ATP-sensitive potassium channel works. Recent studies indicate that other ABC transporters are able to interact with other members of the Kir 6.0 family and other Kir family members. A functional interaction between Kir 6.1 and Kir 1.2 and the cystic fibrosis transmembrane regulator has been demonstrated (19–21) and it has been suggested that SUR1 is able to couple with other Kir subfamily members, namely Kir 1.0 and an inward rectifier endogenous to the cell line studied (22). Additionally, cystic fibrosis transmembrane regulator can interact with the amiloride-sensitive Na⁺ channel and an outwardly rectifying Cl⁻ channel (23). The association between ABC transporters and potassium channels may be a general biological motif manifest in prokaryotes and eukaryotes (23, 24). Thus the elucidation of this problem may shed light on the general question of how ABC transporters interact with the ion channels and the mechanisms governing the selectivity of this interaction.

The data in this study establishes a methodology to examine the interaction between SUR1 and Kir 6.2 and how this relates to $K_{ATP}$ channel physiology and pharmacology. We identify a domain in the proximal C terminus on the pore forming subunit that is responsible for biochemical interaction. Moreover functional studies indicate that while this domain may be necessary for the full reconstitution of $I_{KATP}$ drug responsiveness and nucleotide regulation, it is not sufficient. The presence of both regions in the N and distal C terminus is required.
channel chimera cDNAs into pcDNA3.1+Zeo (Invitrogen). Tagged and chimeric proteins were sequenced to confirm their identity. In addition, the sequencing data were complemented by biochemical and functional studies further confirming the intended changes.

During the planning of these experiments a strategy was devised so that a number of different biochemical approaches could possibly be used. As a result, ChmD and Chm E were tagged with six histidines at the N terminus and FLAG at the C terminus and Chm F was tagged six histidines at the C terminus and FLAG at the N terminus. Channel chimaeras A, B, and C were generated later in the study when an antibody to Kir 6.2 had been characterized and a strategy settled on. Thus these chimaeras were not epitope tagged.

Cell Culture

HEK293 cells (a human embryonic kidney cell line) were cultured in minimal essential medium with Earle's Salts, 1-glutamine supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (from a stock of 10,000 units/ml penicillin and 1 mg/ml streptomycin) at 37 °C in humidified 5% CO2. Cells were transfected with LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. Stable cell lines were established using the appropriate antibiotic selection with 1 mg/ml G418 (Life Technologies, Inc.) and 364 μg/ml Zeocin (Invitrogen). For the establishment of polyclonal lines, cells were grown under selective pressure (727 μg of G418) for 3 weeks after transfection and then frozen down in aliquots for subsequent growth and use in binding studies. For the establishment of monoclonal lines, single colonies were picked after growth under selective pressure (727 μg of G418) every 3 weeks after transfection and propagated to establish monoclonal stable cell lines. The presence of the components in the cell line was established by Western blotting and/or patch clamping. A number of different monoclonal cell lines were isolated for Chms A through E + SUR1-Myc. However, only one line of Chm F + SUR1-Myc could be isolated and then only with an N-terminal FLAG-tagged version.

Biochemistry

Antiserum and Antibodies—The Myc antibody was used from a hybridoma cell line (9E10 epitope, a gift from Dr. S. Moss). The Myc antibody was coupled to protein A under high salt condition. Kir 6.2 antiserum was raised in rabbits to peptides linked to keyhole limpet hemocyanin corresponding to sequences in the distal C terminus (peptide sequence RQLDEDRSLLDALTLASSRGPLRKRSC, cysteine added for coupling purposes). Kir 2.1 antiserum (a gift of Dr. LY Jan) was used as an in a previous study5. Immuno precipitation—Stable cell lines were grown to 100% confluence in a 600-cm2 area dish, washed, and harvested in ice-cold Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Approximately 0.8 mg of protein was homogenized using a 1 ml tight-fitting Dounce homogenizer in the presence of protease inhibitors (10 μg/ml benzamidine, 1 μg/ml antipain, 2 μg/ml aprotinin). Approximately 80 μg of protein sample from the homogenate was lysed using an equal volume of 2% SDS in upper gel buffer, sonicated, and denatured (94 °C for 3 min) prior to being analyzed on a denaturing polyacrylamide gel as a check for expression of the appropriate protein(s). The rest of the homogenate was solubilized in 1% Triton X-100 for 2 h at 4 °C before centrifugation to remove the unsolubilized material. Approximately 3 mg of Protein A-Sepharose-4B (Pharmacia) was added to the supernatant and incubated with gentle rotation for 2 h at 4 °C to preclear the sample. A 1:1 suspension of Myc antibody coupled to Protein A-Sepharose in TBS + 1% Triton (10 μl) was subsequently applied to the precleared sample and incubated for at least 8 h at 4 °C with gentle rotation. After incubation with Myc-Sepharose the sample was washed 4 times with 1 ml of TBS + 1% Triton and once in a final wash with 1 ml of TBS. Bound protein was eluted with 100 μl of 2 times loading buffer and denatured (94 °C for 3 min) prior to analysis on a denaturing polyacrylamide gel.

Metal Chelate Affinity Purification—Cells were harvested, homogenized, and solubilized as above. Unsolubilized material was removed by centrifugation and 50 μl of a 50% slurry of Talon resin (CLONTech) added to the supernatant. The Talon-binding resin is a Co2+-charged tetradeutate chelator coupled to Sepharose beads. The sample was exposed to the resin for at least 2 h with gentle rotation at room temperature and subsequently washed 5 times with 1 ml of 20 mM imidazole + 1% Triton in TBS and once in a final wash with 1 ml of 20 mM imidazole in TBS alone. The bound protein was eluted from the resin with 100 μl of 1 M imidazole in TBS, diluted with an equal volume of 2 times gel loading buffer and denatured as described above prior to analysis on a denaturing polyacrylamide gel.

Western Blotting—Gel electrophoresis and transfer to nitrocellulose membranes was carried out using the minigel system (Bio-Rad) according to the manufacturer's instructions. Membranes were probed as appropriate with Myc antibody (9E10) obtained directly from mouse hybridoma cells, Kir6.2 antibody, or Kir2.1 antibody and the corresponding secondary antibodies before bands were visualized using the ECL Western blot analysis system (Amersham Pharmacia Biotech) according to manufacturer's instructions.

Comments on Optimization of the Biochemical Strategy

A number of practical problems were encountered with the implementation of the intended strategy. Initially, attempts were made to work with transiently transfected cells, however, it was difficult to express sufficient protein for good biochemical studies and a stable transfection approach was adopted. At the start of the study the intention was to use a commercially available FLAG antibody to detect interactions with Kir 6.2-FLAG. However, the product appeared to be a thousand-fold less sensitive than indicated on the data sheet as determined from dot blots to control fusion protein. Detection of Kir 6.2 by this method was poor and variable. A specific antisera to Kir 6.2 was thus generated. The coimmunoprecipitation strategy using Myc monoclonal antibody harvested from hybridoma cells cultured directly in the laboratory proved robust. A copurification strategy based on purification of a His6-tagged Kir 6.2 and copurification of SUR1-Myc proved more troublesome. Of Kir 6.2-His6, Kir 6.2-FLAGHis6, and Kir 6.2-myctagHis6 only the latter would purify with metal chelate affinity chromatography. In addition, a nonspecific interaction of SUR1-Myc with the resin was encountered requiring high imidazole wash concentrations to remove. Because of these factors an interaction was detectable using this method but the final signal was weaker.

Radioligand Binding

Cells were grown, washed, harvested, and homogenized in the presence of protease inhibitors (as described previously) in binding buffer (50 mM Tris-HCl, pH 7.4). A small fraction of this crude homogenate was used directly in each binding reaction (approximately 60 μg of protein). Incubations were done in the presence of 3H glibenclamide (NEN Life Science Products Inc.) to assess specific binding. Nonspecific binding was determined by parallel incubations in the presence of 20 μM unlabeled glibenclamide. Binding reactions were incubated for at least 2 h at room temperature before the reaction was stopped by addition of 1 ml of ice-cold binding buffer. Immediately after stopping the reaction bound ligand was separated from unbound ligand by vacuum filtration onto Whatman GF/B filters. Filters were washed 4 times with 2 ml of ice-cold binding buffer before bound radioactivity was determined by liquid scintillation counting using Ultima Gold MV (Packard) and a Beckman LS6000TA liquid scintillation counter. Points were determined in triplicate.

Radioligand equilibrium binding data were fitted using nonlinear regression (GraphPad Inplot 4.03). Ligand depletion was estimated empirically using a centrifugation binding assay (26) at a low concentration point and was generally less than 20%. In this case data were fitted to the curve,

\[ y = \frac{B_{\text{max}}}{K_r + x} \]

where \( K_r \) is the binding affinity, \( B_{\text{max}} \) is the maximal amount bound, \( y \) is the amount of tritiated glibenclamide bound per milligram of total protein, and \( x \) is the concentration of tritiated glibenclamide in the incubation.

Electrophysiology

Whole cell and inside-out patch clamp recordings of HEK293 cells were performed using the Axopatch 200B amplifier (Axon Instruments) with glass pipettes pulled from borosilicate glass. For whole cell recordings pipettes were pulled with resistance of approximately 1.5–2.5 MΩ. For inside-out patch recordings pipettes were pulled with a resistance of approximately 6 MΩ. Pipettes were coated with a paraffin/ mineral oil suspension after filling. Data were acquired and analyzed using pDial and pClamp 5 and the current programs. Data were filtered at 1 kHz and digitized at 5–10 kHz. Cells had a capacitance of approximately 15 pF and series resistance was at least 80% compensated. Solutions contained in mM, 107 KCl, 1.2 MgCl2, 1 CaCl2, 10 EGTA, 5 Hepes (KOH to pH 7.2 – 140 mM K+) used as the pipette solution in whole cell studies and the bath in inside-out studies and another that contained 140 KCl, 2.6 CaCl2, 1.2 MgCl2, 5 Hepes (pH...
7.4) was used as the bath solution in whole cell studies and pipette solution in the inside-out configuration. ATP containing solutions (magnesium salt) were made up daily from frozen aliquots of concentrated (pH adjusted to 7.2) in 20 mM stock solution in the above whole cell/inside-out solution. ADP (sodium salt, note solutions contain 1.2 mM MgCl2) was added from a concentrated stock. Tolbutamate containing solutions were made up from a concentrated stock in dimethyl sulfoxide and the final dimethyl sulfoxide concentration was at most 0.1%. Diazoxide containing solutions were made up from a concentrated stock in dimethyl sulfoxide and the final dimethyl sulfoxide concentration was 0.2%. When experiments were undertaken with transiently transfected cells (a small number of experiments here), 50 ng of an enhanced variant of the green fluorescent protein (EGFPN1, CLONTECH) was co-transfected with the relevant clone. Transfected cells were identified by epifluorescence and patched as above.

Current expression was screened for using the whole cell configuration in a pipette solution containing 0 ATP. In our hands whole cell currents did not run down so that we were able to detect small levels of channel expression. The pipette was supplemented with 0.3, 0.6, and 3 mM ATP depending on the current density and the nature of the experiment being undertaken. For example, with the SUR1 + Kir 6.2 line used in Fig. 1 currents were so large with 0 and 0.3 mM ATP that they were difficult to adequately voltage clamp and therefore 0.6 mM ATP was used. Dose-response curves were constructed with application of a dose of drug with wash off to steady-state at each dose. The application of one dose after another was randomly chosen. Curves were fitted using non-linear regression (GraphPad Inplot v4.03) to

\[ \frac{I}{IC_50} = \left( \frac{b-a}{1 + \left( \frac{x}{EC_{50}} \right)^n} \right) \]

where \( x \) is the concentration of drug or nucleotide, \( I/IC_50 \) is the effect measured relative to control, the \( EC_{50} \) is the point of 50% effect, and the \( EC_{50} \) is the Hill coefficient, \( b \) is the maximum effect (in this study the curves are all inhibitory and \( I/IC_50 \) is measured thus \( b = 1.0 \)), and \( a \) is the minimum effect (generally constrained to 0 but allowed to vary for the Chm A + SUR1-Myc tolbutamide dose-response curve). The data were fitted and displayed with the \( x \) axis log transformed.

In whole cell studies the cell was dialyzed with the relevant ATP concentration until a steady state was reached generally within 5–10 min. Where necessary nonspecific leak was distinguished from K selective currents by the use of 10 mM BaCl2 in the bath to block the currents. Currents were measured 20 ms after the voltage was stepped into the relevant pulse. The situation was more complex with inside-out studies. Chm B + SUR1-Myc ran down very quickly and it was impossible to establish stable current recording conditions in this mode. Therefore, single-channel conductance was measured in the cell-attached configuration. Upon patch excision with the other cell lines the data were analyzed in two ways. In the majority of current containing patches a large number of channels were present and macroscopic currents were recorded. Five current-voltage relationships were averaged and current was measured 100 ms into the step pulse. Sometimes only 5 or less channels were present in the patch. We never observed a case where we were convinced of the presence of a single channel with the current densities achieved in this study. Data were analyzed by determining NPFs from 30-s sweeps of data.

**RESULTS**

**Stable Expression of SUR1 and Kir 6.2**—It is possible to produce monocolonal cell lines stably transfected with SUR1 and Kir 6.2 in HEK293 cells by using a dual selection strategy (see “Experimental Procedures”). Studies using the whole cell configuration of the patch clamp technique revealed the presence of a K+ selective current that was inhibited by tolbutamide and activated by diazoxide. In addition, by excising inside-out patches and studying macroscopic currents in this stable line it is possible to show that these currents were inhibited by ATP and stimulated by MgADP in the bath solution (see below). Thus it is possible to stably express SUR1 and Kir 6.2 in HEK293 cells and functionally recapitulate the properties of the equivalent native channel found in pancreatic beta cells.

**Epitope Tagging SUR1 and Kir 6.2**—In order to explore biochemically the interaction between SUR1 and Kir 6.2, these proteins have been tagged with short amino acid sequences that are recognized by an antibody or by a metal chelate affinity resin. Using such approaches it is possible to use a co-immunoprecipitation or co-purification strategy to explore protein-protein interactions (27, 28). Using standard molecular techniques a series of epitope tags, including six histidines (His6), Myc, and FLAG epitopes, were introduced onto the C terminus of SUR1 (SUR1-Myc, SUR1-MycHis6) and Kir 6.2 (6.2-His6, 6.2-FLAG, 6.2-MycHis6). Kir 2.1 was tagged at the N terminus with six histidines (Kir 2.1-His6) (25).

The functional consequences of such modification of the proteins was first assessed. Radioligand binding using tritiated glibenclamide was performed on homogenates of polyclonal cell lines expressing SUR1, SUR1-Myc, SUR1-MycHis6, and a monoclonal cell line expressing Kir 6.2 and SUR1. The binding parameters (±S.E., \( B_{max} \) in pmol/mg of protein) determined from a number of such experiments were as follows. For SUR1 (\( n = 4 \), \( K_D = 1.30 \pm 0.05 \text{nM}, B_{max} = 14.09 \pm 2.21 \text{pmol/mg protein} \) and \( n_H = 0.97 \pm 0.08 \). For SUR1-Myc (\( n = 4 \), \( K_D = 1.25 \pm 0.17 \text{pmol/mg protein} \), \( B_{max} = 20.43 \pm 2.92 \text{pmol/mg protein} \), and \( n_H = 1.0 \pm 0.13 \). For SUR1-MycHis6 (\( n = 4 \), \( K_D = 1.95 \pm 0.79 \text{nmol/mg protein} \), \( B_{max} = 19.15 \pm 6.33 \text{pmol/mg protein} \), and \( n_H = 0.90 \pm 0.11 \). For Kir 6.2 + SUR (\( n = 5 \), \( K_D = 1.41 \pm 0.35 \text{pmol/mg protein} \), \( B_{max} = 43.1 \pm 9.98 \text{pmol/mg protein} \), and \( n_H = 0.93 \pm 0.13 \). Thus epitope tagging does not affect the ability of the sulfonylurea receptor to bind tritiated glibenclamide with low nanomolar affinity.

A number of monocolonal stable lines were established expressing various combinations of tagged and untagged proteins. The qualitative response of some of these to diazoxide and tolbutamide was tested and the mean data obtained at -100 mV (nA, ±S.E.) were as follows. For SUR1 + Kir 6.2: control = 2.99 ± 0.51 (n = 11), addition of 0.5 mM tolbutamide = 0.53 ± 0.08 (n = 9), and addition of 0.4 mM diazoxide = 6.70 ± 1.53 (n = 5). For SUR1-Myc + 6.2-MycHis6: control = 3.42 ± 0.48 (n = 12), addition of 0.5 mM tolbutamide = 0.71 ± 0.23 (n = 7), and addition of 0.4 mM diazoxide = 6.34 ± 0.77 (n = 6). For SUR1-MycHis6 + 6.2-FLAG: control = 3.8 ± 0.43 (n = 13), addition of 0.5 mM tolbutamide = 0.84 ± 0.16 (n = 8), and addition of 0.4 mM diazoxide = 7.69 ± 1.78 (n = 8). Thus epitope tagging does not dramatically alter the drug responsiveness.

In contrast in a monocolonal line coexpressing SUR1-Myc and Kir 2.1-His6, the strongly rectifying currents of Kir 2.1 were not affected by tolbutamide and diazoxide. The mean data obtained for SUR1-Myc + Kir 2.1-His6 (nA, ±S.E.): control = 8.49 ± 0.76 (n = 17), addition of 0.5 mM tolbutamide = 7.52 ± 0.88 (n = 12), and addition of 0.4 mM diazoxide = 6.93 ± 0.98 (n = 11). Therefore, Kir 2.1-His6 is not functionally modulated by the sulfonylurea receptor.

**A Biochemical Assay for Protein-Protein Interaction**—The biochemical characteristics of these cell lines were investigated and Fig. 1A shows Western blots with the various antibodies and antisera used. The immunoblots show that the antibody and antisera recognize a specific band of the approximately correct molecular weight present in stably transfected but not wild type HEK293 cells for SUR1-Myc, Kir 2.1, and Kir 6.2. SUR1-Myc was generally identified as a doublet of approximately 170 and 140 kDa in size. This is likely to represent differences in post-translational modification especially glycosylation (29). Kir 2.1-His6 was recognized as a single band with a slightly higher molecular weight than might be expected from its primary sequence as noted previously (25). Kir 2.1 was identified as a single band of approximately 44 kDa. Using this antibody occasional background bands were noted.

Fig. 1B shows the results of co-immunoprecipitation experiments with the Myc antibody against SUR1-Myc in the lines SUR1-Myc + Kir 6.2-FLAG and SUR1-Myc + Kir 6.2-His6.
Immunoprecipitation of SUR1-Myc results in the coimmunoprecipitation of 6.2-FLAG and 6.2-His$_6$. This result is likely to be due to a specific protein-protein interaction as Kir 6.2 does not copurify in immune complexes if SUR1 is not tagged with Myc and Kir 2.1-His$_6$ does not co-immunoprecipitate with SUR1-Myc. These experiments were repeated on at least two other occasions with similar results.

Fig. 1C shows the result of a co-purification strategy based on metal chelate affinity chromatography recognizing a hexahistidine tag in the line SUR1-Myc + Kir 6.2-MycHis$_6$. Purification of SUR1-Myc results in the co-purification of 6.2-FLAG and 6.2-His$_6$. The wash conditions used for the purification are adequate as SUR1-Myc does not purify when expressed alone. Purification of Kir 2.1-His$_6$ does not lead to co-purification of SUR1-Myc.
cation of 6.2-MycHis$_6$ leads to the copurification of SUR1-Myc. This result is likely to indicate a specific protein-protein interaction. The wash conditions are adequate because SUR1-Myc does not purify when expressed alone. Additionally, the purification of Kir 2.1-His$_6$ does not lead to the co-purification of SUR1-Myc. These experiments were repeated on at least two other occasions with similar results. Of the two strategies, that based on immunoprecipitation proved the most robust and was used to explore the interaction domain.

Biochemical Analysis of Kir 6.2/Kir 2.1 Chimaeras—In order to explore the domains responsible on the channel for the biochemical interaction with the sulfonylurea receptor, a chimeric analysis was undertaken between a channel that does not interact with SUR1, namely Kir 2.1 and one that does Kir 6.2. Fig. 2A shows the boundaries of the chimaeras constructed between Kir 6.2 and Kir 2.1. Monoclonal stable lines were constructed expressing these together with SUR1-Myc and subjected to a co-immunoprecipitation strategy outlined in Fig. 1B. The antisera to Kir 2.1 and Kir 6.2 both recognize epitopes in the distal C terminus and can be used to identify the chimaeras biochemically (Fig. 2). The chimaeras selected for analysis represent evenly spaced progressive replacement of segments of one channel with the other.

Fig. 2B shows that immunoprecipitation of SUR1-Myc leads to the copurification of Chm A, Chm B, and Chm C. In addition, immunoprecipitation of SUR1-Myc leads to the copurification of Chm D and F but not Chm E (Fig. 2C). Chm D also immunoprecipitated with SUR1-Myc in another cell line in which there were lower expression levels of the channel chimaera (not shown). The experiments were performed at least three times with similar results.

Functional Analysis of Kir 6.2/Kir 2.1 Chimaeras—A fundamentally important question relates to the functional consequences of this interaction: is the presence of the protein-protein interaction sufficient to confer full sensitivity to nucleotides and drugs? Three of the chimaeras led to the expression of current in the presence of SUR1-Myc (Chm A + SUR1-Myc, Chm B + SUR1-Myc, and Chm F + SUR1-Myc) while the other three did not (Fig. 3A). Chm A, Chm B, and Chm F did not express in the absence of SUR1-Myc (not shown, assayed using transient transfection).

Variations in the magnitude of macroscopic currents or single-channel current deflections (NPo) of these chimaeras in the inside-out configuration were measured in response to perfusion with ATP and ADP in the bath (Fig. 3B). The results show that ChmA + SUR1-Myc is ATP-sensitive (EC$_{50}$ equivalent to 555 $\mu$m) while Chm F + SUR1-Myc is ATP insensitive (Fig. 3C). The activity of Chm B + SUR1-Myc declined rapidly upon patch excision and thus ATP sensitivity of the current in the whole cell configuration was examined. With 0.6 mM in the pipette solution a current density at -50 mV of 0.368 nA/pF (n = 17) was measured and with 3 mM ATP in the pipette solution a current density at -50 mV of 0.077 nA/pF (n = 12) was obtained. No significant decrease in the current density was apparent. Thus ATP sensitivity can be profoundly modulated by modification of either the N or the C terminus. The potential for current enhancement by MgADP was determined (Fig. 3, D and E). ChmA + SUR1-Myc shows sensitivity in the presence of 1 mM ATP but not 0.1 mM and Chm F is ADP insensitive.

The drug sensitivity of the chimaeras was next determined.
in the whole cell configuration (Fig. 4). Chm A + SUR1-Myc was modulated by tolbutamide and diazoxide with 3 mM ATP in the pipette solution. Chm B + SUR1-Myc was unresponsive to diazoxide at either ATP concentration while it was partially sensitive to tolbutamide. Chm F + SUR1-Myc was unresponsive to either agent at both ATP concentrations. Dose-response curves for tolbutamide inhibition of ChmA + SUR1-Myc and of ChmB + SUR1-Myc (Fig. 4D) gave an EC50 of 12.5 and 294 mM, respectively, compared with 10.5 μM for Kir 6.2 + SUR1. The tolbutamide concentrations used in this study vary by an order of magnitude than those that lead to channel inhibition by a direct effect on the pore forming subunit (30).

**DISCUSSION**

The major conclusions of this study are that a domain in the proximal C terminus (at least amino acids 208–279) in the inwardly rectifying potassium channel Kir 6.2 appears to largely determine biochemical interaction with the sulfonylurea receptor. However, the presence of this domain is not simply sufficient for the functional communication of signals between the sulfonylurea receptor and Kir 6.2. It seems likely that a largely intact Kir 6.2 N and distal C terminus are necessary for complete functional reconstitution of the ATP-sensitive channel complex.

Fig. 5A shows a cartoon showing assembly of an inward rectifier potassium channel such as Kir 2.1 that does not interact with an ABC transporter. A region in M2 and the proximal C terminus determines homotypic and heterotypic interactions (25) together with a contribution from other regions (31, 32). The model shown is broadly compatible with recent crystallization studies on a bacterial potassium channel (33) and other recent structural studies on the inward rectifiers (34). This channel bore closer homology to the voltage-gated family despite possessing only two transmembrane segments. A truncated version of the channel was analyzed and the structure of the C terminus was not determined. In an analogous fashion it is likely for the inwardly rectifying family of potassium channels, that H5 only partially spans the membrane and that residues in M2 form an inner helix on the inner side of the membrane.

One potential difference may be that regions in the C terminus determine homotypic and heterotypic interactions for voltage-gated family of potassium channels (35). In an analogous fashion it is likely for the inwardly rectifying family of potassium channels, that H5 only partially spans the membrane and that residues in M2 form an inner helix on the inner side of the membrane. One potential difference may be that regions in the proximal C terminus form part of the pore (35).

Fig. 5B shows a preliminary model for how domains in Kir 6.2 interact with the sulfonylurea receptor. The basic proposal is that the sulfonylurea receptor contacts the pore forming subunit (30).
interaction of Kir 6.2 with SUR1 represents a much more extreme example of the regulation of a potassium channel by an auxiliary protein: expression of current is dependent on coexpression of Kir 6.2 with SUR1 and many of the defining characteristics such as drug sensitivity of the current are accounted for by SUR1. Thus the interaction of auxiliary proteins with domains determining homotypic and heterotypic association may be a general theme in potassium channel assembly.

Furthermore, a series of studies have highlighted the importance of residues in M2 (D172 in Kir 2.1) and the proximal C terminus (E224 in Kir 2.1) in determining rectification, permeation, and single channel properties (39–46) and it seems likely that both regions form part of the pore. Based on the recent crystalization studies the hypothesis was advanced that residues located at the end or just after M2 are a gate while ATP binding occurs at other sites in the distal C terminus or by somehow coordinating the N and C termini (49–54). Our data are consistent with the above studies and do support a model in which both the distal C terminus and N terminus are involved. Other modulators of $K_{ATP}$ channel function, namely tolbutamide, diazoxide, and MgADP, appear to exert their effects by interacting with the sulfonylurea receptor. All the chimeras which lead to current expression are able to interact biochemically with SUR1-Myc. Chm A is able to display full functional coupling but higher ATP concentrations are necessary to fully unmask it. However, for Chm B and Chm F the transduction of signals between the two proteins seems to be more profoundly altered. Chm B shows some modulation by tolbutamide but is not influenced by diazoxide. Chm F has a channel with high intrinsic open probability (40, 49, 54) or functional uncoupling seen. For example, the substitution of the Kir 6.2 N and C terminus with that of Kir 2.1 may result in a channel with high intrinsic open probability (40, 49, 54) or has the possibility that a series of weak protein-protein interactions occur between Kir 6.2 and SUR1 and that these have functional importance. Third, the chimeric channels may not be properly folded and a subtle disruption in structure may have profound functional consequences. The data contains the
important message that using functional readouts to solely examine the interaction between these two proteins should be interpreted with care.

In summary, we have established a method for examining the protein-protein interaction between SUR1 and Kir 6.2. We have established that Kir 2.1 does not interact with SUR1 and examined a number of chimaeras between these two channels. The results point to a domain in the proximal C terminus determining biochemical interaction, a domain that also largely determines homotypic and heterotypic association between different Kir family members. While this region may be necessary for functional communication between the two proteins it is not sufficient and full functional reconstitution of \( I_{K_{\text{ATP}}} \) requires that a largely intact N and distal C terminus are present in Kir 6.2.

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