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Stoichiometry of Sulfonylurea-Induced ATP-Sensitive Potassium Channel Closure

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MOLECULAR PHARMACOLOGY

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/K_{IR}6.2 reconstitutes the neuronal/pancreatic β-cell channel, whereas SUR2A/K_{IR}6.2 and SUR2B/K_{IR}6.1 (or K_{IR}6.2) 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/K_{IR}6.2 and SUR2B/K_{IR}6.2 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, amazingly 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/K_{IR}6.2 and SUR2B/K_{IR}6.2 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 β 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/K_{IR}6.2 has been proposed to reconstitute the neuronal/pancreatic β-cell (Inagaki et al., 1995), SUR2A/K_{IR}6.2 the cardiac (Inagaki et al., 1996; Okuyama et al., 1998), and SUR2B/K_{IR}6.1 (or K_{IR}6.2) 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

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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.

SUR1/K_{IR}6.2 or SUR2B/K_{IR}6.2 channels are significantly higher than affinities of the drugs. These findings and steeper concentration inhibition curves are explained by a model in which channel closure is induced by occupation of one of the four SUR sites per channel complex.

Experimental Procedures

Materials. [³H]P1075 (specific activity 116 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Freiburg, Germany). [³H]glibenclamide (specific activity 51 Ci/mmol) was obtained from NEN (Dreieich, Germany). All other chemicals and drugs were obtained from the sources described elsewhere (Schwanstecher et al., 1992a, 1994). Stock solutions of all drugs were prepared in KOH (20–50 mM) or dimethyl sulfoxide with a final solvent concentration in the media below 1%.

Molecular Biology. SUR2/ct1 and SUR2/ctB were constructed as described (Schwanstecher et al., 1998), substituting the complementary DNA coding for the C-terminal 42 amino acids of rat SUR2A with the corresponding sequences from hamster SUR1 or human SUR2B. Rat SUR2B was obtained from SUR2/ctB by replacing asparagine in position 1538 against asparatic acid. Hamster SUR1 and rat K_{IR}6.2 were fused tail-head through a six glycine linker (SUR1~K_{IR}6.2; Clement et al., 1997). Hamster SUR1 1540X (Table 1) was constructed by substitution of the cDNA triplet coding for histidine in position 1541 by a stop codon. The resulting products were subcloned into the pECE vector and sequenced to verify chimeric constructs or point mutations and PCR fidelity before transfection.

Binding Assays. Transfections and membrane preparations were performed as described (Schwanstecher et al., 1992a, 1998). Briefly, COS-7 cells cultured in Dulbecco's modified Eagle's medium (DMEM) HG (10 mM glucose), supplemented with 10% fetal calf serum, were plated at a density of 5×10^5 cells per dish (94 mm) and allowed to attach overnight. Two hundred micrograms of pECE-SUR with or without pSV-mouse K_{IR}6.2 or pECE-rat K_{IR}6.1 complementary DNA were used to transfect ten 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 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 and were used 60 to 72 h post-transfection to assay binding or prepare membranes as described (Schwanstecher et al., 1992a). To assay binding to the intact cells, they were washed twice, resuspended (final concentration 2–4 · 10⁵ cells/ml) and incubated in "extracellular solution" (final concentrations in mM: 140 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 5 Tris, pH 7.4) containing [³H]P1075 (final concentration 3 nM, nonspecific binding defined by 100 μM pinacidil) or [³H]glibenclamide (final concentration 3 or 10 nM and nonspecific binding defined by 100 nM or 30 μM glibenclamide for SUR1 or SUR2B, respectively) and displacing drugs as indicated in Table 1. Incubations were carried out for 2 h at room temperature and were terminated by rapid filtration through Whatman GF/B filters. To measure binding to membranes from COS-cells or pancreatic islets the resuspended fraction (final protein concentration 5–50 μg/ml) was incubated in Tris-buffer (50 mM, pH 7.4) containing either [³H]glibenclamide (final concentration 0.3 nM, nonspecific binding defined by 100 nM glibenclamide) or [³H]P1075 (final concentration 3 nM, nonspecific binding defined by 100 μM pinacidil) and other additions as shown in the figures and tables. The free Mg^{2+} concentration was kept close to 0.7 mM. ADP (0.3 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 carried out for 1 h at room temperature and were 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 8×10^4 cells per dish (35 mm). Twenty micrograms of pECE-hamster SUR1, rat SUR2A, or rat SUR2B complementary DNA and 20 μg of pSV-mouse K_{IR}6.2 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 as described previously (Schwanstecher et al., 1994). Membrane patches were clamped at −50 mV. The intracellular bath solution contained (mM) 140 KCl, 2 $CaCl_2$, 0.7 free

TABLE 1
Sulfonylurea affinities of SUR isoforms in membranes or intact cells transiently expressed with or without inward rectifier subunit

	Radioligand	Glibenclamide	Glipizide	Meglitinide	Tolbutamide
ha SUR1	glib.	0.72 ± 0.06 nM (0.94)	17 ± 3 nM (0.94)	6.9 ± 0.6 μM (0.93)	29 ± 2 μM (0.98)
ha SUR1, IS	glib.	0.85 ± 0.03 nM (0.98)	22 ± 4 nM (0.96)	6.4 ± 0.3 μM (1.02)	33 ± 4 μM (0.93)
ha SUR1/K_{IR}6.2	glib.	0.78 ± 0.07 nM (1.03)	16 ± 2 nM (0.93)	n.d.	25 ± 3 μM (1.04)
ha SUR1~K_{IR}6.2	glib.	0.83 ± 0.07 nM (0.97)	20 ± 3 nM (1.02)	n.d.	37 ± 2 μM (0.96)
ha SUR1, cells	glib.	0.62 ± 0.04 nM (0.96)	15 ± 5 nM (1.00)	6.3 ± 0.3 μM (0.99)	26 ± 3 μM (1.05)
ha SUR1/K_{IR}6.2, cells	glib.	0.75 ± 0.08 nM (1.01)	14 ± 2 nM (0.98)	n.d.	31 ± 1 μM (0.93)
ha SUR1 1540X	glib.	0.65 ± 0.04 nM (0.97)	19 ± 2 nM (0.92)	n.d.	27 ± 2 μM (0.94)
hu SUR1	glib.	0.55 ± 0.09 nM (0.94)	13 ± 4 nM (0.94)	7.0 ± 1 μM (0.93)	22 ± 2 μM (0.98)
rat SUR2B	P1075	0.25 ± 0.03 μM (0.99)	6.1 ± 0.3 μM (0.99)	9.2 ± 1.3 μM (0.93)	260 ± 10 μM (1.02)
rat SUR2B, IS	P1075	0.30 ± 0.06 μM (1.04)	5.6 ± 0.2 μM (0.95)	n.d.	240 ± 14 μM (0.94)
rat SUR2B/K_{IR}6.2	P1075	0.29 ± 0.05 μM (0.93)	6.3 ± 0.5 μM (0.92)	n.d.	290 ± 10 μM (0.99)
rat SUR2B, cells	P1075	0.32 ± 0.03 μM (0.97)	7.1 ± 0.6 μM (0.98)	7.8 ± 0.7 μM (1.00)	300 ± 12 μM (1.03)
rat SUR2B, cells	glib.	0.35 ± 0.09 μM (1.02)	5.8 ± 0.8 μM (1.02)	n.d.	280 ± 15 μM (1.00)
rat SUR2B/K_{IR}6.1, cells	P1075	0.38 ± 0.07 μM (1.04)	6.4 ± 0.2 μM (0.98)	n.d.	270 ± 12 μM (0.94)
rat SUR2B/K_{IR}6.2, cells	P1075	0.37 ± 0.06 μM (1.01)	5.5 ± 0.4 μM (0.96)	n.d.	250 ± 10 μM (0.98)
hu SUR2B	P1075	0.27 ± 0.04 μM (0.96)	8.0 ± 0.4 μM (0.90)	8.2 ± 0.8 μM (0.94)	310 ± 18 μM (1.06)
rat SUR2A	P1075	0.29 ± 0.04 μM (1.01)	5.3 ± 0.2 μM (1.00)	6.9 ± 0.2 μM (0.93)	230 ± 16 μM (1.05)
rat SUR2A, cells	P1075	0.31 ± 0.08 μM (1.02)	7.1 ± 0.5 μM (1.04)	n.d.	290 ± 19 μM (1.02)
rat SUR2A/K_{IR}6.2, cells	P1075	0.26 ± 0.05 μM (0.97)	5.9 ± 0.3 μM (0.96)	n.d.	280 ± 14 μM (0.95)
SUR2/ct1	P1075	0.23 ± 0.04 μM (0.96)	5.0 ± 0.1 μM (1.03)	7.2 ± 0.4 μM (1.01)	250 ± 16 μM (1.06)

Dissociation constants (K_Ds) for glibenclamide, glipizide, meglitinide, or tolbutamide were assessed by displacement of [³H]glibenclamide (glib.) or [³H]P1075 (P1075) as detailed in *Experimental Procedures*. Hill coefficients are given in brackets. Results shown as mean (± S.E.M. for K_D values) from three to five independent displacement curves. SUR isoforms were transiently expressed in COS-7 cells either alone (e.g. ha SUR1) or in combination with K_{IR}6.1 or K_{IR}6.2 (e.g., ha SUR1/K_{IR}6.2). 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 K_{IR}6.2, ha SUR1 1540X a mutation lacking the C-terminal 42 aminoacids and SUR2/ct1 a chimera consisting of rat SUR2A with the C terminus of hamster SUR1 (see *Experimental Procedures*); ha = hamster, hu = human, n.d. = not determined.

Mg^{2+}, 10 EGTA, 5 HEPES (pH 7.3) and the pipette solution 146 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$ and 10 HEPES (pH 7.4). ADP (0.3 mM) enhances maximal sulfonylurea-induced inhibition of $SUR1/K_{IR}6.2$ but not of $SUR2A/K_{IR}6.2$-channels (Gribble et al., 1998). It was added to
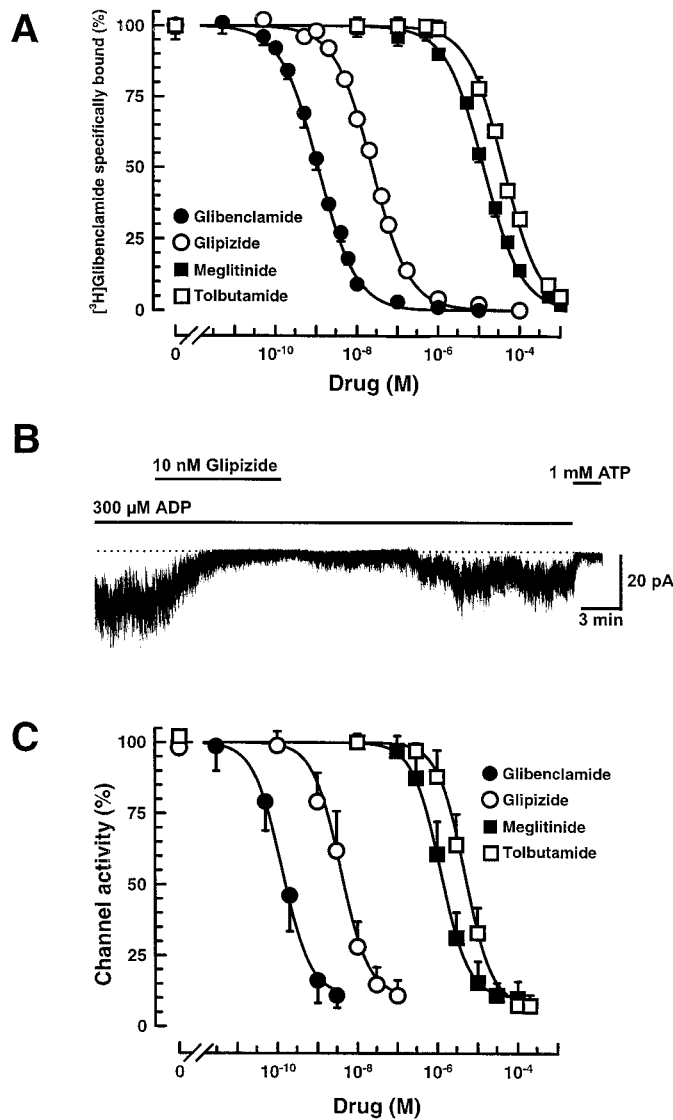

Fig. 1. Binding affinities for SUR1 and potencies of sulfonylureas and meglitinide to inhibit $SUR1/K_{IR}6.2$ channels. A, [^{3}H]glibenclamide (0.3 nM) displacement assays were done with membranes from COS-7 cells expressing wild-type hamster SUR1. All incubations were performed in Tris-buffer (50 mM, pH 7.4) containing 0.3 mM ADP, 0.7 mM free Mg^{2+}, and displacing drugs as indicated. The IC_{50} values (half-maximally inhibitory concentrations) and Hill coefficients are: 1.02 ± 0.09 nM, 0.94 (glibenclamide, ●); 24 ± 4 nM, 0.94 (glipizide, ○); 9.8 ± 1.0 μM, 0.93 (meglitinide, ■); 41 ± 2 μM, 0.98 (tolbutamide, □). B, glipizide-induced inhibition of hamster $SUR1/K_{IR}6.2$ 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 Mg^{2+} 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 *Experimental Procedures*). C, potencies of sulfonylureas and meglitinide to inhibit recombinant hamster $SUR1/K_{IR}6.2$ 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 EC_{50} values (half-maximally effective concentrations) and Hill coefficients are: 0.13 ± 0.06 nM, 1.23 (glibenclamide, ●); 3.8 ± 1.2 nM, 1.26 (glipizide, ○); 1.2 ± 0.3 μM, 1.26 (meglitinide, ■); 4.9 ± 1.6 μM, 1.30 (tolbutamide, □). Results shown are mean $\pm$ S.E.M. ($n = 4$–8).

TABLE 2
Sulfonylurea affinities and potencies in recombinant and native K_{ATP} channels

	Glibenclamide		Glipizide		Meglitinide		Tolbutamide	
	K_D	EC_{50}	K_D	EC_{50}	K_D	EC_{50}	K_D	EC_{50}
ha $SUR1/K_{IR}6.2$	0.72 nM (0.94)	0.13 nM (1.23)	17 nM (0.94)	3.8 nM (1.26)	6.9 μM (0.93)	1.2 μM (1.26)	29 μM (0.98)	4.9 μM (1.30)
pancreatic β cells	1.4 nM (0.96)[a]	0.05 nM (1.51)[b]	21 nM (0.96)	4.0 nM (n.d.)[c]	7.8 μM (0.97)	0.5 μM (1.30)[c]	32 μM (0.93)[d]	2.5 μM (1.53)[e]
rat $SUR2B/K_{IR}6.2$	250 nM (0.96)	42 nM (1.27)	6.1 μM (0.99)	1.2 μM (1.32)	9.2 μM (0.93)	1.6 μM (1.26)	260 μM (1.02)	88 μM (1.29)
aortic rings (rat)	430 nM (1.12)[f]	97 nM (1.20)[f]	6.3 μM (1.00)[f]	1.5 μM (1.25)[f]	n.d.	n.d.	n.d.	n.d.

Dissociation constants (K_Ds) for hamster SUR1 or rat SUR2B and EC_{50} values to inhibit activity of recombinant channels were taken from Figs. 1 and 2. K_Ds for binding of glipizide and meglitinide to pancreatic β cells were assessed in a membrane fraction from mouse pancreatic islets using a [^{3}H]glibenclamide displacement assay (see *Experimental Procedures*). The other data are from: [a]Schwanstecher et al., 1991; [b]Gromada et al., 1995; [c]Zünkler et al., 1988; [d]Schwanstecher et al., 1992b; [e]Gillis et al., 1989; [c]Quast et al., 1993. EC_{50} values to inhibit β cell K_{ATP} channel activity were measured using the cell-attached (e) or the whole-cell configuration (b, c) of the patch-clamp technique. Data for intact aortic rings were assessed using [^{3}H]P1075 displacement and inhibition of ^{86}Rb$^+$ efflux (f). n.d. = not determined.

the bath solution of SUR1/K_{IR}6.2 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 EC_{50} 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 K_{ATP} channels per patch ranged from 15 to 50. Varying channel densities did not affect EC_{50} values or Hill coefficients.

Data. Data analysis (including calculation of K_Ds from IC_{50} 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) $(1 - b)^4 + 4 \cdot b \cdot (1 - b)^3$ (two-site model) (iii) $1 - [b^4 + 4 b^3 \cdot (1 - b)]$ (three-site model) (iv) $1 - b^4$ (four-site model) with b = probability of drug binding at c assuming K_D and Hill coefficient = 1 (see legend to Fig. 3D).

Results

SUR1/K_{IR}6.2 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 constants (K_Ds) of 0.72 nM, 17 nM, 6.9 μM, and 29 μM (Fig. 1A; Table 1).

To assess the functional relevance of the sulfonylurea site on SUR1 the ability of the drugs to close reconstituted SUR1/K_{IR}6.2 channels was tested. All of the drugs strongly inhibited K_{ATP} channel activity in inside-out patches transiently coexpressing SUR1 and K_{IR}6.2 (Fig. 1, B and C). EC_{50} values (glibenclamide, 0.13 nM; glipizide, 3.8 nM; meglitinide, 1.2 μM; tolbutamide, 4.9 μM) were in the same rank order (glibenclamide < glipizide < meglitinide < tolbutamide) but 4.5- to 6-fold lower than K_D values for binding to SUR1 (Figs. 1, A and C, and 3A). Inhibition was reversible for all drugs tested with recovery of channel activity being particularly slow after application of glipizide (Fig. 1B) or glibenclamide. Hill coefficients for drug-induced channel closure were significantly higher than one ranging between 1.23 and 1.30 (Figs. 1C and 3B).

SUR2B/K_{IR}6.2 Channels. The affinity of [³H]glibenclamide for rat SUR2B was too weak to allow direct detection of binding to membrane fractions by use of the filtration assay. Therefore, binding of sulfonylureas and meglitinide to SUR2B was measured indirectly through the allosteric inhibition of high-affinity P1075 binding (Hambrock et al., 1998; Schwanstecher et al., 1998). Similarly to SUR1 all drugs induced complete monophasic displacement with Hill coefficients close to one (0.93–1.02) yielding, however, apparent dissociation constants (glibenclamide, 0.25 μM; glipizide, 6.1 μM; meglitinide, 9.2 μM; tolbutamide, 260 μM; Fig. 2A, Table 1), which were up to 400-fold higher than K_Ds for binding to hamster SUR1. Interestingly, however, meglitinide did not show markedly lower affinity for binding to
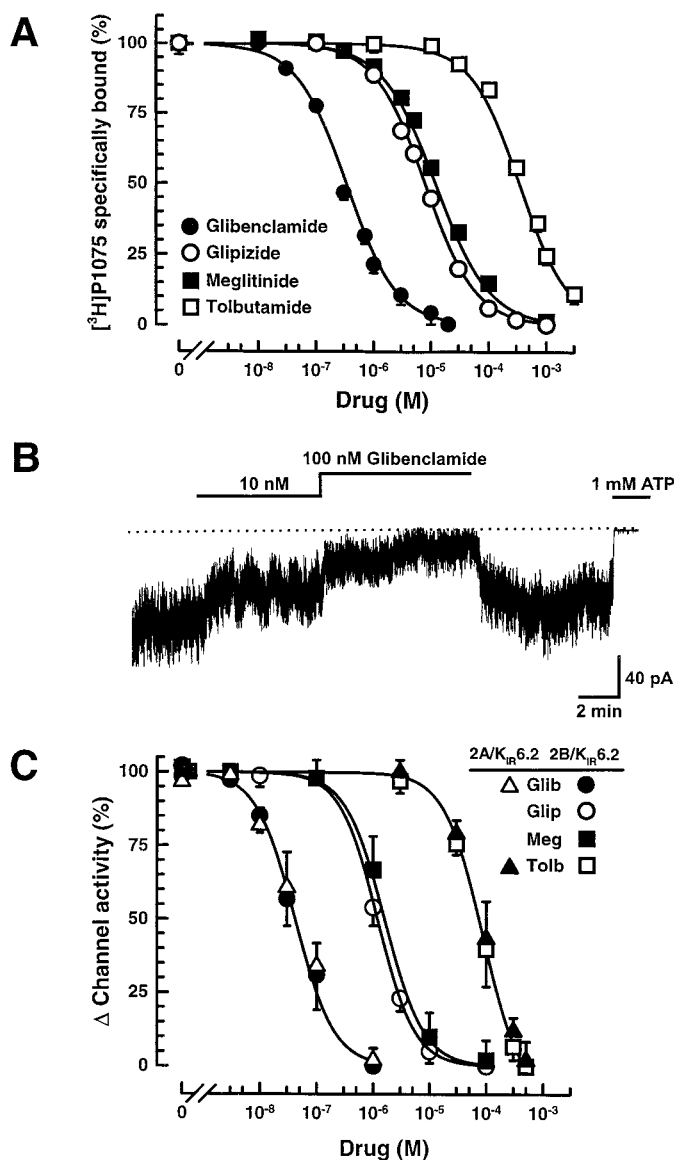

Fig. 2. Binding affinities for SUR2B and potencies of sulfonylureas and meglitinide to inhibit SUR2B/K_{IR}6.2 or SUR2A/K_{IR}6.2 channels. A, [³H]P1075 (3 nM) displacement assays were done with membranes from COS-7 cells expressing wild-type rat SUR2B. All incubations contained 100 μM MgATP (to enable P1075 binding, see *Experimental Procedures*), 0.7 mM free Mg^{2+}, and displacing drugs as indicated. The IC_{50} values (half-maximally inhibitory concentrations) and Hill coefficients are: 0.33 ± 0.04 μM, 0.99 (glibenclamide, ●); 7.9 ± 0.4 μM, 0.99 (glipizide, ○); 12 ± 1.7 μM, 0.93 (meglitinide, ■); 340 ± 20 μM, 1.02 (tolbutamide, □). B, glibenclamide-induced inhibition of rat SUR2B/K_{IR}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 Mg^{2+} was maintained at 0.7 mM in all solutions. The patch was exposed to glibenclamide and ATP as indicated by the lines above the record. C, potencies of sulfonylureas and meglitinide to inhibit recombinant rat SUR2B/K_{IR}6.2 or rat SUR2A/K_{IR}6.2-channels. Channel inhibition was recorded in inside-out patches as shown in part B. Results are expressed as a percentage of maximal drug-induced inhibition of channel activity (60–80% of activity in control solution before and after application of test substances). The EC_{50} values (half-maximally effective concentrations) and Hill coefficients are: 42 ± 14 nM, 1.27 (glibenclamide, SUR2B, ●); 1.2 ± 0.4 μM, 1.32 (glipizide, SUR2B, ○); 1.6 ± 0.7 μM, 1.26 (meglitinide, SUR2B, ■); 88 ± 21 μM, 1.29 (tolbutamide, SUR2B, □); 45 ± 12 nM, 1.35 (glibenclamide, SUR2A, △); 85 ± 18 μM, 1.30 (tolbutamide, SUR2A, ▲). Results shown are mean ± S.E.M. ($n = 3$–8).

SUR2B (K_D = 9.2 μM; Fig. 2A, Table 1) than SUR1 (K_D = 6.9 μM; Fig. 1A, Table 1).

All drugs rapidly and reversibly inhibited activity of transiently expressed SUR2B/K_{IR}6.2 channels with EC_{50} values (glibenclamide, 42 nM; glipizide, 1.2 μM; meglitinide, 1.6 μM; tolbutamide, 88 μM; Fig. 2, B and C), which, similarly to SUR1/K_{IR}6.2 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/K_{IR}6.2 channels (EC_{50} = 45 nM or 85 μM,

respectively; Fig. 2C) were similar to those observed for SUR2B/K_{IR}6.2 channels (Fig. 2C).

Discussion

This study provides a new insight into the mechanism of sulfonylurea-induced closure of K_{ATP} 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/K_{IR}6.2, SUR2A/K_{IR}6.2 or SUR2B/K_{IR}6.2 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 K_{IR}6.2. This was shown by
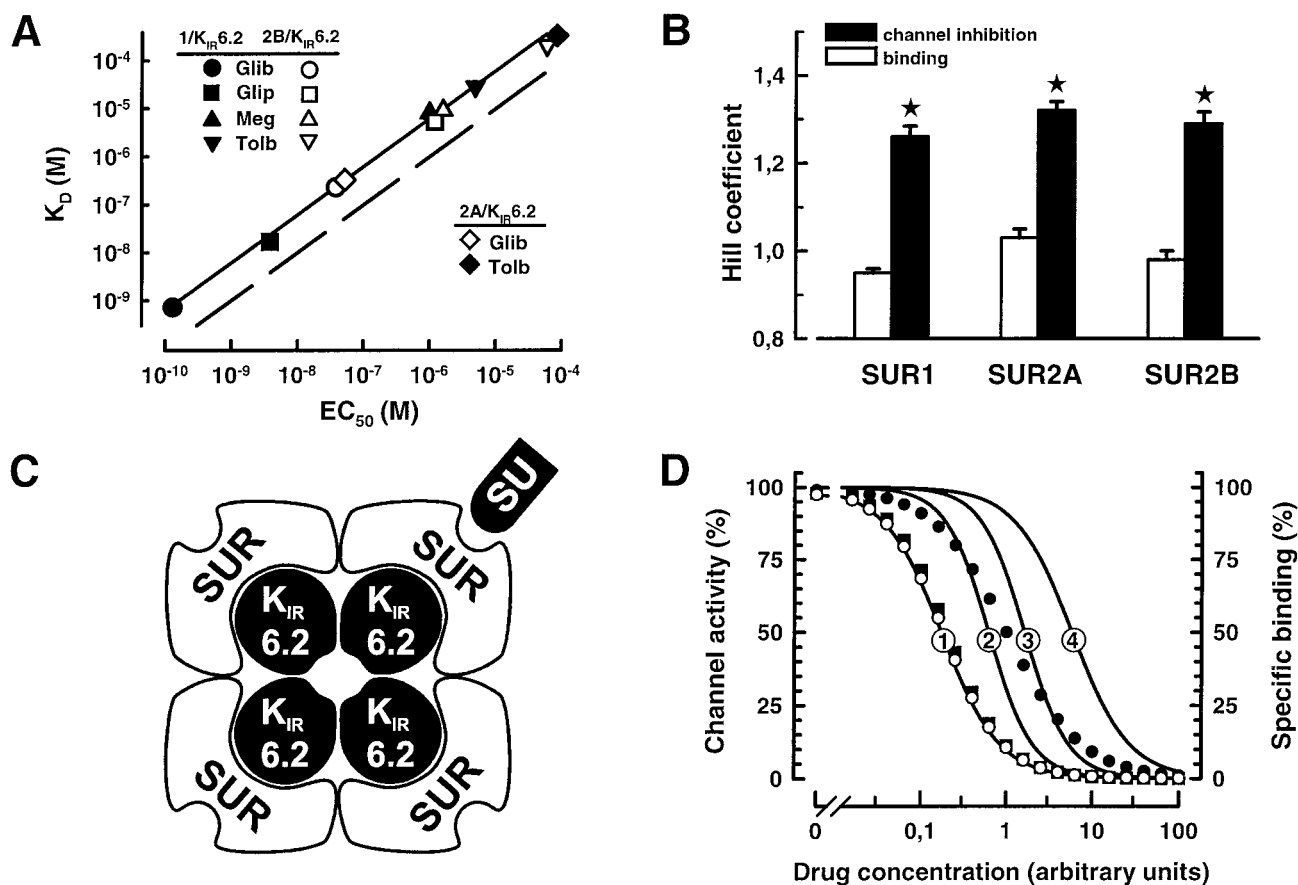

Fig. 3. Stoichiometry of sulfonylurea action. A, potencies of sulfonylureas are higher than their affinities. K_D and EC_{50} values were taken from Fig. 1, 2, and Table 1 (1 = hamster SUR1; 2A = rat SUR2A; 2B = rat SUR2B; Glib = glibenclamide, Glip = glipizide, Meg = meglitinide, Tolb = tolbutamide). 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 B) or drug-induced inhibition of channels reconstituted with K_{IR}6.2 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 K_{ATP} channels (Clement et al., 1997). D, sulfonylurea-induced K_{ATP} 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/K_{IR}6.2, Fig. 1, Table 1; the corresponding curve is represented by open circles, ○) or 4.93 ± 0.67 and 1.29 ± 0.02 (rat SUR2B/K_{IR}6.2, Fig. 2, Table 2; the corresponding curve is represented by closed squares, ■).

controls indicating that substitution of Tris-buffer with intracellular solution, assay of affinities in intact cells, and coexpression with or fusion to K_{IR} subunits do not alter K_Ds (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 $K_{IR}6.2$ 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 $K_{IR}6.2$ directly.

The most likely explanation for the apparent discrepancy between drug binding and action results from the subunit architecture of K_{ATP} channels. These channels require four SUR molecules to be active (Fig. 3C; Clement et al., 1997; Shyng and Nichols, 1997). Although stoichiometry of sulfonylurea binding to SUR1 is not yet clear, the displacement studies suggest one binding site per molecule and accordingly four sites per channel. Any combination of these sites might mediate channel closure, and we have constructed some of the resulting theoretical concentration-inhibition curves (Fig. 3D). One of these models, assuming inhibition from binding to just one and any of the four sites, almost precisely describes our findings (Fig. 3D). EC_{50} values for inhibition of SUR1/$K_{IR}6.2$ or SUR2B/$K_{IR}6.2$ channels were 5.42 ± 0.33- or 4.93 ± 0.67-fold lower than the corresponding dissociation constants with Hill coefficients of 1.26 ± 0.02 or 1.29 ± 0.02, respectively. The one-site model predicts a K_D/EC_{50} ratio of 5.75 and a slope factor of 1.27 (Fig. 3D). When using the inside-out configuration of the patch-clamp technique we expect a reduced concentration of lipophilic drugs at the cytoplasmic side of membrane patches due to diffusion into the pipette solution. Thus EC_{50} values are probably slightly overestimated, which would provide a plausible explanation for the small difference between measured K_D/EC_{50} ratios (5.42 or 4.93, see above) and the theoretical value (5.75). We conclude that binding of sulfonylureas to any of the four sites per channel is sufficient to induce closure of K_{ATP} channels.

K_{ATP} channel activity could not be suppressed completely by sulfonylurea concentrations 10- to 80-fold higher than EC_{50} values (Figs. 1C and 2C). This finding is consistent with previous reports for native pancreatic β-cells and SUR1/$K_{IR}6.2$ or SUR2A/$K_{IR}6.2$ channels (Schwanstecher et al., 1994; Gribble et al., 1997, 1998). The reason for the failure to suppress channel openings entirely is unknown. However, presumably probability that sulfonylurea binding results in channel closure is lower than one. Consistent with that idea, the maximal amount of suppressable channel activity was found to depend on the SUR subtype, being higher for SUR1/$K_{IR}6.2$ (85–95%, Fig. 1C) than SUR2(A or B)/$K_{IR}6.2$ channels (60–80%, Fig. 2C).

K_D values for binding to hamster SUR1 correspond well to those in membranes from mouse pancreatic islets (Table 2) strongly supporting the conclusion that SUR1 represents the

SUR of pancreatic β cells. Affinity of rat SUR2A and SUR2B was too low to allow direct detection of [³H]glibenclamide binding to membranes and thus interaction with SUR2 isoforms was measured indirectly via negative allosteric coupling of the receptor sites for sulfonylureas and KCOs (Hambrock et al., 1998; Schwanstecher et al., 1998). Displacement of low-affinity [³H]glibenclamide binding to intact COS-cells transiently expressing SUR2B yielded K_Ds that did not differ significantly from those obtained by use of the [³H]P1075 assay either in membranes or intact cells (Table 1). These data validate use of allosteric P1075 displacement to measure sulfonylurea affinities of SUR2 isoforms.

[³H]P1075 displacement gave regular curves for all drugs tested with Hill coefficients near one proposing binding to the same noncooperative site (Fig. 2A; Table 1). Sulfonylureas had the same rank order of affinities found for SUR1 (glibenclamide > glipizide > tolbutamide) with, however, significantly higher K_Ds. Identical rank orders (Table 1, Fig. 3A), negative allosteric coupling to the KCO site (SUR1, Schwanstecher et al., 1998; SUR2A, Table 1; SUR2B, Hambrock et al., 1998, Fig. 2A and Table 1), and similar EC_{50}/K_D ratios (Fig. 3A) indicate a high degree of similarity within binding sites, suggesting that small sequence differences might be responsible for either high or low sulfonylurea affinity. Amazingly, the benzoic acid derivative meglitinide did not show markedly lower affinity for SUR2 isoforms and thus this structure could represent a basis for the development of SUR2-specific drugs.

Affinities and potencies were strictly correlated (Fig. 3A) indicating that the sulfonylurea binding sites detected on SUR1 or SUR2B represent the functionally relevant receptor sites. Interestingly, affinities for human SUR1 or SUR2B did not differ significantly from those for the corresponding hamster or rat isoforms (Table 1) supporting the hypothesis that conservation of the receptor sites might be important for regulation by endogenous ligands (Heron et al., 1998).

Affinities of sulfonylureas for SUR2A did not differ significantly from those for SUR2B and a SUR2 construct containing the C terminus of SUR1 (SUR2/ct1; Table 1). We conclude that the C-terminal 42 amino acids are not essential for sulfonylurea binding and thus most probably are not involved in formation of the binding pocket. Consistently, deletion of the C-terminal 42 amino acids (ha SUR1 1540X) does not affect sulfonylurea affinity of hamster SUR1 (Table 1). The data predict identical sulfonylurea sensitivities of SUR2A/$K_{IR}6.2$ and SUR2B/$K_{IR}6.2$ channels and, according to that idea, similar potencies were observed for glibenclamide (42 or 45 nM, respectively; Fig. 2C), meglitinide (1.6 or 0.5 μM; Fig. 2C, Gribble et al., 1998), and tolbutamide (88 or 85 μM; Fig. 2C). The conclusion that the two channel subtypes don't differ in sulfonylurea sensitivity also conforms with published data for native cardiac and vascular K_{ATP} channels (Belles et al., 1987; Venkatesh et al., 1991; Findlay, 1992; Xu and Lee, 1994; Quayle et al., 1995).

Recently, sulfonylurea potencies have been reported that were significantly weaker than sensitivities determined in this study (EC_{50} for inhibition of SUR1/$K_{IR}6.2$ by glibenclamide = 4 nM; EC_{50} for inhibition of SUR2A/$K_{IR}6.2$ by tolbutamide = 1.7 mM; Gribble et al., 1998), suggesting that drug action might be underestimated using the Xenopus expression system.

Our data present new insight into molecular pharmacology

of K_{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_{ATP} channel closure.

References

Aguilar-Bryan L, Clement JP IV, Gonzalez G, Kunjilwar K, Babenko A and Bryan J (1998) Toward understanding the assembly and structure of K_{ATP} channels. *Physiol Rev* **78:**227–245.

Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP IV, Boyd AE 3rd, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J and Nelson DA (1995) Cloning of the beta cell high-affinity sulfonylurea receptor: A regulator of insulin secretion. *Science (Wash DC)* **268:**423–426.

Ashcroft FM and Rorsman P (1991) Electrophysiology of the pancreatic β-cell. *Prog Biophys Mol Biol* **54:**87–143.

Belles B, Hescheler J and Trube G (1987) Changes of membrane currents in cardiac cells induced by long whole-cell recordings and tolbutamide. *Pflügers Arch* **409:**582–588.

Chutkow WA, Simon MC, Le Beau MM and Burant CF (1996) Cloning, tissue expression, and chromosomal localization of SUR2, the putative drug-binding subunit of cardiac, skeletal muscle, and vascular K_{ATP} channels. *Diabetes* **45:**1439–1445.

Clement JP IV, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar-Bryan L and Bryan J (1997) Association and stoichiometry of K_{ATP} channel subunits. *Neuron* **18:**827–838.

Edwards G and Weston AH (1993) The pharmacology of ATP-sensitive potassium channels. *Annu Rev Pharmacol Toxicol* **33:**597–637.

Findlay I (1992) Inhibition of ATP-sensitive K^+ channels in cardiac muscle by the sulphonylurea drug glibenclamide. *J Pharmacol Exp Ther* **261:**540–545.

Gillis KD, Gee WM, Hammoud A, McDaniel ML, Falke LC and Misler S (1989) Effects of sulfonamides on a metabolite-regulated ATP$_i$-sensitive K^+ channel in rat pancreatic B-cells. *Am J Physiol* **257:**C1119–C1127.

Gribble FM, Tucker SJ and Ashcroft FM (1997) The interaction of nucleotides with the tolbutamide block of cloned ATP-sensitive K^+ channel currents expressed in *Xenopus* oocytes: A reinterpretation. *J Physiol* **504:**35–45.

Gribble FM, Tucker SJ, Seino S and Ashcroft FM (1998) Tissue specificity of sulfonylureas: Studies on cloned cardiac and beta-cell K(ATP) channels. *Diabetes* **47:**1412–1418.

Gromada J, Dissing S, Kofod H and Frøkjaer-Jensen J (1995) Effects of the hypoglycemic drugs repaglinide and glibenclamide on ATP-sensitive potassium channels and cytosolic calcium levels in β TC3 cells and rat pancreatic beta cells. *Diabetologia* **38:**1025–1032.

Hambrock A, Löffler-Walz C, Kurachi Y and Quast U (1998) Mg^{2+} and ATP dependence of K(ATP) channel modulator binding to the recombinant sulphonylurea receptor, SUR2B. *Br J Pharmacol* **125:**577–583.

Heron L, Virsolvy A, Peyrollier K, Gribble FM, Le Cam A, Ashcroft FM and Bataille

D (1998) Human alpha-endosulfine, a possible regulator of sulfonylurea-sensitive K_{ATP} channel: Molecular cloning, expression and biological properties. *Proc Natl Acad Sci USA* **95:**8387–8391.

Inagaki N, Gonoi T, Clement JP IV, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S and Bryan J (1995) Reconstitution of IK_{ATP}: An inward rectifier subunit plus the sulfonylurea receptor. *Science (Wash DC)* **270:**1166–1170.

Inagaki N, Gonoi T, Clement JP IV, Wang CZ, Aguilar-Bryan L, Bryan J and Seino S (1996) A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. *Neuron* **16:**1011–1017.

Isomoto S, Kondo C, Yamada M, Matsumoto S, Higashiguchi O, Horio Y, Matsuzawa Y and Kurachi Y (1996) A novel sulfonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive K^+ channel. *J Biol Chem* **271:**24321–24324.

Okuyama Y, Yamada M, Kondo C, Satoh E, Isomoto S, Shindo T, Horio Y, Kitakaze M, Hori M and Kurachi Y (1998) The effects of nucleotides and potassium channel openers on the SUR2A/Kir6.2 complex K^+ channel expressed in a mammalian cell line, HEK293T cells. *Pflügers Arch* **435:**595–603.

Quast U, Bray KM, Andres H, Manley PW, Baumlin Y and Dosogne J (1993) Binding of the K^+ channel opener [^{3}H]P1075 in rat isolated aorta: Relationship to functional effects of openers and blockers. *Mol Pharmacol* **43:**474–481.

Quayle JM, Bonev AD, Brayden JE and Nelson MT (1995) Pharmacology of ATP-sensitive K^+ currents in smooth muscle cells from rabbit mesenteric artery. *Am J Physiol* **269:**C1112–C1118.

Schwanstecher M, Brandt C, Behrends S, Schaupp U and Panten U (1992a) Effect of MgATP on pinacidil-induced displacement of glibenclamide from the sulphonylurea receptor in a pancreatic β-cell line and rat cerebral cortex. *Br J Pharmacol* **106:**295–301.

Schwanstecher M, Löser S, Brandt C, Scheffer K, Rosenberger F and Panten U (1992b) Adenine nucleotide-induced inhibition of sulphonylureas to their receptor in pancreatic islets. *Br J Pharmacol* **105:**531–534.

Schwanstecher M, Löser S, Rietze I and Panten U (1991) Phosphate and thiophosphate group donating adenine and guanine nucleotides inhibit glibenclamide binding to membranes from pancreatic islets. *Naunyn-Schmiedeberg's Arch Pharmacol* **343:**83–89.

Schwanstecher M, Schwanstecher C, Dickel C, Chudziak F, Moshiri A and Panten U (1994) Location of the sulphonylurea receptor at the cytoplasmic face of the β-cell membrane. *Br J Pharmacol* **113:**903–911.

Schwanstecher M, Sieverding C, Dörschner H, Gross I, Aguilar-Bryan L, Schwanstecher C and Bryan J (1998) Potassium channel openers require ATP to bind to and act through sulfonylurea receptors. *EMBO J* **17:**5529–5535.

Shyng S and Nichols CG (1997) Octameric stoichiometry of the K_{ATP} channel complex. *J Gen Physiol* **110:**655–664.

Venkatesh N, Lamp ST and Weiss JN (1991) Sulfonylureas, ATP-sensitive K^+ channels, and cellular K^+ loss during hypoxia, ischemia, and metabolic inhibition in mammalian ventricle. *Circ Res* **69:**623–637.

Xu X and Lee KS (1994) Characterization of the ATP-inhibited K^+ current in canine coronary smooth muscle cells. *Pflügers Arch* **427:**110–120.

Yamada M, Isomoto S, Matsumoto S, Kondo C, Shindo T, Horio Y and Kurachi Y (1997) Sulphonylurea receptor 2B and Kir6.1 form a sulphonylurea-sensitive but ATP-insensitive K^+ channel. *J Physiol* **499:**715–720.

Zünkler BJ, Lenzen S, Männer K, Panten U and Trube G (1988) Concentration-dependent effects of tolbutamide, meglitinide, glypizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic B-cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **337:**225–230.

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