

Characterization of a Mutant Sulfonylurea Receptor SUR2B with High Affinity for Sulfonylureas and Openers: Differences in the Coupling to Kir6.x Subtypes

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ABSTRACT

ATP-dependent K^+ channels are composed of pore-forming subunits of the Kir6.x family and of sulfonylurea receptors (SURs). SUR1, expressed in pancreatic β -cells, has a higher affinity for sulfonylureas, such as glibenclamide, than SUR2B, expressed in smooth muscle. This difference is mainly caused by serine 1237 in SUR1 corresponding to tyrosine 1206 in SUR2B. To increase the affinity of SUR2B for glibenclamide, the mutant SUR2B(Y1206S) was constructed. In whole-cell patch-clamp experiments, glibenclamide inhibited the channel formed by coexpression of mutant SUR2B with Kir6.1 or 6.2 in human embryonic kidney cells with IC_{50} values of 2.7 and 13 nM, respectively (wild-type, 43 and 167 nM). In intact cells, [3H]glibenclamide bound to mutant SUR2B with a K_D value of 4.7 nM (wild-type, 32 nM); coexpression with Kir6.1 or 6.2

increased affinity by 4- and 8-fold, respectively. Binding of the opener [3H]P1075 to SUR2B(Y1206S) was the same as to wild-type and was unaffected by coexpression. In cells, the ratio of glibenclamide:P1075 sites was $\approx 1:1$; in membranes, it varied with the MgATP concentration. Heterologous competition curves were generally biphasic; the shape of the curve depended on the Kir-subtype. The effects of coexpression were weakened or abolished when binding assays were conducted in membranes. It is concluded that the mutation Y1206S increases the affinity of SUR2B for and the channel sensitivity toward glibenclamide by 7- to 15-fold. The interaction of glibenclamide (but not opener) with mutant SUR2B is modified by coexpression with Kir6.x in a manner depending on the Kir subtype and on the integrity of the cell.

ATP-dependent K^+ channels (K_{ATP} channels) are a class of K^+ channels that are gated by the intracellular ATP/ADP ratio; functionally, these channels couple the metabolic state of the cell to membrane potential and excitability (Ashcroft and Ashcroft, 1990; Edwards and Weston, 1993). Pharmacologically, K_{ATP} channels are closed by the hypoglycemic sulfonylureas (SUs) used in the treatment of diabetes type II; they are activated by the K^+ channel openers, a chemically heterogeneous group of compounds that induce hypotension (Ashcroft and Ashcroft, 1990; Edwards and Weston, 1993).

K_{ATP} channels are heteromeric complexes composed of pore-forming α -subunits belonging to the family of inwardly rectifying K^+ channels (Kir6.x) and sulfonylurea receptors (SURs) as β -subunits (Inagaki et al., 1995; Sakura et al., 1995; for reviews, see Ashcroft and Gribble, 1998; Aguilar-Bryan and Bryan, 1999). SUR is a member of the ATP-binding cassette protein superfamily (Aguilar-Bryan et al.,

1995; Sakura et al., 1995). It carries binding sites for nucleotides (Ueda et al., 1999), sulfonylureas, and openers (Aguilar-Bryan et al., 1995; Hambrock et al., 1998; Schwanstecher et al., 1998). The K_{ATP} channels in different tissues combine different subtypes of SUR and Kir6.x. The SUR in the pancreatic β -cell (SUR1) exhibits high affinity for the SUs and a low affinity for the openers, whereas the SUR found in the various muscle types (SUR2) shows high affinity for the openers but lower affinity for the SUs (Hambrock et al., 1998; Schwanstecher et al., 1998; Dörschner et al., 1999; Hambrock et al., 1999; Russ et al., 1999). Two isoforms of SUR2 have been characterized, SUR2A and SUR2B, that differ only in the last carboxyl-terminal exon. SUR2A is expressed in skeletal and heart muscle; SUR2B in smooth muscle (Inagaki et al., 1996; Isomoto et al., 1996). SUR2B combines with Kir6.1 to form the K_{ATP} channel in vascular smooth muscle and with Kir6.2 in nonvascular smooth muscle (Isomoto et al., 1996; Yamada et al., 1997). Kir6.2 carries a high-affinity site for ATP that mediates channel block by micromolar concentrations of ATP (Tucker et al., 1997) whereas Kir6.1-contains

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ABBREVIATIONS: K_{ATP} channel, ATP-sensitive K^+ channel; SU, sulfonylurea; SUR, sulfonylurea receptor; P1075, *N*-cyano-*N'*-(1,1-dimethylpropyl)-*N'*-3-pyridylguanidine; Kir, inwardly rectifying K^+ channel; HEK, human embryonic kidney; GBC, glibenclamide; B_{NS} , nonspecific binding; B_{TOT} , total binding; B_{max} , maximum concentration of binding sites; B_S , specific binding; AZ-DF 265, 4-[[*N*-(α -phenyl-2-piperidino-benzyl)carbamoyl]methyl]benzoic acid; PIP₂, phosphatidylinositol-4,5-bisphosphate.

ing K_{ATP} channels are inhibited by ATP only in the millimolar concentration range (Yamada et al., 1997).

Essential parts of the binding sites for SUs and openers are located on the third domain of SUR (Ashfield et al., 1999; Uhde et al., 1999; Babenko et al., 2000). Uhde et al. (1999) have shown that the binding site for the opener *N*-cyano-*N'*-(1,1-dimethylpropyl)-*N''*-3-pyridylguanidine (P1075) consists of two stretches of amino acids directly flanking the binding site for glibenclamide. Ashfield et al. (1999), comparing the SU binding domains of SUR1 and SUR2A, have identified a critical amino acid in the intracellular loop connecting transmembrane segments 15 and 16 that is important in determining SU-sensitivity: if serine 1237 in rat SUR1 is replaced by tyrosine, which is the corresponding residue in SUR2, the high affinity for tolbutamide and glibenclamide is lost. Conversely, one might expect that replacement of Tyr 1206 in SUR2 (mouse numbering) by Ser would increase the SU affinity of SUR2. Indeed, in a preliminary communication, it has been reported that rat SUR2B(Y1205S) has a 25-fold higher affinity for glibenclamide than for wild-type SUR2B (Toman et al., 2000).

The new mutant is useful because it exhibits high affinity for both openers and glibenclamide. This allows [3 H]glibenclamide and [3 H]P1075 binding to be performed with the same SUR and thereby a more precise analysis of the relationship between sulfonylurea and opener binding than previously possible. Using wild-type SUR2B, [3 H]glibenclamide binding studies in cells were difficult to interpret because of the high level of intrinsic (non-SUR2B) glibenclamide binding (Russ et al., 1999); in membranes, they were impossible to quantify (Dörschner et al., 1999; Russ et al., 1999). There is agreement that sulfonylurea and opener binding is mutually exclusive by a negative allosteric coupling of the two binding sites (Bray and Quast, 1992; Schwanstecher et al., 1992); however, details of this interaction remain unknown. In addition, several aspects of the interaction of glibenclamide with SUR2, such as the equilibrium dissociation constant of the radioligand (K_D) are still in doubt and there is disagreement whether or not the affinity of glibenclamide binding to SUR can be determined quantitatively by inhibition of opener binding (Dörschner et al., 1999; Russ et al., 1999). We have made use of the SUR2B(Y1206S) mutant to clarify these questions. In addition, we compare here for the first time the differential effect of coexpression of (mutant) SUR with Kir6.1 and Kir6.2 on the interaction with glibenclamide and P1075.

Experimental Procedures

Cell Culture and Transfections. Human embryonic kidney (HEK) 293 cells were cultured as described previously (Hambrock et al., 1998, 1999) in minimum essential medium containing glutamine, supplemented with 10% fetal bovine serum and 20 μ g/ml gentamycin. Cells were transfected with mammalian expression vector pcDNA3.1 (Invitrogen, Karlsruhe, Germany) containing the coding sequence of murine SUR2B (GenBank accession number D86038; Isomoto et al., 1996), mutant SUR2B (see below), murine Kir6.1 (D88159; Yamada et al., 1997) or Kir6.2 (D50581; Inagaki et al., 1996). As a control they were transfected with pcDNA3.1 vector alone. Cotransfection of SUR2B (wild-type or mutant) with Kir6.1 or Kir6.2 was done transiently at a molar plasmid ratio of 1:1, if not stated otherwise, and using LipofectAMINE and OptiMEM (Invitrogen) as described previously (Hambrock et al., 1998). In cotransfec-

tions used for electrophysiological experiments, the pEGFP-C1 vector (CLONTECH, Palo Alto, CA), encoding for green fluorescent protein, was added for easy identification of transfected cells. Two to 4 days after transfection, cells were used for binding studies and electrophysiological experiments. Cells stably transfected with wild-type or mutant SUR2B were isolated in the presence of 700 μ g of geneticin/ml of medium within the first 3 weeks and 300 μ g of geneticin/ml of medium thereafter; 1 week before experiments, the antibiotic was withdrawn.

Site-Directed Mutagenesis. The mutant SUR2B(Y1206S) was constructed using the QuikChange Site-Directed Mutagenesis System (Stratagene, Amsterdam, The Netherlands). Murine SUR2B-cDNA, inserted into pcDNA3.1, was used as the template. Two completely complementary primers (31-mer oligonucleotides) were designed, containing the desired mutation (TAC to TCC) in the middle region. For each reaction, 125 ng of forward and reverse mutagenic primers was combined with 100 ng of wild-type murine SUR2B-cDNA in pcDNA3.1 and 2.5 U of *Pfu Turbo* DNA polymerase in a 18 cycle PCR reaction (denaturation, 30 s at 95°C; annealing, 60 s at 55°C; extension, 29 min at 68°C). Parental methylated DNA was destroyed by digestion with *DpnI* and the newly synthesized DNA was transformed into Max Efficiency DH5 α competent cells (Invitrogen). The presence of the desired mutation was confirmed by nucleotide sequencing of the relevant DNA region.

Patch-Clamp Experiments. The patch-clamp technique was used in the whole-cell configuration as described in detail in Russ et al. (1999). Bath solution was 142 mM NaCl, 2.8 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 11 mM D-(+)-glucose, and 10 mM HEPES, titrated to pH 7.4 with NaOH at 37°C. Patch pipettes were filled with 132 mM K-glutamate, 10 mM NaCl, 2 mM MgCl₂, 10 mM HEPES, 1 mM EGTA, 1 mM Li₂GDP, and 0.3 mM Na₂ATP, titrated to pH 7.2 with NaOH, and had a resistance of 3 to 5 M Ω . Data were recorded with an EPC 9 (HEKA, Lambrecht, Germany) amplifier using the "Pulse" software (HEKA). Series resistance was compensated by 70%. Isolated cells showing green fluorescence were clamped to -60 mV; every 12 s, seven square pulses ranging from -110 to 10 mV (0.5 s each) were applied (Fig. 1). For evaluation of the inhibition by glibenclamide (GBC), the current at -60 mV was used and traces were individually corrected for run down.

Equilibrium Binding Experiments in Cells. Experiments were conducted at 37°C with an incubation time of 30 min as described previously (Hambrock et al., 1998; Russ et al., 1999). Cells were suspended by rinsing with a HEPES-buffered physiological salt solution containing 139 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 1.25 mM CaCl₂, 11 mM D-(+)-glucose; 5 mM HEPES, gassed with 95% O₂/5% CO₂, and titrated to pH 7.4 with NaOH at 37°C. Binding experiments were started by addition of cells (final concentration, 0.8–2 \times 10⁶ cells/ml corresponding to 0.2–0.5 mg of protein/ml) to physiological salt solution containing the radiolabel (for competition studies, 2 to 4 nM [3 H]GBC/1 to 3 nM [3 H]P1075 and the inhibitor of interest). After 30 min, incubation was stopped by diluting 0.3-ml aliquots in triplicate into 8 ml of ice-cold quench solution (50 mM Tris-(hydroxymethyl)-aminomethane, 154 mM NaCl, pH 7.4) and rapid filtration under vacuum over Whatman GF/C filters. Filters were washed twice with 8 ml of ice-cold quench solution and counted for 3 H in the presence of 6 ml of scintillant (Ultima Gold; Packard, Meriden, CT). Nonspecific binding of [3 H]GBC/[3 H]P1075 binding was determined in the presence of 100/10 μ M P1075. P1075 completely and specifically inhibits [3 H]P1075 and [3 H]GBC binding to (mutant and wild-type) SUR2B, whereas GBC also binds to non-transfected HEK 293 cells with low affinity (Russ et al., 1999).

Equilibrium Binding Experiments in Membranes. Membranes were prepared as described previously (Hambrock et al., 1998). Briefly, cells at \approx 80% confluence (16 million cells per dish) were suspended by rinsing with medium and centrifuged for 6 min at 500 g at 37°C. The pelleted cells were lysed by addition of 5 ml (per dish) of ice-cold hypotonic buffer containing, 10 mM HEPES, 1 mM EGTA, pH 7.4, and the lysate centrifuged at 100,000g and 4°C for 60

min. The resulting membrane pellet was resuspended in a buffer containing 5 mM HEPES, 5 mM KCl, 139 mM NaCl; 0 or 2.2 mM MgCl_2 at pH 7.4 and 4°C at a protein concentration of ≈ 0.7 mg/ml and frozen at -80°C. In the binding assay, membranes (final protein concentration, 0.2–0.5 mg/ml) were added to the incubation buffer (139 mM NaCl, 5 mM KCl, 5 mM HEPES, 2.2 mM MgCl_2) supplemented with 1 mM Na_2ATP , the radiolabel (for competition, [^3H]GBC ≈ 3 nM or [^3H]P1075 = 1–3 nM), and the inhibitor of interest at 37°C. At equilibrium (15 min for [^3H]GBC and 25 min for [^3H]P1075 binding), incubation was stopped as described above and aliquots were filtered over Whatman GF/B filters.

Data Analysis, Modeling and Statistics. In saturation experiments, nonspecific binding (B_{NS}) was proportional to the free label concentration, L , and was fitted to the equation, $B_{\text{NS}} = a \times L$, where a denotes the proportionality constant. Total binding (B_{TOT}) was then analyzed as the sum of specific and nonspecific binding and was fitted to the equation,

$$B_{\text{TOT}} = B_{\text{max}} \times L \times (L + K_D)^{-1} + a \times L, \quad (1)$$

to estimate K_D and B_{max} (fmol/mg of protein) values by the least-squares method. Experiments were performed over a large range of radioligand concentrations so that all parameters including B_{NS} ,

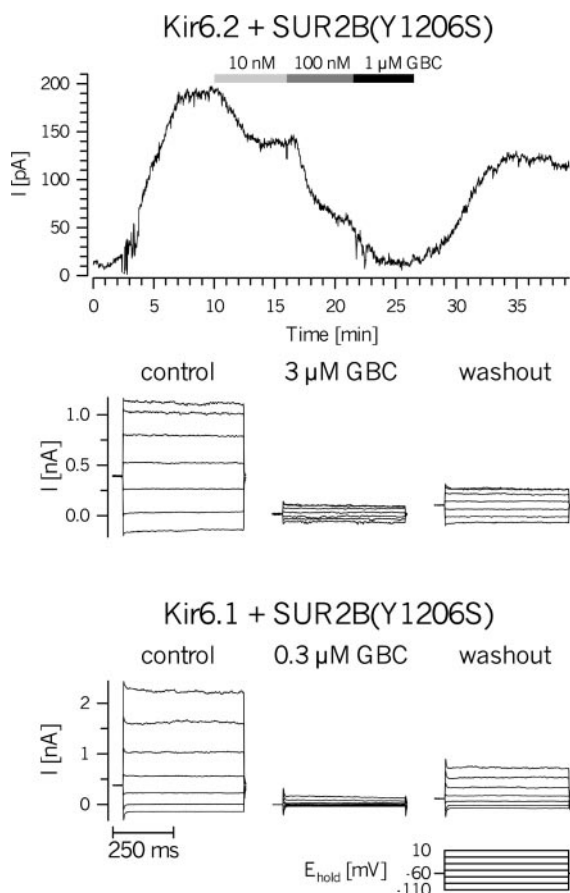


Fig. 1. Whole-cell current recordings from HEK 293 cells transfected with Kir6.2 or Kir6.1 together with SUR2B(Y1206S). Top, time course of the current at -60 mV and 37°C from a cell expressing Kir6.2 + SUR2B(Y1206S). After establishing the whole-cell configuration, the K_{ATP} current developed within some minutes; thereafter, GBC was applied cumulatively to the bath and washed out. Block by 1 μM GBC was fully reversible if the rundown of current ($\approx 3\%$ /min) is taken into account. Center and bottom, currents at different test potentials. Cells were clamped at -60 mV and the pulse protocol shown in the lower right corner was started every 12 s. The control traces (left) were recorded at ≈ 7 min; middle traces, GBC at 3 μM (Kir6.2/SUR) or 0.3 μM (Kir6.1/SUR) produced an almost complete block of the K_{ATP} current; right, currents after washout for ≈ 15 min.

could be determined from the B_{TOT} curve. B_{NS} was determined independently in the presence of 100 μM P1075 giving the same result and thus validating this approach.

Equilibrium inhibition curves were analyzed according to the logistic equation for up to three components,

$$y = 100 - \sum_{i=1}^3 A_i (1 + 10^{n_{\text{H},i}(\text{px} - \text{pIC}_{50,i})})^{-1}; \quad i = 1 - 3 \quad (2)$$

Here A_i denotes the amplitude, $n_{\text{H},i}$ the Hill coefficient, and $\text{IC}_{50,i}$ the midpoint of component i with $\text{pIC}_{50,i} = -\log \text{IC}_{50,i}$; x is the concentration of the compound under study with $\text{px} = -\log x$. IC_{50} values were converted to K_i by correcting for the presence of the radioligand L according to the Cheng-Prusoff equation

$$K_i = \text{IC}_{50}(1 + L/K_D)^{-1}, \quad (3)$$

In case of homologous competition experiments, K_i is identical to K_D . Although the binding sites for glibenclamide and openers are not identical, binding of the two ligands to SUR is mutually exclusive (Bray and Quast, 1992; Dörschner et al., 1999; Russ et al., 1999). Hence, the Cheng-Prusoff correction was also applied in case of heterologous competition experiments and generally did not exceed a factor of 2.

The concentration of binding sites of the radioligand (B_{max}) was estimated from the specific binding (B_S) at the radioligand concentration L in the absence of competitor according to the Law of Mass Action:

$$B_{\text{max}} = B_S(1 + K_D/L) \quad (4)$$

Fits of the equations to the data were performed according to the least-squares method using the FigP program (Biosoft, Cambridge, UK) or SigmaPlot (SPSS Inc., Chicago, IL). Individual competition experiments were analyzed according to logistic Hill equation with one component. If the presence of more than 1 component was evident, multicomponent analysis was used with $n_{\text{H}} = 1$ (eq. 2). The number of components was then determined by the 'extra sum of squares principle' (F-test) and by the 'Minimum Akaike Information Criterion' as described (Quast and Mählmann, 1982); the tests gave identical results. Amplitudes and pIC_{50} values are normally distributed (Christopoulos, 1998) and were compared by one way analysis of variance. Differences versus the control group were assessed by Dunnett's test and pairwise multiple comparisons were performed by the Tukey-Kramer test using the InStat program (v.2.2; GraphPad Software, San Diego, CA). In the case of only two groups, a two-tailed unpaired Student's t test was used. In the text, IC_{50} values are given followed by the 95% confidence interval in parentheses. In calculations involving two mean values with standard errors, propagation of errors was taken into account.

Secondary structure predictions were made according to the algorithms of Garnier et al. (1978) using the computer program Gene-Runner (ver. 3.04; Hastings Software Inc., Hastings on Hudson, NY) or programs GOR1 and GOR4 supplied by the ExPASy proteomics server of the Swiss Institute of Bioinformatics (Geneva, Switzerland). Sequence alignments were performed with the program BLAST 2 Sequences supplied by the National Center of Biotechnology Information (Bethesda, MD).

Materials. [^3H]P1075 [specific activity, 4.5 TBq (117 Ci)/mmol] was purchased from Amersham Buchler (Braunschweig, Germany) and [^3H]GBC [specific activity, 1.85 TBq (50 Ci)/mmol] from PerkinElmer Life Science Products (Bad Homburg, Germany). The reagents and media used for cell culture and transfection were from Invitrogen. Na_2ATP and Li_2GDP were from Roche Molecular Biochemicals (Mannheim, Germany), glibenclamide from Sigma (Deisenhofen, Germany) and cytochalasin D from Fluka (Neu-Ulm, Germany). The following drugs were kind gifts of the pharmaceutical companies indicated in parentheses: AZ-DF 265 (Thomae, Biberach,

Germany), levromakalim (SmithKline-Beecham, Harlow, UK), meglitinide (Aventis, Frankfurt, Germany), and P1075 (Leo Pharmaceuticals, Ballerup, Denmark). K_{ATP} channel modulators were dissolved in dimethyl sulfoxide/ethanol [50/50 (v/v)] and further diluted with the same solvent or with incubation buffer. In binding studies, the final solvent concentration in the assays was always below 0.3%, in electrophysiological studies $\leq 0.1\%$.

Results

Whole-Cell Current Measurements. Fig. 1 shows whole-cell currents from HEK 293 cells transfected with SUR2B(Y1206S) and Kir6.2 or 6.1. After establishing the whole-cell patch-clamp configuration and dialyzing the cell with GDP (1 mM) + ATP (0.3 mM) in the presence of Mg^{2+} , an outward current developed which was sensitive to inhibition by glibenclamide. No such current was seen in control cells. These observations and the reversal of the slowly developing currents in transfected cells at ≈ -100 mV (Fig. 1) identified the currents as typical K_{ATP} currents.

Glibenclamide inhibited the currents flowing through SUR2B(Y1206S)/Kir6.1 and Kir6.2 channels with IC_{50} values of 2.7 (2.1, 3.5) and 13 (6, 28) nM (95% confidence intervals in parentheses); in experiments with wild-type SUR2B, values of 43 (33, 55) and 167 (103, 270) nM were obtained (Fig. 2). It was also observed that the glibenclamide sensitivity of Kir6.2-containing channels varied considerably from cell to cell. For example, in case of the Kir6.2/mutant SUR2B channel, the data could be divided into two groups with IC_{50} values of 7 and 70 nM and Hill coefficients of 0.8 in both cases (not shown); pooled together, the above-mentioned IC_{50} value of 13 nM was obtained with $n_H \approx 0.7$. For the Kir6.2/SUR2B wild-type channel, the extreme IC_{50} values were ≈ 0.1 and 1 μ M; pooled data gave an IC_{50} value of 167 nM and $n_H \approx 0.7$. This was in contrast to Kir6.1 containing channels, where scatter was much smaller and n_H was not significantly different from 1.

The glibenclamide data reported in the literature are generally obtained using isolated inside/out patches at room temperature. Carrying out such experiments with the Kir6.2/SUR2B channel, the fraction of the ATP-sensitive current that was inhibited by glibenclamide (1 μ M) in the absence of nucleotides was determined to be $69 \pm 3\%$ ($n = 30$), and this value was set to 100%. In these experiments, a much smaller variability of the glibenclamide sensitivity than in intact cells was observed. The glibenclamide inhibition curve (not illustrated) gave an IC_{50} value of 27 (14, 50) nM and $n_H = 0.99 \pm 0.25$ in agreement with the value of 42 nM obtained in COS cell patches by Dörschner et al. (1999).

Affinity of SUR2B(Y1206S) for Glibenclamide and P1075 in Intact Cells. Fig. 3a shows a [3H]glibenclamide saturation binding experiment in intact HEK 293 cells expressing mutant SUR2B. A mean K_D value of 4.7 nM was obtained from four such experiments (for confidence intervals, see Table 1); B_{max} was 574 ± 39 fmol/mg protein. In homologous competition experiments, glibenclamide displaced its tritiated analog in a multiphasic manner (Fig. 4). It is known from earlier experiments that control HEK 293 cells (Russ et al., 1999) and HEK 293 cells transfected only with pcDNA3.1 vector lacking the insert (A. Piehl, C. Löffler-Walz, and A. Hambrock, unpublished observations) have endogenous glibenclamide binding components with K_D values around 300 nM and 16 μ M. If we keep the value of 300 nM

constant and analyze for three otherwise free floating components, a high-affinity component is obtained with $52 \pm 2\%$ of total binding and a K_i value of 5.4 nM. This K_i value is in excellent agreement with the result of the saturation binding experiments. Without keeping the K_D value of the first endogenous component fixed, similar parameters were obtained; however, the errors in the parameters were much larger because of the overlap of the binding sites (not shown). In heterologous competition experiments, the two carbox-amido-benzoate analogs of the SUs ('glitnides'), AZ-DF 265 and meglitinide, inhibited [3H]glibenclamide binding in a triphasic manner (Fig. 4); for the specific component (i.e., binding to mutant SUR2B, $\approx 50\% B_{TOT}$) K_i values of 0.63 (0.60, 0.66) and 6.8 (6.2, 7.7) μ M were obtained, respectively.

In HEK 293 cells coexpressing mutant SUR2B with Kir6.1 or Kir6.2, [3H]glibenclamide saturation binding experiments gave K_D values of 1.1 and 0.58 nM, respectively (Table 1 and Fig. 3b). Hence, coexpression with Kir6.x increased the affinity of glibenclamide binding to SUR2B(Y1206S) by 4- and 8-fold, depending on the Kir subtype.

Opener binding to SUR2B(Y1206S) was studied using the tritiated opener [3H]P1075 (Bray and Quast, 1992) as the radiolabel. The P1075 inhibition curve (not illustrated) was monophasic ($n_H = 0.97 \pm 0.05$) with a K_i ($= K_D$) value of 2.9 nM. From the amount of specific [3H]P1075 binding at 1.5

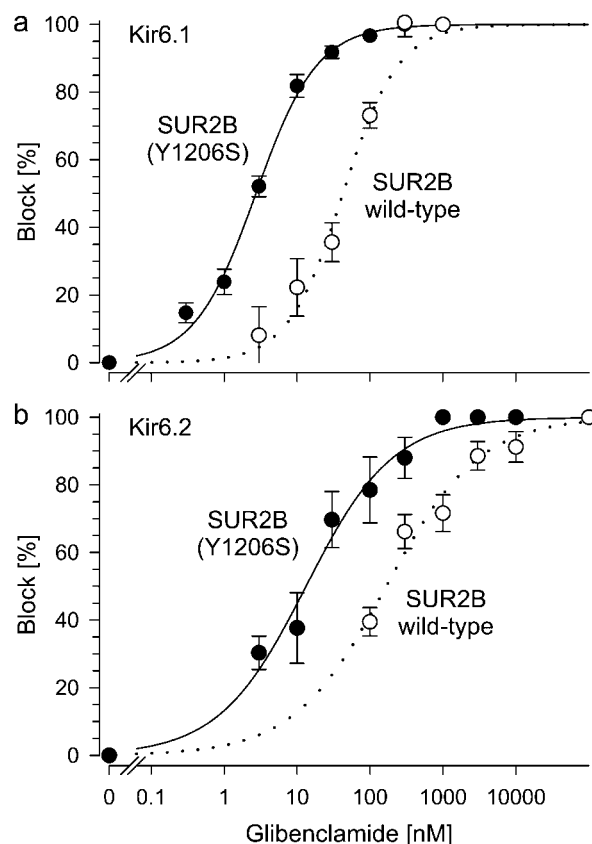


Fig. 2. Concentration-dependent inhibition by glibenclamide of whole cell currents from HEK 293 cells transfected with Kir6.1 (a) or Kir6.2 (b) together with SUR2B(Y1206S) or SUR2B. Experiments were performed as in Fig. 1, $n = 3$ to 12 per point; data for Kir6.1 + SUR2B are from Russ et al. (1999). Regression curves represent the fit of the logistic equation (eq. 2) to the data with the following parameters [SUR2B(Y1206S)/SUR2B wild-type]: a, $pK = 8.56 \pm 0.05/7.37 \pm 0.05$, $n_H = 1.03 \pm 0.14/1.14 \pm 0.16$; b, $pK = 7.88 \pm 0.17/6.78 \pm 0.11$, $n_H = 0.73 \pm 0.18/0.68 \pm 0.15$, respectively.

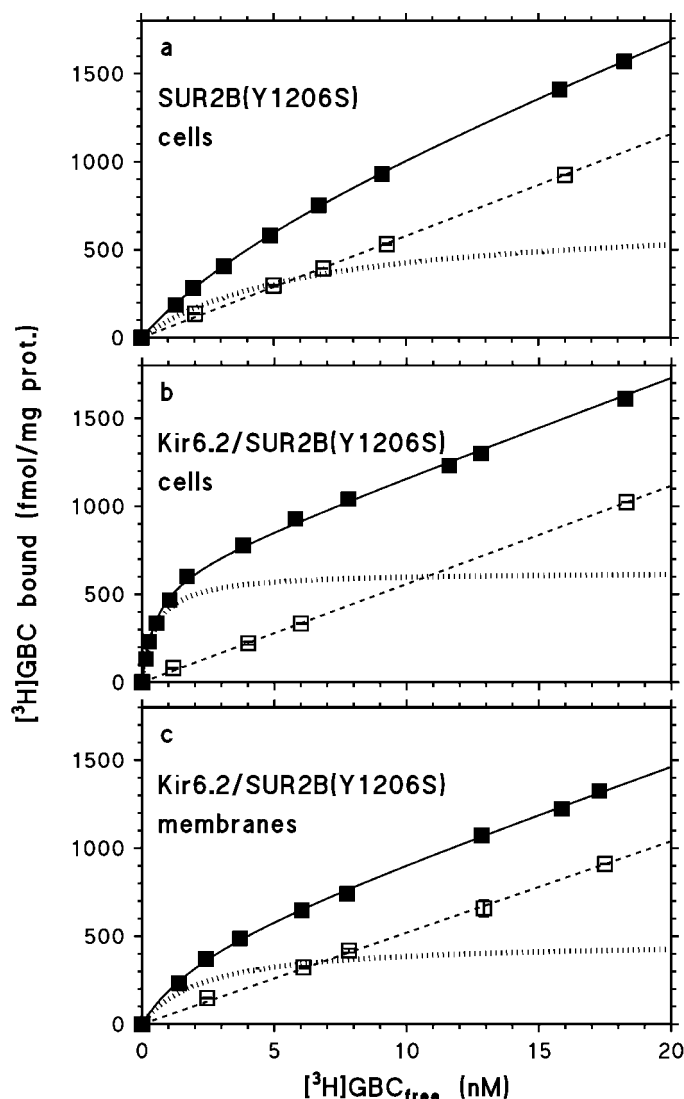


Fig. 3. [^3H]glibenclamide saturation binding experiments in HEK 293 cells expressing mutant SUR2B (Y1206S) (a), Kir6.2/SUR2B(Y1206S) (b), and membranes containing Kir6.2/SUR2B(Y1206S) in the presence of MgATP (1 mM) (c). Total binding (B_{TOT} , \blacksquare) and nonspecific binding (B_{NS} , \square) are shown; B_{NS} was determined in the presence of P1075 (100 μM ; see *Experimental Procedures*). Specific binding (B_{S}), calculated as $B_{\text{TOT}} - B_{\text{NS}}$ is represented by the dotted curves. The fit of eq. (1) under *Experimental Procedures* gave K_{D} values of 6.3, 0.5, and 2.7 nM and B_{max} values of 694, 628, and 400 fmol/mg protein in a, b, and c, respectively. For mean values from at least three experiments, see Tables 1 and 2.

nM (236 ± 12 fmol/mg protein), one estimates by correction for incomplete saturation (eq. 4) a B_{max} value of 711 ± 25 fmol/mg protein for the opener. Together with the result from Fig. 3a, one calculates the ratio of glibenclamide to P1075 sites to 0.79 ± 0.06 (taking propagation of errors into account). Cotransfection with Kir6.x did not affect the results of homologous [^3H]P1075 competition assays (Table 1).

Interaction of Glibenclamide and P1075 Sites. In HEK 293 cells transfected with mutant SUR2B, P1075 inhibited total [^3H]glibenclamide binding to $\approx 50\%$ (i.e., exactly the amount of glibenclamide binding to SUR; see above); no inhibition was found in nontransfected HEK 293 cells. Figure 5a shows the inhibition curve in transfected cells expressed as percentage of specific binding. The curve was biphasic with the high-affinity component making up about two thirds

of the total amplitude and a having a K_{i} value of 2.6 nM; the low-affinity component was in the micromolar range. A similarly biphasic curve was found for the opener, levromakalim (not illustrated). Coexpression with Kir6.2 left the high-affinity component of the [^3H]glibenclamide-P1075 curve unchanged and shifted the low-affinity component to the left by a factor of 7, thus reducing the biphasic nature of the curve (Table 1 and Fig. 5c). Coexpression with Kir6.1 led to the disappearance of the low-affinity component, rendering the curve monophasic. The K_{i} value of 5.5 nM corresponded to the true affinity of P1075 to SUR2B(Y1206S) (Fig. 5b and Table 1).

We also studied the inhibition of [^3H]P1075 binding by glibenclamide. In HEK 293 cells expressing mutant SUR2B alone, the inhibition curve was monophasic ($n_{\text{H}} = 0.93 \pm 0.05$) with $K_{\text{i}} = 21$ nM [i.e., a value more than four times higher than that determined in [^3H]glibenclamide saturation and homologous competition experiments (Table 1, Fig. 5d)]. Cotransfection with Kir6.x rendered these curves biphasic (Fig. 5, e and f). In the case of Kir6.1, the competition curve consisted of two components of similar size with K_{i} values of 0.7 and 39 nM (Fig. 5e, Table 1). In the presence of Kir6.2, there was a dominant high-affinity component (81%) with $K_{\text{i}} = 0.3$ and a small low-affinity component with $K_{\text{i}} = 22$ nM (Fig. 5f, Table 1). It is intriguing that the high-affinity K_{i} values of these curves are similar to the K_{D} values determined in the corresponding [^3H]glibenclamide saturation experiments (1 and 0.6 nM, respectively, see Table 1); in addition, the K_{i} values of the low-affinity components resemble the value found in the [^3H]P1075-glibenclamide competition curve with mutant SUR2B expressed alone (21 nM; Table 1). This raised the suspicion that cotransfection, performed at a molar plasmid ratio of Kir to SUR = 1:1 was insufficient to complex all SUR and that, because of different expression rates of Kir6.1 and 6.2, different proportions of mutant SUR remained free. To test this possibility, the strongly biphasic

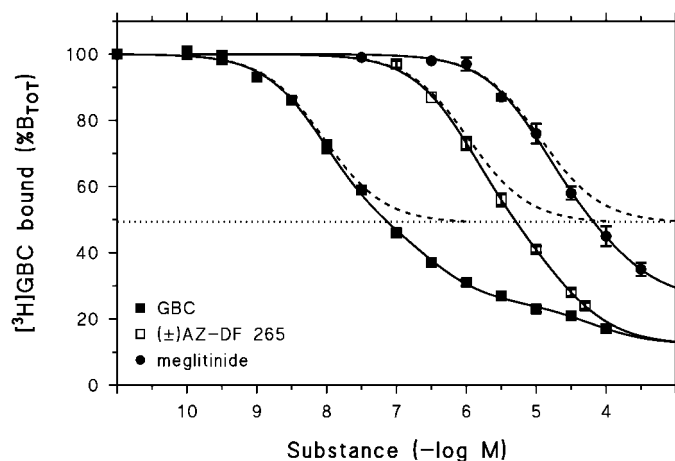


Fig. 4. Inhibition of [^3H]glibenclamide binding in HEK 293 cells expressing SUR2B(Y1206S) by glibenclamide, AZ-DF265, and meglitinide. The data, expressed as percentage of total binding (B_{TOT}) are means from three experiments; [^3H]glibenclamide was ≈ 3 nM. To account for [^3H]glibenclamide binding to proteins in addition to SUR2B(Y1206S), a logistic equation with three components (eq. 2 under *Experimental Procedures*) was fitted to the data (see text) and gave the parameters (glibenclamide/AZ-DF 265/meglitinide) A_1 (% B_{TOT}), $52 \pm 2/52/52$; $\text{pIC}_{50,1}$, $8.05 \pm 0.04/5.97 \pm 0.02/4.94 \pm 0.03$; A_2 (% B_{TOT}), $24 \pm 3/24/24$; $\text{pIC}_{50,2}$, $6.51/4.9 \pm 0.1/3.8 \pm 0.1$; A_3 (% B_{TOT}), $12 \pm 5/12/0$; $\text{pIC}_{50,3}$, $4.2 \pm 0.5/4.4 \pm 0.2$ /not determined (due to solubility problems). The broken curves represent binding to SUR2B(Y1206S) (high-affinity component).

TABLE 1

Binding experiments with [³H]GBC and [³H]P1075 in intact cells*K_D/K_i* values are geometric means from *n* independent experiments; A denotes the amplitudes in percentage of specific binding. Experiments were performed as described in Figs. 3 to 5. Data for SUR2B wild-type are from Russ et al. (1999).

	[³ H]GBC					[³ H]P1075				
	GBC		P1075			P1075		GBC		
	<i>K_D</i>	<i>n</i>	<i>K_i</i>	A	<i>n</i>	<i>K_D</i>	<i>n</i>	<i>K_i</i>	A	<i>n</i>
	<i>nM</i>		<i>nM</i>	% <i>B_S</i>		<i>nM</i>		<i>nM</i>	% <i>B_S</i>	
SUR2B(Y1206S)	4.7 (3.2,6.8)	5	2.6 (2.1,3.1)	65 ± 2	3	2.9 (2.6,3.2)	3	21 (20,22)	100	3
Kir6.1/SUR2B(Y1206S)	1.1 (0.9,1.2) ^a	3	794 (661,955)	35 ± 2	5	3.5 (3.2,3.8)	3	0.66 (0.55,0.79) ^b	55 ± 2 ^c	6
Kir6.2/SUR2B(Y1206S)	0.58 (0.50,0.66) ^a	3	5.5 (4.4,6.9)	100	5			43 (35,51) ^c	45 ± 2	
			112 (102,123)	42 ± 2	3	3.2 (2.9,3.5)	3	0.31 (0.23,0.41) ^b	81 ± 3 ^c	3
SUR2B	32 (16,65)	4	6.6 (5.0,8.7) ^d	100	4	4.3 (3.5,5.1)	4	22 (17,28) ^c	19 ± 3	6
Kir6.1/SUR2B	6.3 (3.5,12)	4	3.2 (1.6,6.4) ^d	100	4	5.8 (5.2,6.3)	4	1000 (830,1200)	100	6
								17 (13,22) ^e	32 ± 6 ^f	6
Kir6.2/SUR2B	N.D.		N.D.			3.9 (3.8,4.0)	3	600 (460,800) ^e	68 ± 6	3
								13 (10,17) ^e	76 ± 4 ^f	
								870 (440,1740) ^e	24 ± 4	

^a Values different from one another (*P* < 0.01, *t* test).^{b,c} Significant differences in parameters for cotransfection with Kir6.1 and Kir6.2: ^b *P* < 0.05; ^c *P* < 0.01 (*t* test).^d Signal too small to resolve two components (Russ et al., 1999).^e Differences not significant.^f Differences in amplitudes: *P* < 0.01.

N.D., not determined.

[³H]P1075-glibenclamide inhibition curve for Kir6.1/SUR was also performed using cells transfected with a Kir:SUR ratio of 4:1. The results were identical to those obtained at the 1:1 ratio and fit parameters were pooled (Table 1). One must conclude that the biphasic shape of these heterologous competition curves in cells cotransfected with mutant SUR2B and Kir6.*x* (Fig. 5) is not caused by some proportion of SUR remaining uncomplexed by Kir6.*x*.

In addition, [³H]P1075-glibenclamide competition experiments were performed using wild-type SUR2B (Table 1); they showed a pattern similar to that obtained with the mutant. In cells expressing SUR2B alone, inhibition curves were monophasic (*n_H* = 0.99 ± 0.04), with *K_i* = 1.0 (0.8, 1.2) μM. Again, coexpression with Kir6.*x* at a molar plasmid ratio of Kir:SUR = 4:1 rendered the curves biphasic, with the high-affinity components giving *K_i* values of roughly 15 nM and the low-affinity components values of 600 and 870 nM (Table 1). The contribution of the high-affinity component to the total inhibition curve was 32% in case of cotransfection with Kir6.1 and 76% with Kir6.2 (Table 1).

Experiments in the Presence of Cytochalasin D. We have shown previously that disruption of the actin cytoskeleton abolished high affinity binding of [³H]glibenclamide binding to the vascular K_{ATP} channel in rat aortic rings (Löffler-Walz and Quast, 1998).

To test the possibility that the effects of coexpression described above were caused by coupling of the recombinant channel on the actin cytoskeleton, experiments were performed in the presence of cytochalasin D (3 μM). In cells expressing mutant SUR2B alone, cytochalasin significantly affected neither glibenclamide binding nor the [³H]glibenclamide-P1075 inhibition curve (*n* = 3, not shown). This may not be too surprising because SUR1 alone is not transported to the cell membrane (Sharma et al., 1999; Zerangue et al., 1999). However, in cells coexpressing Kir6.1 + SUR2B(Y1206S), the affinity of glibenclamide was also not affected by cytochalasin. Similarly, whole-cell current measurements showed no significant effect of cytochalasin (3 and 10 μM, 30 min) on the glibenclamide sensitivity of the channels formed by Kir6.1 + SUR2B

wild-type (*n* = 8). However, cells rapidly lost their irregular shape and became roundish, showing that cytochalasin D was active.

Binding Assays in Membranes. These experiments are summarized in Table 2. Nucleotides strongly affect glibenclamide and opener binding to SURs (Hambrock et al., 1998, 1999; Schwanstecher et al., 1998) and K_{ATP} channels (for review, see Ashcroft and Ashcroft, 1992). To facilitate comparison with the studies in intact cells, experiments were performed in the presence of 1 mM MgATP; at the end of the incubation period, 50 to 100 μM ADP was also present (Hambrock et al., 1999). Binding of [³H]glibenclamide to mutant SUR2B gave a *K_D* value of 3.4 nM, similar to that in cells. The [³H]glibenclamide-P1075 inhibition curve was again strongly biphasic, with the *K_i* value of the high-affinity component (5.6 nM) in perfect agreement with the result of the [³H]P1075 experiments in membranes (see below and Table 2). On order to examine whether or not the biphasic nature of this curve depended on the radioligand concentration, experiments were performed at [³H]glibenclamide concentrations ranging from 0.6 to 6 nM. No change in amplitudes and *K_i* values was observed (data not shown). Coexpression with Kir6.1 and 6.2 did not increase the affinity of glibenclamide for SUR in membranes (see Fig. 3c for Kir6.2/SUR2B(Y1206S)); however, it increased the amplitude of the high-affinity component (see Fig. 5, b and c; Table 2).

[³H]P1075 binding, as assessed in homologous competition experiments, gave a *K_D* value of 6.5 nM. This was about two times higher than that in cells and resembled the *K_i* value from the high-affinity component of the [³H]glibenclamide-P1075 competition curve (Table 2). As in cells, the affinity for the opener was unaffected by cotransfection of mutant SUR2B with Kir6.*x* (Table 2). [³H]P1075-glibenclamide competition experiments in membranes containing mutant SUR2B alone were monophasic (Fig. 5d) and gave a *K_i* value of 245 nM. Coexpression with Kir6.*x* rendered the [³H]P1075-glibenclamide competition curves strongly biphasic with amplitudes similar to those found in cells; however, the *K_i* val-

ues for both components were generally 10× higher (Tables 1 and 2).

The number of binding sites of the two radioligands in membranes at 1 mM MgATP, determined from saturation experiments or estimated from specific binding using eq. 4, were 292 ± 17 and 759 ± 38 fmol/mg for [3 H]glibenclamide and [3 H]P1075, respectively, resulting in an apparent ratio of glibenclamide sites:P1075 sites of 0.38 ± 0.03 ($n = 27$). In the absence of MgATP, B_{\max} of glibenclamide increased 2.7 ± 0.2 -fold to 727 ± 42 fmol/mg, whereas K_D remained unchanged ($n = 6$; C. Löffler-Walz, A. Hambrock, and U. Quast, in preparation). In paired experiments, the maximum number of binding sites of SUR2B(Y1206S) was determined for each ligand under optimized conditions (glibenclamide, 0 MgATP; P1075, 1 mM MgATP) and gave a ratio of glibenclamide sites to P1075 sites of 1.00 ± 0.05 ($n = 9$).

Discussion

Properties of SUR2B(Y1206S). In this study, we have investigated the impact of the substitution Y1206S in SUR2B on the interaction with sulfonylureas and openers and have

assessed the effects of coexpression of mutant SUR2B with Kir6.1 and 6.2.

In intact cells, wild-type SUR2B binds [3 H]P1075 with $K_D = 4$ nM and [3 H]glibenclamide with $K_D = 32$ nM (Table 1; Russ et al., 1999). This study has shown that SUR2B(Y1206S) binds [3 H]P1075 with unchanged affinity ($K_D = 4$ nM); however, the K_D value for glibenclamide was 4.7 nM, indicating that the point mutation increased the affinity of SUR2B for glibenclamide ≈ 7 -fold (see also Toman et al., 2000). There are several potential explanations for this increase in affinity. Because of the smaller volume of the serine side chain compared with tyrosine, the binding pocket of mutant SUR2B for glibenclamide may better accommodate the ligand sterically (Ashfield et al., 1999). The effect could also be more indirect. Secondary structure predictions showed that the exchange of Tyr by Ser at position 1206 in the intracellular loop between transmembrane segments 15 and 16 increases the probability of the surrounding region to form an α -helix instead of a β -sheet. The comparison of this region from SUR1 and SUR2B (amino acids 1181–1251 in rat SUR1 and 1150–1220 in mouse SUR2B) reveals 78% identity. Scanning the databases SWISS-PROT and TrEMBL for different patterns from this region shows that the

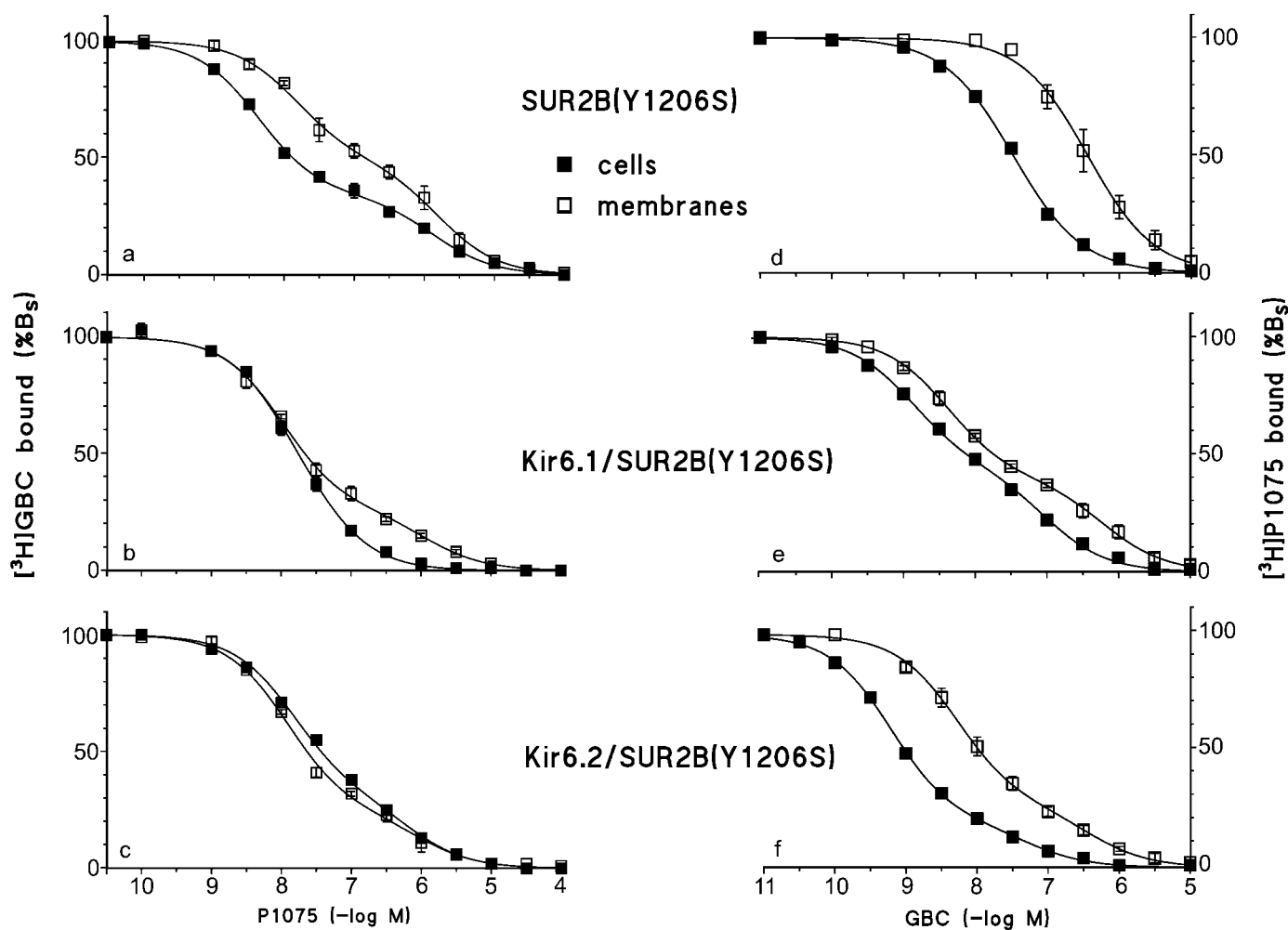


Fig. 5. Inhibition of [3 H]glibenclamide binding by P1075 (left) and [3 H]P1075 by glibenclamide in cells (■) and membranes (□). a and d, SUR2B(Y1206S) alone. b and e, coexpression with Kir6.1 c and f, coexpression with Kir6.2. Data are expressed as percentage of specific binding (% B_s); $n = 3$ to 6. The parameters listed in Table 1 and 2 were obtained from the fit of a two-component logistic function with Hill coefficients of 1 to the data. Experiments in membranes were conducted in the presence of MgATP (1 mM); [3 H]glibenclamide concentration was 1.5 to 3.0 nM and [3 H]P1075 was 2.7 to 3.0 nM.

pattern NNIA directly preceding Tyr/Ser (position 1234–1237 in rat SUR1 and 1202–1205 in mouse SUR2B) occurs in a variety of ATP-synthases from different species and is part of various other nucleotide binding proteins (e.g., ADP-ribosylation-factors, DNA-polymerases, GTP-binding proteins) as well as diverse cytoskeletal proteins or proteins with structural function (e.g., dynein, fimbrin, clathrin-assembly protein). Therefore, the mutation Y1206S in SUR2B could affect not only glibenclamide affinity but also the interaction with the cytoskeleton and, possibly, the nucleotides (see also below).

Coexpression of SUR2B(Y1206S) with Kir6.1 or 6.2.

Upon coexpression of mutant SUR2B with the Kir6.x subtypes, functional K_{ATP} channels were formed. Glibenclamide inhibited these channels with approximately 15-fold higher potency than the corresponding wild-type channels; this fits reasonably well with the 7-fold difference in affinity of glibenclamide binding. Interestingly, the glibenclamide sensitivity depended on the Kir6.x subtype: under otherwise identical conditions, glibenclamide inhibited Kir6.1/SUR2B or Kir6.1/SUR2B(Y1206S) channels with 4-fold higher potency than those formed with Kir6.2. These results predict that the vascular K_{ATP} channel (Kir6.1/SUR2B) exhibits a higher sensitivity toward glibenclamide than that in nonvascular smooth muscle (Kir6.2/SUR2B). Furthermore and in contrast to Kir6.1, the glibenclamide sensitivity of the Kir6.2 containing channels was highly variable in intact cells but not in isolated patches, and the corresponding concentration-response curves showed a low Hill coefficient. This points to the modulation of glibenclamide sensitivity by an additional component that was poorly controlled in our experiments and that affected Kir6.2-containing channels more than those with Kir6.1. Possible candidates are phospholipids such as phosphatidylinositol-4,5-bisphosphate (PIP₂; Krauter et al., 2001) or nucleotides that might be incompletely clamped by dialysis of the cell with the pipette solution.

In binding studies using intact cells, the affinity of the opener P1075 for mutant SUR2B was not affected by cotransfection. In contrast, glibenclamide affinity was increased 4-fold by coexpression with Kir6.1, thus corroborating a result obtained with wild-type SUR2B (Table 1). With Kir6.2, however, affinity was increased 8-fold. These affinity shifts

were not observed in membranes, suggesting that they depend on additional components. The actin cytoskeleton, which is required for high glibenclamide affinity in rat aortic rings (Löffler-Walz and Quast, 1998), is probably not involved here because cytochalasin D affected neither glibenclamide binding to Kir6.1/SUR2B(Y1206S) nor the glibenclamide sensitivity of the Kir6.1/SUR2B wild-type channel. These results suggest that the lack of cytochalasin D effect on the channel observed here may be caused primarily by an as-yet-unidentified component that is different or missing in the recombinant system and not by the mutation per se.

The state of SUR determines the state of the Kir subunit; however, little attention is paid to how Kir affects the properties of SUR. As shown here, Kir6.x affects the glibenclamide affinity of SUR and the relationship between glibenclamide and opener binding (see below) in a manner depending on the Kir6.x subtype. Various portions of Kir6.2 have been proposed to be important for the interaction with SUR1 (Giblin et al., 1999; Mikhailov et al., 2000; Schwappach et al., 2000); data for Kir6.1 are missing. Kir6.1 is larger than Kir6.2 (424 versus 390 amino acids) with 70% identity between both proteins. Alignment reveals a gap at the extracellular loop close to M1 and a major difference at the distal C terminus (Sakura et al., 1995). Therefore, it is no surprise that Kir6.x affects SUR in a subtype specific way.

It is interesting to compare the *affinities* of glibenclamide binding with the *potencies* for channel block. The affinity of mutant SUR2B for glibenclamide was increased more by complex formation with Kir6.2; Kir6.1 containing channels were, however, more sensitive to glibenclamide than Kir6.2 channels. This discrepancy is quite unexpected and shows that the cross talk between SUR and Kir is reciprocal and complex; additional factors, such as ATP, ADP, Mg²⁺, and PIP₂, may also play a role.

Relationship between Opener and Sulfonylurea Binding. A first result from this study is that in cells expressing SUR2B(Y1206S) alone, the stoichiometry of glibenclamide to P1075 sites is $\approx 1:1$. This holds true also in membranes, if the maximum number of glibenclamide sites at 0 MgATP is compared with the P1075 sites at 1 mM MgATP. If both radioligands are measured in the presence of

TABLE 2

Binding experiments with [³H]GBC and [³H]P1075 in membranes

K_D/K_i values are geometric means from n independent experiments; A denotes the amplitudes in percentage of specific binding. Experiments were performed as described in Fig. 5. Experiments on SUR2B are from Hambrock et al. (1998).

	[³ H]GBC					[³ H]P1075				
	GBC		P1075			P1075		GBC		
	K_D	n	K_i	A	n	K_D	n	K_i	A	n
	nM		nM	%B _S		nM		nM	%B _S	
SUR2B2B(Y1206S)	3.4 (2.9,3.9)	4	5.6 (3.4,9.3)	45 ± 4	6	6.5 (6.2,6.8)	3	245 (162,372)	100	3
Kir6.1/SUR2B(Y1206S)	2.5 (2.2,2.9)	3	660 (480,910)	55 ± 4	3	6.8 (5.6,8.1)	3	2.7 (1.9,3.7) ^b	59 ± 1 ^b	6
Kir6.2/SUR2B(Y1206S)	2.7 (2.3,3.0)	3	690 (600,790)	27 ± 2 ^a	3	7.2 (4.4,12.0)	3	360 (230,540) ^b	41 ± 1 ^b	4
			7.1 (6.5,7.8)	75 ± 2 ^a				4.4 (2.8,6.9)	75 ± 3	
			650 (340,1230)	25 ± 2 ^a				288 (191,437)	25 ± 3	
SUR2B	N.D.		N.D.			3.3 (2.4,4.6)	4	2400 (1900,3000)	100	4
Kir6.1/SUR2B	N.D.		N.D.			4.4		72 (50,105)	62 ± 3	3
								2400 (2000,2900)	38 ± 3	
Kir6.2/SUR2B	N.D.		N.D.			4.4		50 (21,120)	42 ± 3	3
								200 (1000,4000)	58 ± 3	

^a Amplitudes but not pK_i values are different from those of curve with SUR2B(Y1206S) alone ($P < 0.01$, analysis of variance followed by Tukey-Kramer).

^b Parameters pooled from experiments with plasmid ratio Kir:SUR = 1:1 and 4:1 (see text).

N.D., not determined (see text).

1 mM MgATP, however, the ratio is 0.38 ± 0.03 . The fact that MgATP reduces B_{\max} but does not affect the affinity K_D value of glibenclamide rules out the possibility that SUR2B(Y1206S), expressed alone, exists predominantly as a monomer with a single binding site for glibenclamide, which is negatively allosterically linked to MgATP binding. In this case, MgATP should reduce only the affinity of glibenclamide binding. Instead, MgATP, at saturating concentrations (C. Löffler-Walz, A. Hambrock, and U. Quast, in preparation), induces a nonequivalence of the glibenclamide sites leaving about one third of them unaffected and shifting the other ones to very low affinity or rendering them inaccessible. Therefore, the glibenclamide sites are coupled. Assuming one site per monomer, SUR2B(Y1206S) expressed alone must then form homomultimers. Because K_{ATP} channels are octamers [(Kir/SUR)₄; Clement et al., 1997; Shyng and Nichols, 1997] and NBF1 of SUR1, expressed alone, forms tetramers (Mikhailov and Ashcroft, 2000), one may speculate that SUR alone also forms tetramers.

The nonequivalence of the glibenclamide sites in the presence of MgATP compared with the apparently homogenous P1075 sites may account for the complexity of the heterologous competition curves in membranes; in cells, the reasons are less obvious. [³H]P1075-glibenclamide inhibition curves with mutant SUR2B alone were monophasic but gave K_i values considerably higher than that expected from the affinity of glibenclamide (cells 4-fold; membranes 72-fold). With wild-type SUR2B in intact cells, this difference is 30-fold, and for Kir6.1/SUR2B, 44-fold (Table 1; Russ et al., 1999). Collectively, these results show that this type of assay is inappropriate to determine the affinity of glibenclamide binding (but see Dörschner et al., 1999). However, it certainly provides important insight into the coupling of the P1075 and glibenclamide binding sites. In both cells and membranes, [³H]P1075-glibenclamide and [³H]glibenclamide-P1075 inhibition curves with Kir6.x/SUR2B(Y1206S) were biphasic in a manner depending on the Kir subtype. [³H]P1075-glibenclamide competition experiments with wild-type SUR2B + Kir6.x gave biphasic inhibition curves similar to those obtained with the mutant. This showed that these biphasic curves are not caused by the point mutation Y1206S in SUR2B; however, one cannot exclude that signaling within the protein may have been distorted by the mutation and that some of the results obtained with the mutant do not necessarily apply to native SUR2B.

The heterologous competition experiments in cells suggest that [³H]glibenclamide abolishes the equivalence and independence of opener sites in the multimeric complex and vice versa. Hence, the negative allosteric coupling of opener and glibenclamide sites is complex, and cooperativity of sites and possibly mixed complexes, binding both opener and glibenclamide simultaneously, may exist. The sequential model of allosteric proteins (in which all states of an allosteric protein can exist) may be more appropriate than the concerted transition model, which predicts symmetrical states (i.e., mutually exclusive binding of openers and SUs) (for reviews, see Henis and Levitzki, 1979; Ricard and Cornish-Bowden, 1987). The negative allosteric interaction between P1075 and glibenclamide at SUR2B(Y1206S) is modulated by coexpression depending on the Kir6.x subtype and by nucleotides, Mg²⁺, Kir6.x, and PIP₂.

In conclusion, we have characterized here the properties of a novel SUR2B mutant that has high affinity for both gliben-

clamide and P1075. The study has produced three major results: 1) The interaction of Kir6.x with SUR modifies glibenclamide affinity in a manner depending on the Kir6.x subtype and requiring the intact cell; 2) SUR2B(Y1206S), expressed alone, may form homomultimers; and 3) the negative allosteric interaction between glibenclamide and P1075 sites at mutant SUR2B is complex and induces heterogeneity of the reciprocal binding sites. Therefore, the allosteric theory of the K_{ATP} channel still needs to be developed.

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