Stoichiometry of Sulfonylurea-Induced ATP-Sensitive Potassium Channel Closure

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ABSTRACT

Hypoglycemic sulfonylureas (e.g., glibenclamide, glipizide, and tolbutamide) exert their stimulatory effect on excitatory cells by closure of ATP-sensitive potassium ($K_{ATP}$) channels. These channels are heteromultimers composed with a 4:4 stoichiometry of an inwardly rectifying $K^+$ channel ($K_{IR}$) subunit 6.x plus a sulfonylurea receptor (SUR). SUR1/KIR6.2 reconstitutes the neuronal/pancreatic $\beta$-cell channel, whereas SUR2A/KIR6.2 and SUR2B/KIR6.1 (or KIR6.2) are proposed to reconstitute the cardiac and the vascular smooth muscle-type $K_{ATP}$ channels, respectively. SUR2A and SUR2B are splice variants of a single gene differing only in their C-terminal 42 amino acids. Affinities of sulfonylureas for rat SUR2A, rat or human SUR2B, and a SUR2 chimera containing the C-terminal 42 amino acids of SUR1 did not differ significantly, implying that the C terminus does not form part of the binding pocket. Consistent with these findings, reconstituted SUR2A/KIR6.2 and SUR2B/KIR6.2 channels revealed similar sensitivities for glibenclamide and tolbutamide. Dissociation constants of sulfonylureas for SUR2A and SUR2B were 10- to 400-fold higher than for SUR1, however, surprisingly the benzoic acid derivative meglitinide did not show lower affinity for SUR2 isoforms. Potencies of glibenclamide, glipizide, tolbutamide, and meglitinide to inhibit activity of SUR1/KIR6.2 and SUR2B/KIR6.2 channels were 3- to 6-fold higher than binding affinities of these drugs with concentration-inhibition relations being significantly steeper (Hill coefficients 1.23–1.32) than binding curves (Hill coefficients 0.93–1.06). The data establish that the C terminus of SURs does not affect sulfonylurea affinity and sensitivity. We conclude that occupation of one of the four SUR sites per channel complex is sufficient to induce $K_{ATP}$ channel closure.

Hypoglycemic sulfonylureas (e.g., glibenclamide, glipizide, and tolbutamide) are widely used in the therapy of noninsulin-dependent diabetes mellitus. These drugs exert their stimulatory effect on insulin secretion by interaction with a high-affinity sulfonylurea receptor in the plasma membrane of pancreatic $\beta$ cells (SUR1). Occupation of this receptor induces closure of the ATP-sensitive potassium ($K_{ATP}$) channel of these cells thereby depolarizing the plasma membrane and initiating the events finally leading to exocytosis of insulin (Ashcroft and Rorsman, 1991; Edwards and Weston, 1993; Aguilar-Bryan et al., 1998). Recent progress resulted in cloning of $K_{ATP}$ channels and elucidation of their subunit composition (Aguilar-Bryan et al., 1995, 1998; Inagaki et al., 1995, 1996; Isomoto et al., 1996; Clement et al., 1997; Yamada et al., 1997). These channels are assembled with a tetradimeric stoichiometry, (SUR/KIR6.x)$4$, from two structurally distinct subunits, the regulatory SUR plus a pore-forming inwardly rectifying $K^+$ channel ($K_{IR}$) subunit 6.1 or 6.2. Three isoforms of SURs have been cloned, SUR1 and two splice products of a single gene, SUR2A and SUR2B, differing only in their C-terminal 42 to 45 amino acids (Isomoto et al., 1996; Chutkow et al., 1996; Aguilar-Bryan et al., 1998). SUR1/KIR6.2 has been proposed to reconstitute the neuronal/pancreatic $\beta$-cell (Inagaki et al., 1995), SUR2A/KIR6.2 the cardiac (Inagaki et al., 1996; Okuyama et al., 1998), and SUR2B/KIR6.1 (or KIR6.2) the vascular smooth muscle-type $K_{ATP}$ channels (Isomoto et al., 1996; Yamada et al., 1997; Schwanstecher et al., 1998).

Still, important questions of sulfonylurea action have not been addressed. SURs have been shown to represent the receptors for potassium channel openers (KCOs) and the C terminus to be critical for binding of these drugs (Schwanstecher et al., 1998). However, the role of the C terminus in sulfonylurea binding and action has yet not been defined. Although it seems clear that sulfonylureas exert their effect by interaction with the SUR subunit, it is unknown how many of the four subunits per complex have to be occupied to induce channel closure.

In this report, we establish that the C terminus of SURs does not affect sulfonylurea affinity and sensitivity. The data indicate that potencies of sulfonylureas to close recombinant

ABBREVIATIONS: $K_{ATP}$, channel, ATP-sensitive potassium channel; SUR, sulfonylurea receptor; $K_{IR}$, inwardly rectifying $K^+$ channel; KCO, potassium channel opener; DMEM, Dulbecco’s modified Eagle’s medium.
curves. SUR isoforms were transiently expressed in COS-7 cells either alone (e.g., ha SUR1) or in combination with KIR6.1 or KIR6.2 (e.g., ha SUR1/KIR6.2). Assays were performed using a membrane fraction of these cells (e.g., ha SUR1) or intact cells (e.g., ha SUR1, cells). “IS” indicates use of intracellular solution instead of Tris-buffer as incubation medium (see Experimental Procedures); ha SUR1 – KIR6.2 is a fusion of hamster SUR1 and rat KIR6.2, ha SUR1/KIR6.2, cells P1075 0.26

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>Glibenclamide</th>
<th>Glipizide</th>
<th>Meglitidine</th>
<th>Tolbutamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ha SUR1</td>
<td>glib.</td>
<td>0.72 ± 0.06 nM (0.94)</td>
<td>17 ± 3 nM (0.94)</td>
<td>6.9 ± 0.6 pM (0.93)</td>
</tr>
<tr>
<td>ha SUR1, IS</td>
<td>glib.</td>
<td>0.85 ± 0.03 nM (0.98)</td>
<td>22 ± 4 nM (0.96)</td>
<td>6.4 ± 0.3 pM (1.02)</td>
</tr>
<tr>
<td>ha SUR1/KIR6.2</td>
<td>glib.</td>
<td>0.78 ± 0.07 nM (1.03)</td>
<td>16 ± 2 nM (0.93)</td>
<td>n.d.</td>
</tr>
<tr>
<td>ha SUR1 – KIR6.2</td>
<td>glib.</td>
<td>0.83 ± 0.07 nM (0.97)</td>
<td>20 ± 3 nM (1.02)</td>
<td>n.d.</td>
</tr>
<tr>
<td>ha SUR1, cells</td>
<td>glib.</td>
<td>0.62 ± 0.04 nM (0.96)</td>
<td>15 ± 5 nM (1.00)</td>
<td>6.3 ± 0.3 pM (0.99)</td>
</tr>
<tr>
<td>ha SUR1/KIR6.2, cells</td>
<td>glib.</td>
<td>0.75 ± 0.08 nM (1.01)</td>
<td>14 ± 2 nM (0.98)</td>
<td>n.d.</td>
</tr>
<tr>
<td>ha SUR1 1540X</td>
<td>glib.</td>
<td>0.65 ± 0.04 nM (0.97)</td>
<td>19 ± 2 nM (0.92)</td>
<td>n.d.</td>
</tr>
<tr>
<td>hu SUR1</td>
<td>glib.</td>
<td>0.55 ± 0.09 nM (0.94)</td>
<td>13 ± 4 nM (0.94)</td>
<td>7.0 ± 1 µM (0.93)</td>
</tr>
<tr>
<td>rat SUR2B</td>
<td>P1075</td>
<td>0.25 ± 0.03 µM (0.09)</td>
<td>6.1 ± 0.3 µM (0.99)</td>
<td>9.2 ± 1.3 µM (0.93)</td>
</tr>
<tr>
<td>rat SUR2B, IS</td>
<td>P1075</td>
<td>0.30 ± 0.06 µM (1.04)</td>
<td>5.6 ± 0.2 µM (0.95)</td>
<td>n.d.</td>
</tr>
<tr>
<td>rat SUR2B/KIR6.2</td>
<td>P1075</td>
<td>0.29 ± 0.05 µM (0.93)</td>
<td>6.3 ± 0.5 µM (0.92)</td>
<td>n.d.</td>
</tr>
<tr>
<td>rat SUR2B, cells</td>
<td>P1075</td>
<td>0.32 ± 0.03 µM (0.97)</td>
<td>7.1 ± 0.6 µM (0.98)</td>
<td>7.8 ± 0.7 µM (1.00)</td>
</tr>
<tr>
<td>rat SUR2B, cells</td>
<td>glib.</td>
<td>0.35 ± 0.09 µM (1.02)</td>
<td>5.8 ± 0.8 µM (1.02)</td>
<td>n.d.</td>
</tr>
<tr>
<td>rat SUR2B/KIR6.1, cells</td>
<td>P1075</td>
<td>0.38 ± 0.07 µM (1.04)</td>
<td>6.4 ± 0.2 µM (0.98)</td>
<td>n.d.</td>
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<tr>
<td>rat SUR2B/KIR6.2, cells</td>
<td>P1075</td>
<td>0.37 ± 0.06 µM (1.01)</td>
<td>5.5 ± 0.4 µM (0.96)</td>
<td>n.d.</td>
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<tr>
<td>hu SUR2B</td>
<td>P1075</td>
<td>0.27 ± 0.04 µM (0.96)</td>
<td>8.0 ± 0.4 µM (0.90)</td>
<td>8.2 ± 0.8 µM (0.94)</td>
</tr>
<tr>
<td>rat SUR2A</td>
<td>P1075</td>
<td>0.29 ± 0.04 µM (1.01)</td>
<td>5.3 ± 0.2 µM (1.00)</td>
<td>6.9 ± 0.2 µM (0.93)</td>
</tr>
<tr>
<td>rat SUR2A, cells</td>
<td>P1075</td>
<td>0.31 ± 0.09 µM (1.02)</td>
<td>7.1 ± 0.5 µM (1.02)</td>
<td>n.d.</td>
</tr>
<tr>
<td>rat SUR2A/KIR6.2, cells</td>
<td>P1075</td>
<td>0.26 ± 0.05 µM (0.97)</td>
<td>5.9 ± 0.3 µM (0.96)</td>
<td>n.d.</td>
</tr>
<tr>
<td>SUR2/c1</td>
<td>P1075</td>
<td>0.23 ± 0.04 µM (0.97)</td>
<td>5.0 ± 0.1 µM (1.03)</td>
<td>7.2 ± 0.4 µM (1.01)</td>
</tr>
</tbody>
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Dissociation constants (Kd;M) for glibenclamide, glipizide, meglitinide, or tolbutamide were assessed by displacement of [3H]Glibenclamide (glib.) or [3H]P1075 ([P1075] = 0.06–0.13 nM) by a range of concentrations of the nine sulfonylureas to enable [3H]P1075 binding (Schwanstecher et al., 1992a, 1994). Membrane patches were left at –50 mV. The intracellular bath solution contained (mM) 140 KCl, 2 CaCl2, 0.7 free Mg2+ concentration was kept close to 0.7 mM. ADP (10 mM) was added to the incubations with SUR1 to warrant identical nucleotide concentrations in binding and patch-clamp experiments (Fig. 1, Tables 1 and 2). ATP (0.1 mM) was added to incubation media for SUR2 isoforms to enable [3H]P1075 binding (Schwanstecher et al., 1998). Incubations were performed at 37 °C and terminated by rapid filtration through Whatman GF/B filters. Membranes from pancreatic islets of obese-hyperglycemic mice were prepared as described (Schwanstecher et al., 1991).

Electrophysiology. Transfections were performed as described above with the following modification. COS cells were plated at a density of 8 × 10^6 cells per dish (35 mm). Twenty micrograms of pECE-hamster SUR1, rat SUR2A, or rat SUR2B complementary DNA and 20 µg of pSV-mouse KIR6.2 complementary DNA were mixed and used to transfect six 35-mm plates. Experiments in the inside-out configuration of the patch-clamp technique were performed at room temperature and were terminated by rapid filtration through Whatman GF/B filters.
Mg⁡²⁺, 10 EGTA, 5 HEPES (pH 7.3) and the pipette solution 146 KCl,
2.6 CaCl₂, 1.2 MgCl₂ and 10 HEPES (pH 7.4). ADP (0.3 mM) en-
hances maximal sulfonylurea-induced inhibition of SUR1/KIR6.2 but
not of SUR2A/KIR6,6.2-channels (Gribble et al., 1998). It was added to

Fig. 1. Binding affinities for SUR1 and potencies of sulfonylureas and
meglitinide to inhibit SUR1/KIR6.2 channels. A, [³H]glibenclamide (0.3
nM) displacement assays were done with membranes from COS-7 cells
expressing wild-type hamster SUR1. All incubations were performed in
Tris-buffer (50 mM, pH 7.4) containing 0.3 mM ADP, 0.7 mM free Mg⁡²⁺,
and displacing drugs as indicated. The IC₅₀ values (half-maximally in-
hibitory concentrations) and Hill coefficients are: 1.02 ± 0.09 nM, 0.94
(glibenclamide, ○); 24 ± 4 nM, 0.94 (glipizide, □); 9.8 ± 1.0 µM, 0.83
(meglitinide, △); 41 ± 2 µM, 0.98 (tolbutamide, □). B, glipizide-induced
inhibition of hamster SUR1/KIR6,6.2 channels transiently expressed in
COS-7 cells. Representative current recorded from an inside-out patch at
−50 mV. Inward currents are shown as downward deflections. Free Mg²⁺
was maintained at 0.7 mM in all solutions. The patch was exposed to
nucleotides and glipizide as indicated by the lines above the record. ADP
was added to enhance maximal drug-induced inhibition (see Experimen-
tal Procedures). C, potencies of sulfonylureas and meglitinide to inhibit
recombinant hamster SUR1/KIR6,6.2 channels. Channel inhibition was
recorded in inside-out patches as shown in part B. Results are expressed
as percentage of channel activity in control solution before and after
application of test substances. The EC₅₀ values (half-maximally effective
concentrations) and Hill coefficients are: 0.13 ± 0.06 nM, 1.23 (gliben-
clamide, ○); 3.8 ± 1.2 nM, 1.26 (glipizide, □); 1.2 ± 0.3 µM, 1.26 (meg-
litinide, △); 4.9 ± 1.6 µM, 1.30 (tolbutamide, □). Results shown are
mean ± S.E.M. (n = 4–8).
the bath solution of SUR1/KIR6.2 registrations to expedite registration of concentration-response curves. For construction of these curves patches were chosen with little “run-down” over the measuring period and drug effects were corrected for this constitutive loss of channel activity by use of linear interpolation. Artifacts due to incomplete drug wash-out 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 channel being in the open state (p). (A) was calculated by dividing the mean current (I) by the single-channel current amplitude (i). Density of KATP channels per patch ranged from 15 to 50. Varying channel densities did not affect EC50 values or Hill coefficients.

**Data.** Data analysis (including calculation of IC50 values) and statistics were performed as described (Schwanstecher et al., 1992a, 1994). Results shown are mean ± S.E.M. (n = 3–16). Theoretical channel activity (A) in the presence of a given concentration of test drug (c) was calculated as follows: (i) (1 − b)4 (one site model) (ii) (1 − b)4 + 4 b (1 − b)2 (two-site model) (iii) 1 − b4 + 4 b3 (1 − b) (three-site model) (iv) 1 − b4 (four-site model) with b = probability of drug binding at c assuming K0 and Hill coefficient = 1 (see legend to Fig. 3D).

**Results**

**SUR1/KIR6.2 Channels.** Competition binding experiments were performed to characterize the properties of the high-affinity sulfonylurea binding site on hamster SUR1. Unlabeled glibenclamide, glipizide, meglitinide, and tolbutamide induced complete monophasic inhibition curves with Hill coefficients close to 1 (0.93–0.98) yielding dissociation of test drug (c) as calculated as follows: (i) (1 − b)4 (one site model) (ii) (1 − b)4 + 4 b (1 − b)2 (two-site model) (iii) 1 − b4 + 4 b3 (1 − b) (three-site model) (iv) 1 − b4 (four-site model) with b = probability of drug binding at c assuming K0 and Hill coefficient = 1 (see legend to Fig. 3D).

![Image](molpharm.aspetjournals.org)
SUR2B ($K_D = 9.2 \, \mu M$; Fig. 2A, Table 1) than SUR1 ($K_D = 6.9 \, \mu M$; Fig. 1A, Table 1).

All drugs rapidly and reversibly inhibited activity of transiently expressed SUR2B/KIR6.2 channels with EC$_{50}$ values (glibenclamide, 42 nM; glipizide, 1.2 $\mu M$; meglitinide, 1.6 $\mu M$; tolbutamide, 88 $\mu M$; Fig. 2, B and C), which, similarly to SUR1/KIR6.2 channels, were significantly lower than $K_D$ values for binding to SUR2B (3–6 fold; Figs. 2, A and C, and 3A). Hill coefficients for the concentration-inhibition curves ranged between 1.26 and 1.32 (Figs. 2C and 3B).

**Role of C Terminus in Sulfonylurea Binding.** To analyze the importance of the SUR C terminus for sulfonylurea binding we assessed the affinities of glibenclamide, glipizide, meglitinide, and tolbutamide for rat SUR2A and a chimera containing the rat SUR2 “backbone” and the 42 C-terminal residues of hamster SUR1 (SUR2/ct1). The dissociation constants of these two isoforms did not differ significantly from those of rat or human SUR2B (Table 1). Consistent with these findings potencies of glibenclamide or tolbutamide to close SUR2A/KIR6.2 channels (EC$_{50} = 45$ nM or 85 $\mu M$, respectively; Fig. 2C) were similar to those observed for SUR2B/KIR6.2 channels (Fig. 2C).

**Discussion**

This study provides a new insight into the mechanism of sulfonylurea-induced closure of KATP channels strongly supporting the idea that interaction with only one of four receptor sites per channel is sufficient for the drugs to exert their effect. This conclusion is based on two findings: 1) potencies of sulfonylureas to inhibit activity of SUR1/KIR6.2, SUR2A/KIR6.2 or SUR2B/KIR6.2 channels were significantly (3.0- to 6.4-fold) higher than binding affinities (Fig. 3A) and 2) for all drugs tested, Hill coefficients for channel inhibition were notably higher than one (1.23–1.35; Fig. 3B).

The leftward shift of potencies versus affinities was neither induced by differences in the composition of the media in binding and patch-clamp experiments nor by loss of associated proteins (e.g., cytoskeletal elements) in the membrane preparation or coexpression with KIR6.2. This was shown by

![Stoichiometry of sulfonylurea action](image)

**Fig. 3.** Stoichiometry of sulfonylurea action. A, potencies of sulfonylureas are higher than their affinities. $K_D$ and EC$_{50}$ values were taken from Fig. 1, 2, and Table 1 (1 = hamster SUR1; 2A = rat SUR2A; 2B = rat SUR2B; Glib = glibenclamide, Glip = glipizide, Meg = meglitinide, Tolb = tolbutamide). The dashed line represents the relation expected for $K_D = EC_{50}$. B, slope factors for drug action are higher than those for drug binding. Hill coefficients for drug binding to SUR isoforms (hamster SUR1; rat SUR2A and 2B) or drug-induced inhibition of channels reconstituted with KIR6.2 were taken from Fig. 1, 2, and Table 1. Results shown are mean ± S.E.M. ($n = 6–18$). *$p < .01$ for comparison with the corresponding Hill coefficient for drug binding. C, tetradimeric architecture of KATP channels (Clement et al., 1997). D, sulfonylurea-induced KATP channel closure is probably due to occupation of a single site. Theoretical concentration-inhibition curves were constructed assuming: 1) noncooperative binding (Hill coefficient = 1) with $K_D = 1$ (the corresponding curve is represented by closed circles, ○, right ordinate) and 2) channel inhibition induced by occupation of one (1), two (2), three (3), or four (4) binding sites per channel (no matter which; see Experimental Procedures). $K_D/EC_{50}$ ratios and Hill coefficients resulting from these models are: 1) $5.75$ and $1.27$, 2) $1.62$ and $1.71$, 3) $0.62$ and $1.71$, and 4) $0.17$ and $1.27$. Arithmetic means of $K_D/EC_{50}$ ratios and Hill coefficients of channel inhibition for the four drugs tested were $5.42 ± 0.33$ and $1.26 ± 0.02$ (hamster SUR1/KIR6.2, Fig. 1, Table 1; the corresponding curve is represented by open circles, ○) or $4.93 ± 0.67$ and $1.29 ± 0.02$ (rat SUR2B/KIR6.2, Fig. 2, Table 2; the corresponding curve is represented by closed squares, □).
controls indicating that substitution of Tris-buffer in intracellular solution, assay of affinities in intact cells, and coexpression with or fusion to KIR subunits do not alter $K_{\text{D}}$ (Table 1). The conclusion that our data obtained in membranes and inside-out patches reflect the properties of the physiologic channel complexes is reinforced by close correlation with results from native tissues (Table 2).

Slope factors (Hill coefficients) for binding of sulfonylureas to SUR1, SUR2A, and SUR2B were entirely close to one, pointing to homogenous populations of noncooperative binding sites (Figs. 1A, 2A, 3B; Tables 1 and 2). Thus, slope factors higher than one in channel-inhibition curves can not be explained by positive cooperativity of drug binding but strongly suggest positive functional interaction of the sites. High concentrations of sulfonylureas have been shown to directly act on a low-affinity site residing on KIR6.2 thereby closing the channel (Gribble et al., 1998). However, significant effects via this site were ruled out by choosing drug concentrations too low to affect $K_{\text{H}}$.6.2 directly.

The most likely explanation for the apparent discrepancy between drug binding and action results from the subunit architecture of KATP channels. These channels require four interacting sites (Fig. 1D; Tables 1 and 2). Thus, slope factors higher than one in channel-inhibition curves can not be explained by positive cooperativity of drug binding but strongly suggest positive functional interaction of the sites. High concentrations of sulfonylureas have been shown to directly act on a low-affinity site residing on KIR6.2 thereby closing the channel (Gribble et al., 1998). However, significant effects via this site were ruled out by choosing drug concentrations too low to affect $K_{\text{H}}$.6.2 directly.

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of K\textsubscript{ATP} channels establishing that the C terminus of SURs does not affect sulfonylurea affinity and sensitivity. We conclude that occupation of one of the four SUR sites per channel complex is sufficient to induce K\textsubscript{ATP} channel closure.

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