Identification of the Potassium Channel Opener Site on Sulfonylurea Receptors*

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Diversity of sulfonylurea receptor (SUR) subunits underlies tissue specific pharmacology of K<sub>ATP</sub> channels, which represent critical regulators of electrical activity in numerous cells. Notably, the neuronal/pancreatic β-cell receptor, SUR1, imparts high sensitivity to hypoglycemic sulfonylureas (SUs; e.g. glibenclamide) and low to potassium channel openers (KCOs; e.g. P1075), whereas the opposite drug sensitivities are conferred by cardiovascular receptors, SUR2A and SUR2B. By exchanging domains between SUR1 and SUR2B, we identify two regions (KCO I: Thr<sup>1059</sup>–Leu<sup>1067</sup> and KCO II: Arg<sup>1218</sup>–Asn<sup>1230</sup>, rat SUR2 numbering) within the second set of transmembrane domains (TMDII) as critical for KCO binding. Swapping both regions reconstitutes KCO affinities and sensitivities of the donor SUR isofrom. High glibenclamide affinity of SUR1 is not reduced by transfer of KCO I plus II from SUR2B, demonstrating that high SU and KCO affinity can coexist in the same SUR molecule. Consistently, high SU affinity was imparted on SUR2B by substituting the region separating KCO I and II (Ile<sup>1086</sup>–Val<sup>1217</sup>) with the corresponding domain of SUR1. We infer the receptor sites for KCOs and SUs to be closely associated within a regulatory domain (Thr<sup>1059</sup>–Asn<sup>1230</sup>) in TMDII of SURs.

Potassium channel openers (KCOs) comprise a structurally diverse group of drugs with a broad spectrum of potential therapeutic applications (e.g. hypoglycemia, hypertension, arrhythmias, angina pectoris, asthma) (1). These drugs (e.g. P1075, pinacidil, levcromakalim, diazoxide) exert their effects on secretory cells, neurones, vascular and nonvascular smooth muscle, and on cardiac and skeletal muscle by opening ATP-sensitive potassium channels (K<sub>ATP</sub> channels), thus shifting the membrane potential toward the reversal potential for potassium and reducing cellular electrical activity (2).

Recent progress resulted in cloning of K<sub>ATP</sub> channels and elucidation of their subunit composition (see Ref. 3 for a review). These channels are assembled with a tetradimeric stoichiometry, (SUR/Kir<sub>6</sub>)<sub>2</sub>, from two structurally distinct subunits, an inwardly rectifying potassium channel subunit (Kir<sub>6.1</sub> or Kir<sub>6.2</sub>) forming the pore and a regulatory subunit, a sulfonylurea receptor (SUR), belonging to the ATP-binding cassette superfamily with multiple transmembrane domains (TMDs) and two nucleotide binding folds (NBFs) (4–11).

Three isoforms of SUs have been cloned, SUR1 and two splice products of a single gene, SUR2A and SUR2B, differing only in their C-terminal 42–45 amino acids (4, 6, 8, 12). SUR1/Kir<sub>6.2</sub> have been proposed to reconstitute the neuronal/pancreatic β-cell (5), SUR2A/Kir<sub>6.2</sub>, the cardiac (6, 13, 14), and SUR2B/Kir<sub>6.1</sub> (or Kir<sub>6.2</sub>), the vascular smooth muscle-type K<sub>ATP</sub> channels (8, 11, 15, 16).

Notably, diversity of SURs confers tissue-specific pharmacology, with SUR2 isoforms imparting high sensitivity to KCOs and low to sulfonylureas (SUs) and SUR1 mediating inverse sensitivities (5, 6, 8, 16–18). Unraveling the molecular basis for these divergent drug sensitivities and understanding the mechanisms involved in drug-induced modulation of K<sub>ATP</sub> channel activity is of key importance for design of tissue specific compounds.

Here, we report two regions within the second set of transmembrane domain (TMDII) of SUs to be essential for KCO binding and action.

EXPERIMENTAL PROCEDURES

Materials and Solutions—[3H]P1075 (specific activity 116 Ci mmol<sup>−1</sup>) was purchased from Amersham Pharmacia Biotech Freiburg, Germany. [3H]Glibenclamide (specific activity 51 Ci mmol<sup>−1</sup>) was from NEN Life Science Products (Dreieich, Germany). All other chemicals and drugs were obtained from the sources described elsewhere (16, 18–20). Stock solutions of drugs were prepared in KOH (50 mM) or dimethyl sulfoxide with a final solvent concentration in the media below 1%.

Molecular Biology—Chimerae comprising segments from hamster SUR1 (GenBank<sup>TM</sup> accession number AF087838) were constructed using standard molecular biology techniques. Products were subcloned into the pECE vector (4) and sequenced to verify constructs and polymerase chain reaction fidelity before transfection.

Composition of chimeras was as follows (numbers indicate amino acid boundaries of SUR2B or SUR1 as indicated; see also Fig. 1A): chimera I (1–675, SUR2B)–(675–1088, SUR1)–(1088–1545, SUR2B); chimera II (1–1087, SUR2B)–(1121–1256, SUR1)–(1256–1545, SUR2B); chimera III (1–1320, SUR2B)–(1358–1582, SUR1); chimera IV (1–919, SUR2B)–(942–1091, SUR1)–(1095–1545, SUR2B); chimera V (1–868, SUR1)–(676–1545, SUR2B); chimera VI (1–1058, SUR2B)–(1092–1200, SUR1)–(1088–1545, SUR2B); chimera VII (1–1217, SUR2B)–(1251–1357, SUR1)–(1321–1545, SUR2B); chimera VIII (1–1091, SUR1)–(1059–1087, SUR2B)–(1121–1582, SUR1); chimera IX (1–1250, SUR1)–(1218–1320, SUR2B)–(1358–1582, SUR1); and chimera X (1–1091, SUR1)–(1059–1087, SUR2B)–(1121–1250, SUR1)–(1218–1320, SUR2B)–(1358–1582, SUR1).

Binding Experiments—Transfections and membrane preparations were performed as described previously (16, 19). Briefly, COS-7 cells cultured in DMEM-HG (10 mM glucose), supplemented with 10% fetal calf serum, were plated at a density of 5 × 10<sup>5</sup> cells per dish (94 mm) and allowed to attach overnight. 200 μg of pECE-SUR complementary DNA were used to transfect 10 plates. For transfection the cells were incubated 4 h in a Tris-buffered salt solution containing DNA (5–10 μg/ml) plus DEAE-dextran (1 mg/ml), 2 min in HEPES-buffered salt.

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solution plus dimethyl sulfoxide (10%) and 4 h in DMEM-HG plus chloroquine (100 μM). Cells were then returned to DMEM-HG plus 10% fetal calf serum. Membranes were prepared 60–72 h posttransfection as described (19). For binding experiments resuspended membranes (final protein concentration 5–50 μg/ml) were incubated in Tris buffer (50 mM, pH 7.4) containing either [3H]P1075 (final concentration 3 nM, nonspecific binding defined by 100 μM pinacidil) or [3H]glibenclamide (final concentration 0.3 nm, nonspecific binding defined by 100 nm glibenclamide) and other additions as shown in the figure. The free Mg²⁺ concentration was kept close to 0.7 mM. In P1075 assays (Fig. 1, A and B), MgATP (0.1 mM) was added to incubation media to enable KCO binding (16). Low affinity P1075 binding to SUR1 isoform (Fig. 1A) was measured via allosteric displacement of [3H]glibenclamide as described previously (16). Incubations were carried out for 1 h at room temperature and were terminated by rapid filtration through Whatman GF/B filters.

**Electrophysiology**—Transfections were performed as described above with the following modification. COS-7 cells were plated at a density of 8 × 10⁴ cells per dish (35 mm). 20 μg of pECE-SUR complementary DNA and 20 μg of pCEC-mouse K₆.₅.₂ complementary DNA (GenBank™ D50581) were mixed and used to transfect six 35-mm plates. Experiments in the inside-out configuration of the patch-clamp technique were performed 1–2 days after transfection at room temperature as described previously (20). Membrane patches were clamped at −50 nV, bathing solutions (in mM) 140 KCl, 0.7 free Mg²⁺, 10 EGTA, 5 HEPES (pH 7.3) and the pipette solution 146 KCl, 2 CaCl₂, 1.2 MgCl₂, and 10 HEPES (pH 7.4). For registration of concentration-response curves (Fig. 1D) patches were chosen with little “run-down” over the measuring period and drug effects were corrected for this loss of channel activity by use of linear interpolation. Artifacts due to incomplete drug washout or slow reversibility were excluded by making sure that cumulative experiments with stepwise increase or decrease of the drug concentration yielded identical EC₅₀ values and slope factors. Channel activity (A) was defined as the product of the number of functional channels (n) and the probability of the channels being in the open state (p). A was calculated by dividing the mean current (I) by the single-channel current amplitude (i). Density of K₆.₅.₂ channels per patch ranged from 15 to 50. Varying density conditions did not affect EC₅₀ values or Hill coefficients.

**Data—Data analysis (including calculation of KD values from IC₅₀ values), and statistics were performed as described (19, 20). Results shown as mean ± S.E. (n = 3–16).**

**RESULTS**

The pharmacological hallmark of SUR2B is its high affinity for KCOs, the KD for P1075 (rat; 11 ± 2 nM) being approximately 100,000-fold lower than that of SUR1 (hamster; 1.06 ± 0.11 M; see also Ref. 16). Based on this huge affinity difference the KCO receptor site was localized by systematically substituting corresponding domains between both isoforms (Fig. 1A).

Whereas both NBFs (chimera I and III; KD = 13 ± 2 nM or 10 ± 1 nM, respectively) and TMDs 14–15 (chimera II; KD = 14 ± 2 nM) did not contribute to discrepant affinities, small, 3–5-fold reductions of SUR2B's P1075 affinity were paralleled by corresponding sensitivity changes of channels transiently reconstituted from chimera II plus KIR6.2 (EC₅₀ = 0.22 ± 0.09 nm; n = 4; results not shown in a figure) was 190-fold higher than that of SUR2B/KIR6.2 channels (EC₅₀ = 42 nm; Ref. 18) coinciding with the drug's potency to inhibit activity of SUR1/KIR6.2 channels (EC₅₀ = 0.13 nm; Ref. 18).

Expression rates of the chimeras did not differ markedly from that of the wild type receptors, ranging from 10 to 50 pmol/mg of membrane protein as calculated from maximal number of binding sites (chimera I–V and VIII–X) or estimated from reconstituted channel activity (chimera VI and VII).

**DISCUSSION**

This study is the first to localize regions in SURs critical for formation of the KCO binding pocket and to establish that high affinity KCO and SU binding can coexist within the same isoform. These conclusions are based on the following findings.

1) Substitution of two regions within TMDII of SUR2B (KCO I and II, Fig. 1F) with the corresponding domains of SUR1 (chimera VI and VII) induced a complete loss of detectable [3H]P1075 binding (Fig. 1A).
2) Simultaneous transfer of these regions into SUR1 (chimera X) strongly increased KCO affinities (6,200-fold for P1075) reproducing the SUR2B characteristic pattern of high affinity, additional regions might be involved (Fig. 1A).
3) High glibenclamide affinity of SUR1 was not reduced by this transfer (Fig. 1E).
4) Loss or gain of KCO affinity were paralleled by corresponding sensitivity changes of channels reconstituted with K₆.₅.₂ (Fig. 1, C and D).

The regions critical for KCO binding reside in TMDII forming part of the putative intracellular loop connecting TMD 13 and 14 (KCO I: Thr₁₀₅₉–Leu₁₀₈₇; SUR2 numbering; Ref. 18) and the domain preceding NBF2 (KCO II: Arg₁₂₁₈–Asn₁₂₃₀; SUR2 numbering) (Fig. 1F). Either of the two regions proved essential for reconstitution of the SUR2B characteristic pattern of high KCO affinities, strongly arguing that both domains interact in formation of the binding pocket. However, since TMDs 1–11 and 12–13 chimera IV) were required for full KCO affinity, additional regions might be involved (Fig. 1A).

Identification of KCO I in a putative intracellular loop suggests localization of the receptor site at the internal face of the plasma membrane implying that, equivalent to SUs (20), KCOs...
have to cross the membrane to exert their effect. This finding also hints the putative intracellular part of KCO II (Ala1026–Ile1088, SUR2 numbering; Fig. 1, F and G) to act as counterpart of KCO I in formation of the site. Albeit, limiting substitutions to this part of KCO II (plus KCO I) did not lead to reconstitution of high KCO affinity (results not shown).

Importantly, high SU affinity could be conferred on SUR2B by substituting the region separating KCO I and II (SUBR: Ile1088–Val1217, SUR2 numbering; Fig. 1P) by the corresponding domain of SUR1 (chimera II). Since this transfer did perfectly reconstitute affinities of SUR1 for glibenclamide, glipizide, and tolbutamide (Fig. 1E), the results strongly suggest SUBR to form the SU binding site, thus supporting conclusions from a recent study (21).

We infer TMDs 14–17 (Thr1059–Asn1320, SUR2 numbering) within TMDII of SURs to be of key importance for drug-induced KATP channel modulation with the core region of this regulatory domain forming the binding site for SUs (SUBR) and the flanking regions (KCO I and KCO II) constituting main parts of the receptor site for KCOs (Fig. 1F). The idea of distinct (although closely associated) sites is supported by substitution of SUBR lacking an effect on P1075 affinity (chimera II, Fig. 1A)
and transfer of KCO I and II not affecting the $K_D$ for glibenclamide (chimera X, Fig. 1, A and E). Close local association of SU and KCO binding regions, on the other hand, conforms with evidence for negative allosteric coupling of the sites (15, 16, 18, 19, 22–24).

Notably, pharmacological properties of SUR1 and SUR2B were combined in either chimera II and X (Fig. 1, A and E), thus establishing for the first time high affinity for KCOs and SUs not to be mutually exclusive. Hence, native SUR isoforms with similar properties might exist, and accordingly evidence for a receptor with high P1075 and glibenclamide affinity has been presented recently in vascular smooth muscle (25). Both chimeras provide excellent tools for further analysis of functional interaction between the drug sites.

High KCO and SU affinities of chimeras II and X matched sensitivities of channels transiently reconstituted with $K_{in}^{62}$ (see “Results” and Fig. 1, B–E). This finding implies that SUR isoforms use identical mechanisms to transduce drug binding to the regulatory domain (KCO I + SUBR + KCO II; Thr$^{1059}$–Asn$^{1320}$; SUR2 numbering; Fig. 1F) into modulation of channel activity.

It might be argued that the regulatory domain does not form the receptor sites itself but indirectly affects KCO or SU binding in other regions. Although this possibility cannot be ruled out, it is unlikely to explain restitution of the correct rank order of affinities (Fig. 1, B and E).

The study provides new insight into the molecular mechanisms of drug-induced $K_{ATP}$ regulation. We conclude the receptor sites for KCOs and SUs to be closely associated within a regulatory domain in TMDII of SURs.

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