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Interaction of K_{ATP} Channel Modulators with Sulfonylurea Receptor SUR2B: Implication for Tetramer Formation and Allosteric Coupling of Subunits

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ABSTRACT

Sulfonylurea receptors (SURs) are subunits of ATP-sensitive K^+ channels (K_{ATP} channels); they mediate the channel-closing effect of sulfonylureas such as glibenclamide and the channel-activating effect of K_{ATP} channel openers such as the pinacidil analog P1075. We investigated the inhibition by MgATP and P1075 of glibenclamide binding to SUR2B, the SUR subtype in smooth muscle. To increase specific binding, experiments were also performed using SUR2B(Y1206S), a mutant with higher affinity for glibenclamide than for the wild-type (K_D = 4 versus 22 nM, respectively) but otherwise exhibiting similar pharmacological properties. In the absence of MgATP, [^{3}H]glibenclamide binding to both SURs was homogenous. MgATP inhibited [^{3}H]glibenclamide binding to both SURs to 25% by reducing the apparent number of glibenclamide binding sites, leaving the affinity unchanged. In the absence of MgATP, P1075 inhibited [^{3}H]glibenclamide binding in a monophasic manner with K_i ≈1 μM. In the presence of MgATP (1 mM), inhibition was biphasic with one K_i value resembling the true affinity of P1075 for SUR2B (2–6 nM) and the other resembling K_i in the absence of MgATP (≈1 μM). The data show that (1) MgATP induces heterogeneity in the glibenclamide sites; (2) the high-affinity glibenclamide sites remaining with MgATP are linked to two classes of P1075 sites; and (3) P1075 interacts specifically with SUR2B also in the absence of MgATP. The data are discussed with the assumption that SUR2B, expressed alone, forms tetramers; that MgATP induces allosteric interactions between the subunits; and that mixed SUR2B-glibenclamide-P1075 complexes can exist at equilibrium.

K_{ATP} channels are a class of K^+ channels composed of pore-forming subunits of type Kir6.x and sulfonylurea receptors (SURs) arranged as a hetero-octameric complex (Kir6.x/SURx)$_4$ (Clement et al., 1997; Shyng and Nichols, 1997; Gonoi and Seino, 2000). These channels are closed by intracellular ATP and are opened by MgADP, thereby linking the metabolic state of the cell to membrane potential and excitability (Ashcroft and Ashcroft, 1990). In addition, they are the target of the hypoglycemic sulfonylureas (SUs) such as glibenclamide, which induce channel closure, and of the K_{ATP} channel openers, a structurally heterogeneous class of compounds (Ashcroft and Ashcroft, 1990), which is exemplified here by the pinacidil analog P1075 (Quast et al., 1993).

SUR, a member of the ATP-binding cassette proteins, is endowed with two nucleotide-binding domains that exhibit ATPase activity and control channel opening (Aguilar-Bryan et al., 1995; Bienengraeber et al., 2000; Zingman et al., 2001). SUR also affords the binding sites for SUs and openers (Hambrock et al., 1998; Schwanstecher et al., 1998). SUR1 is very sensitive to the SUs but shows little sensitivity to most openers, whereas the contrary is true for the two SUR2 subtypes, which differ only in the last 42 amino acids (Inagaki et al., 1996; Isomoto et al., 1996). Essential parts of the binding sites for P1075 and for glibenclamide are located in close proximity on the last transmembrane domain of SUR (Ashfield et al., 1999; Uhde et al., 1999; Babenko et al., 2000; Moreau et al., 2000; Mikhailov et al., 2001).

These structural features are the foundation of allosteric coupling effects that exist between nucleotide, SU, and opener binding to SUR. First, there is the positive allosteric interaction between MgATP and opener binding. MgATP and hydrolyzable analogs induce high-affinity opener binding to SUR2 and sensitivity of SUR1 to pinacidil and diazoxide (Niki and Ashcroft, 1991; Schwanstecher et al., 1991, 1992a, 1998; Quast et al., 1993; Hambrock et al., 1998, 1999). Conversely, openers enhance the ATPase activity of SUR2A, thereby promoting channel activation (Bienengraeber et al., 2000). However, high concentrations of openers activate K_{ATP} channels also in the absence of MgATP, and MgATP sensitizes the channel for the opener

ABBREVIATIONS: SUR, sulfonylurea receptor; P1075, *N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*′′-3-pyridylguanidine); SU, sulfonylurea; K_{ATP} channel, ATP-sensitive K^+ channel; B_{NS}, nonspecific binding; B_{TOT}, total binding; HEK, human embryonic kidney; B_{MAX}, maximum concentration of binding sites; B_S, specific binding; K_D, equilibrium dissociation constant of the radioligand; Kir, inwardly rectifying K^+ channel.

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by slowing down the rate of channel closing upon washout of pinacidil, an effect that is much more pronounced at the Kir6.2/SUR2B than at the Kir6.2/SUR2A channel (Ashcroft and Gribble, 2000; Reimann et al., 2000). However, binding of openers such as P1075 in the absence of MgATP has not yet been studied.

Second, there is the negative allosteric interaction between nucleotide and SU binding. Nucleotides reduce glibenclamide binding to SUR1-containing channels by decreasing the affinity without affecting the number of binding sites (Niki et al., 1990; Schwanstecher et al., 1991, 1992b). For the SUR2 subtypes, the nucleotide regulation of glibenclamide binding has not yet been determined. Third, there is a negative allosteric coupling between opener and SU binding [SUR1 (Niki and Ashcroft, 1991; Schwanstecher et al., 1992a, 1998) and SUR2 (Bray and Quast, 1992; Hambrock et al., 1998, 1999)]. Generally, this leads to mutually exclusive binding between the two. However, we have observed that in rat glomeruli and in A10 cells (a cell line derived from rat aorta), glibenclamide was unable to completely inhibit binding of [^{3}H]P1075 (Metzger and Quast, 1996; Russ et al., 1997). Hence, the relationship between opener and SU binding to SUR requires re-examination.

Here, we study the regulation of glibenclamide binding to SUR2B by MgATP and the inhibition of this binding by P1075. We show that the relatively low affinity of glibenclamide for SUR2B ($K_D \approx 22$ nM), together with a high level of nonspecific binding, makes the analysis of experiments, particularly in the presence of MgATP, difficult. Therefore, experiments were also performed using a SUR2B mutant in which Tyr 1206 of SUR2 (mouse numbering) is replaced by Ser, the amino acid located in the corresponding position of SUR1 (Ashfield et al., 1999; Toman et al., 2000). This mutation leads to a ≈5-fold increase in affinity for glibenclamide ($K_D \approx 4$ nM) but keeps the high affinity of SUR2B for P1075 almost unchanged (6.5 nM) (Toman et al., 2000; Hambrock et al., 2001). Hence, this mutant allows radioligand binding studies with both [^{3}H]glibenclamide and [^{3}H]P1075 to be performed with sufficient precision. Examining the properties of [^{3}H]glibenclamide binding in the presence of MgATP to SUR2B (wild-type and mutant) revealed unexpected complexities, which are discussed while assuming that SUR2B forms homomultimers with intersubunit interactions.

Materials and Methods

Site-Directed Mutagenesis and Cell Transfection. The mutant SUR2B(Y1206S) was constructed from the mouse clone (GenBank accession number D86038; Isomoto et al., 1996) using the QuikChange Site-Directed Mutagenesis System (Stratagene, Amsterdam, The Netherlands) as described previously (Hambrock et al., 2001). Human embryonic kidney (HEK) 293 cells were cultured in minimal essential medium containing glutamine, supplemented with 10% fetal bovine serum and 20 μg/ml gentamicin. Cells were transfected with the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) containing the coding sequence of wild-type or mutant SUR2B using LipofectAMINE and Opti-MEM (Invitrogen) according to the manufacturer's instructions (Hambrock et al., 1998). Stably transfected cells were isolated in the presence of 700 μg of geneticin/ml of medium for the first 3 weeks and 300 μg/ml later; 1 week before membrane preparation, the antibiotic was withdrawn. Membranes were prepared as described previously (Hambrock et al., 1998).

Equilibrium Binding Experiments. For saturation binding experiments of [^{3}H]glibenclamide to SUR2B(Y1206S), membranes (0.1–0.4 mg of protein/ml) were incubated with [^{3}H]glibenclamide (0.7–20 nM) in a total volume of 1 ml at 37°C and pH 7.4 for 15 min in an incubation buffer containing 5 mM HEPES, 139 mM NaCl, and 5 mM KCl; the buffer was supplemented with $MgCl_2$ (0/2.2 mM), EDTA (1/0 mM), and Na_2ATP (0/1 mM). For competition experiments, membranes (0.4–0.5 and 0.1–0.2 mg of protein/ml for wild-type and mutant, respectively) were added to the same incubation buffer (+MgATP or EDTA), supplemented with the radioligand [^{3}H]-glibenclamide ≈ 5 nM (wild-type) or 2.5 nM (mutant), [^{3}H]P1075 ≈ 1.5 nM], and the inhibitor of interest. After equilibrium had been reached (15 min for [^{3}H]glibenclamide and 25 min for [^{3}H]P1075), incubation was stopped by diluting 0.3-ml aliquots (in triplicate) in 8 ml of ice-cold quench solution [50 mM Tris, 154 mM NaCl, pH 7.4]. Bound and free ligands were separated by rapid filtration over GF/B filters (Whatman, Clifton, NJ), washed twice with quench solution, and counted for ^{3}H in the presence of 6 ml of scintillant (Ultima Gold; Packard Instrument Co., Meriden, CT). Nonspecific binding (B_{NS}) of [^{3}H]glibenclamide/[^{3}H]P1075 was determined in the presence of 100/10 μM P1075 (Hambrock et al., 2001). In the absence of MgATP, specific [^{3}H]glibenclamide binding to wild-type SUR2B was approximately 30% of total binding (B_{TOT}) (Fig. 1), to mutant SUR2B, approximately 75%.

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, $B_{NS} = a \times L$, where a is the proportionality constant. Total binding (B_{TOT}) was then analyzed as the sum of specific and nonspecific binding and was fitted to the equation,

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

to estimate the values of the equilibrium dissociation constant (K_D) and the maximum concentration of binding sites (B_{MAX}, fmol/mg of protein) by the least-squares method. Experiments were performed over a wide range of radioligand concentrations so that all parameters, including B_{NS}, 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 two components,

$$y = 100 - \sum_{i=1}^{2} A_i(1 + 10^{n_i(px - pIC_{50,i})})^{-1}. \quad (2)$$

Here, A_i denotes the amplitude of component i, n_i ($n_{H,i}$) is the Hill coefficient, and $IC_{50,i}$ is the midpoint of component i with $pIC_{50,i} = -\log IC_{50,i}$; x is the concentration of the compound under study with $px = -\log x$. IC_{50} values were converted into inhibition constants (K_i) by correcting for the presence of the radioligand, L, according to the equation

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

where K_D is the equilibrium dissociation constant of the radioligand. In case of homologous competition experiments, the inhibition constant K_i is identical with the K_D value. This correction reached maximally a value of 2.

Data are shown as mean ± S.E.M. Fits of the equations to the data were performed according to the least-squares method using the SigmaPlot program (SPSS Inc., Chicago, IL). Individual binding experiments were analyzed, and the parameters were averaged assuming that amplitudes and pIC_{50} values are normally distributed. Competition experiments were first analyzed using the logistic Hill equation with one component; if necessary, a two-component analysis was used with $n_H = 1$ (eq. 2). The number of components was then determined by the extra sum-of-squares principle (F test) and by the

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Minimum Akaike Information Criterion as described previously (Quast and Mählmann, 1982), with both tests producing identical results. Amplitudes and pIC_{50} values are normally distributed and were compared by the use of a one-way analysis of variance. In the case of only two groups, a two-tailed unpaired Student's t test was used. In this article, IC_{50} values are given with the 95% confidence interval in parentheses. In calculations involving two mean values with standard errors, propagation of errors was taken into account.

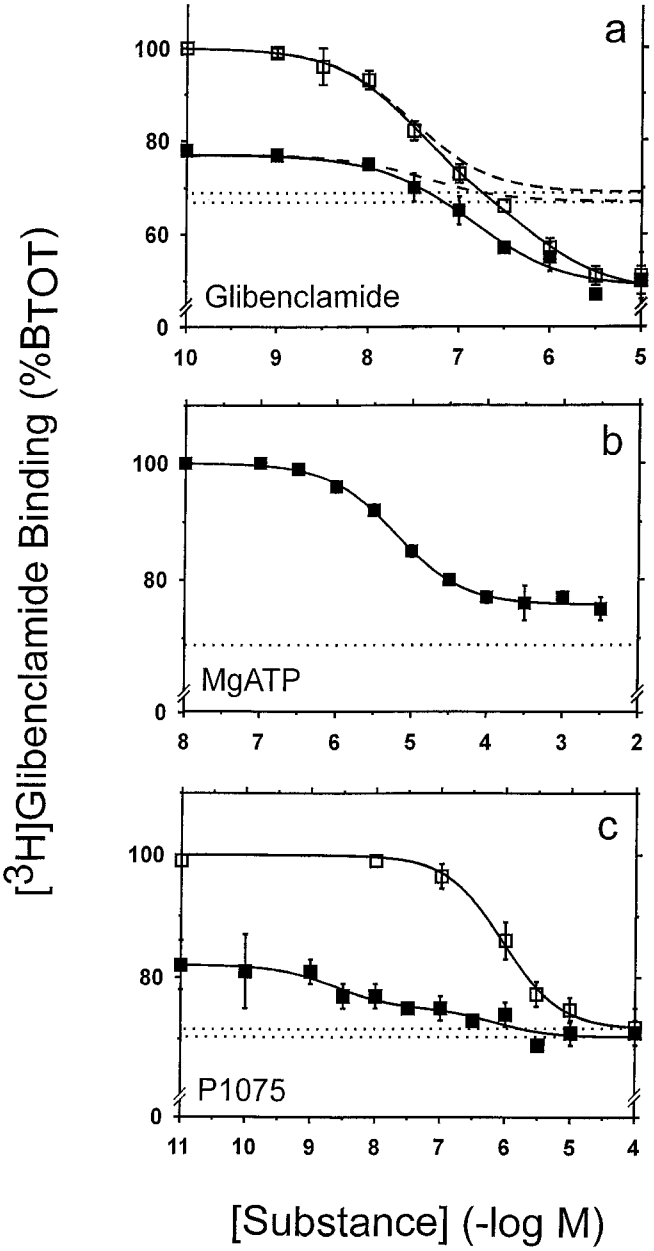

Fig. 1. Properties of [^{3}H]glibenclamide binding to wild-type SUR2B. Data are presented as the percentage of B_{TOT}; B_{NS} was determined in the presence of 100 μM P1075 and is indicated by the dotted lines. Radioligand concentration was 5 nM and B_{TOT} in the absence of MgATP (=100%) was 241 ± 8 fmol/mg of protein (n = 12). Data points in the absence (□) and presence (■) of MgATP (1 mM) are pooled from 4 experiments. a, inhibition of [^{3}H]glibenclamide binding by glibenclamide. Two-component analysis of the curves as indicated in the text gave the parameters (−MgATP/+MgATP), A_1 (% B_{TOT}), 31/10% (fix); $pIC_{50,1}$, 7.57 ± 0.23/7.57 ± 0.12; and A_2 (% B_{TOT}), 25 ± 4/18 ± 2%; $pIC_{50,2}$, 6.21 ± 0.04/6.60 ± 0.23, respectively. The broken curves represent the high-affinity component (binding to SUR2B; Table 1). b, effect of MgATP on [^{3}H]glibenclamide binding. For fitting parameters, see Table 1. c, inhibition by P1075 (fitting parameters in Table 1).

Materials and Solutions. [^{3}H]P1075 [specific activity, 4.5 TBq (117 Ci)/mmol] was purchased from Amersham Buchler (Braunschweig, Germany) and [^{3}H]glibenclamide [specific activity, 1.85 TBq (52 Ci)/mmol] from PerkinElmer Life Sciences (Boston, MA). The reagents and media used for cell culture and transfection were from Invitrogen. Na_2ATP was from Roche Molecular Biochemicals (Mannheim, Germany); glibenclamide was from Sigma Chemie (Deisenhofen, Germany); and P1075 was a kind gift from Leo Pharmaceuticals (Ballerup, Denmark). Glibenclamide and P1075 were dissolved in dimethyl sulfoxide/ethanol (50:50, v/v) to produce stock solutions of 50 mM. These were further diluted with the same solvent or with incubation buffer; the final solvent concentration was lower than 0.3%.

Results

MgATP and [^{3}H]Glibenclamide Binding to SUR2B. Fig. 1a (□) shows the inhibition of [^{3}H]glibenclamide binding by unlabeled glibenclamide in the absence of MgATP (1 mM EDTA, no Mg^{2+}, no ATP added) in membranes from HEK 293 cells expressing SUR2B. Glibenclamide displaced approximately 55% of the radioactivity bound. For an analysis of the inhibition curve, one has to consider that membranes from nontransfected HEK 293 cells and from cells transfected with the expression vector alone possess endogenous glibenclamide binding sites with $K_D \approx 300$ nM (Hambrock et al., 2001). Hence, the [^{3}H]glibenclamide inhibition curve is expected to be biphasic with a specific component representing displacement of the radioligand from SUR2B and a second component representing the endogenous glibenclamide sites. The opener P1075 completely displaces [^{3}H]glibenclamide from the K_{ATP} channel in rat aorta (presumably Kir6.1/SUR2B) and from SUR2B(Y1206S), but it does not affect glibenclamide binding to the endogenous sites (Löffler and Quast, 1997; Hambrock et al., 2001; Fig. 1c). The inhibition curve in Fig. 1a was therefore analyzed for two binding components, with the amplitude of one component kept fixed at the level of P1075-sensitive binding; Hill coefficients were set to 1. The P1075-sensitive component, which represents binding to SUR2B, contributed 30 ± 2% to total binding (Fig. 1a) with a K_D value of 22 nM (95% confidence interval: 8,63) (Table 1). This K_D value, obtained in membranes in the absence of MgATP, is in excellent agreement with the value of 32 nM (16,65) obtained from intact HEK 293 cells stably expressing SUR2B (i.e., in the presence of MgATP) (Russ et al., 1999). The low-affinity component (i.e., the binding to the endogenous HEK 293 cell membrane proteins) contributed approximately 25 ± 4% to total binding (Fig. 1a).

Analogous experiments were performed in the presence of MgATP (1 mM). Figure 1a (■) shows that MgATP reduced glibenclamide-sensitive [^{3}H]glibenclamide binding to 50 ± 10% of that in the absence of MgATP. Although this was quite small in amplitude, two-component analysis was performed as described above, and a K_D value of 22 nM (13,38) was obtained for glibenclamide binding to SUR2B at 1 mM MgATP (i.e., the same value as that obtained in the absence of MgATP). A comparison of the amplitudes of the specific binding component showed that MgATP reduced [^{3}H]glibenclamide binding to SUR2B to 25 ± 5%. Because MgATP did not affect the K_D value, it seems that MgATP reduced [^{3}H]glibenclamide binding to SUR2B by decreasing the apparent number of binding sites without affecting the affinity of the remaining sites. Direct confirmation of this point requires

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saturation binding experiments; in such experiments, however, nonspecific binding would totally obscure specific binding at the radioligand concentrations required.

Fig. 1b presents the concentration dependence of the MgATP effect. In these experiments, MgATP reduced [^{3}H]glibenclamide binding to SUR2B to 26 ± 8% of that in the absence in MgATP, with IC_{50} = 6.2 μM and Hill coefficient ≈ 1 (Table 1).

MgATP and [^{3}H]Glibenclamide Binding to SUR2B-(Y1206S). To obtain more precise information on glibenclamide binding to SUR2B in the presence of MgATP, an improved ratio of specific to nonspecific binding was required. Therefore, further experiments were conducted using the mutant SUR2B(Y1206S). We showed previously that this mutant binds glibenclamide in the presence of MgATP (1 mM) with K_D = 3.4 nM (Hambrock et al., 2001) (i.e., with an approximately 7-fold higher affinity than wild-type SUR2B). Figure 2 shows that MgATP reduced [^{3}H]glibenclamide binding to 26 ± 3% (n = 3), with an IC_{50} of 8.7 μM (6.3,12.0) and a Hill coefficient of 1.20 ± 0.02 (>1, $p < 0.05$). Table 1 shows that these values are in excellent agreement with those obtained with wild-type SUR2B. In 15 additional experiments, it was confirmed that MgATP (1 mM) reduced [^{3}H]glibenclamide binding to 24.0 ± 0.1%.

Using the mutant, it was now possible to perform [^{3}H]glibenclamide saturation binding experiments in the absence and presence of MgATP (1 mM). The inset in Fig. 2 shows these experiments in the Scatchard representation in which the abscissa intercept gives the concentration of binding sites (B_{MAX}) and the slope $1/K_D$. MgATP reduced B_{MAX}, leaving the affinity of the remaining sites for glibenclamide unchanged. Analysis of the individual experiments (untransformed data) gave the following values (−MgATP/+MgATP): K_D= 4.1 (3.0,5.6)/3.9 (3.0,4.8) nM and B_{MAX} = 1150 ± 176/350 ± 29 fmol/mg, respectively (n = 4). Additional experiments (not illustrated) showed that in the absence of Mg^{2+} (presence of 1 mM EDTA), neither ATP nor ADP (1 mM) affected [^{3}H]glibenclamide binding, nor did Mg^{2+} (1.2 mM) in the absence of nucleotides.

Inhibition of [^{3}H]Glibenclamide Binding to Wild-Type and Mutant SUR2B by P1075. The experiments using wild-type SUR2B are illustrated in Fig. 1c. In the absence of MgATP, the inhibition curve was regular, with Hill coefficient ≈ 1 and K_i = 720 nM (300,1740), showing that the opener bound to SUR2B also in the absence of MgATP. In the presence of MgATP, the amplitude was small and the curve very shallow, giving an approximate IC_{50} of ≈100 nM and Hill coefficient ≈ 0.3. The latter indicated either negative cooperativity or heterogeneity of binding sites. The two-component fit shown in Fig. 1c was statistically superior to the Hill fit and gave two amplitudes of similar size with K_i values of 1.7 (0.5,6.3) and 1100 (980,1290) nM (Table 1).

Experiments using mutant SUR2B gave similar results (Fig. 3, □ and ■, and Table 1). In the absence of MgATP, the

TABLE 1

Inhibition of [^{3}H]GBC binding to wild-type and mutant SUR2B by GBC, P1075, and MgATP

$K_D/K_i/IC_{50}$ values are geometric means from three to four independent experiments. A denotes the amplitudes in percentage of specific binding (B_S). Experiments were performed as described in Figs. 1 to 3. With the exception of the K_D values for glibenclamide binding, respective parameters for wild-type and mutant are not different.

Inhibitor	SUR2B Wild-Type			SUR2B(Y1206S)		
	$K_D/K_i/IC_{50}$	A	n_H	$K_D/K_i/IC_{50}$	A	n_H
		%B_S			%B_S	
GBC (MgATP = 0 mM)	22 (8,63) nM	100	1	4.1 (3.0,5.6) nM	100	1
(MgATP = 1 mM)	22 (13,38) nM	100[a]	1	3.9 (3.0,4.8) nM	100[b]	1
P1075 (MgATP = 0 mM)	0.72 (0.30,1.74) μM	100	0.9 ± 0.1	1.2 (1.1,1.3) μM	100	1.1 ± 0.1
(MgATP = 1 mM)	1.7 (0.5,6.3) nM	54 ± 9	1	5.9 (3.5,9.8) nM	46 ± 4	1
	1.1 (1.0,1.3) μM	46 ± 9	1	0.71 (0.49,1.0) μM	54 ± 4	1
MgATP	6.2 (3.4,11) μM	74 ± 8	1.1 ± 0.1	8.7 (6.3,12) μM	74 ± 3	1.2 ± 0.1

100% B_S in the presence of MgATP (1 mM) corresponds to:
[a], 25 ± 5 %B_S in the absence of MgATP.
[b], 24 ± 1 %B_S in the absence of MgATP.

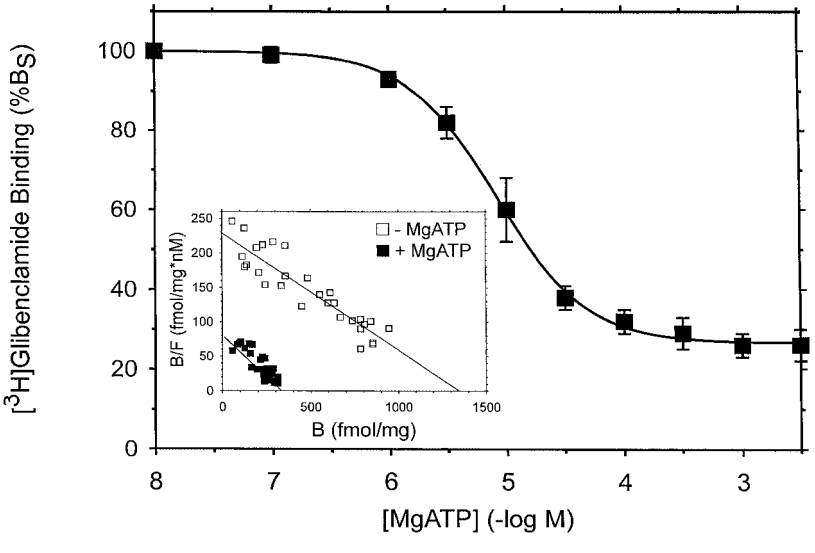

Fig. 2. Effect of MgATP on [^{3}H]glibenclamide binding to SUR2B(Y1206S). Data are means ± S.E.M. from three experiments and expressed as the percentage of specific binding. Total binding was 518 ± 13 fmol/mg of protein, and nonspecific binding was 25 ± 12% of B_{TOT}; free Mg^{2+} concentration was 1.2 mM, and the [^{3}H]glibenclamide concentration was 2.5 nM. The fit of the Hill equation to the data gave an IC_{50} of 8.7 μM and a Hill coefficient of 1.2 (Table 1). Inset, Scatchard representation of [^{3}H]glibenclamide saturation experiments in the absence (□) and presence (■) of 1 mM MgATP (pooled data from four experiments). The fit gave K_D values of 5.8 (5.0,7.0)/4.1 (3.4,5.3) nM and B_{MAX} values of 1337 and 367 fmol/mg of protein in absence and presence of 1 mM MgATP, respectively. See *Results* for parameters from untransformed data.

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inhibition curve was monophasic (Hill coefficient, 1.1 ± 0.1), with K_i = 1.2 μM (Table 1). In the presence of MgATP, the inhibition curve was strongly biphasic with the high-affinity component comprising 46 ± 4% of the total amplitude with a K_i value of 5.9 nM; the low-affinity component gave a K_i value of 710 nM (Fig. 3 and Table 1) (Hambrock et al., 2001).

For mechanistic reasons, it was of interest to determine the state of the [^{3}H]glibenclamide binding sites when the high-affinity component of the P1075 inhibition curve in the presence of MgATP had reached completion (i.e., was the reduction in [^{3}H]glibenclamide binding induced by P1075 in this component because of a decrease in affinity or number of sites for the radioligand). To answer this, [^{3}H]glibenclamide saturation binding experiments were performed in the presence and absence of P1075 (100 nM); at this concentration, the high-affinity component was complete (Fig. 3). The mean parameters from three such experiments were (−/+P1075): K_D = 3.9 (3.2,4.7)/9.3 (7.1,12.3) nM and B_{MAX} = 454 ± 24/395 ± 18 fmol/mg of protein. Figure 4 gives a typical example of these experiments, which were difficult because in the presence of MgATP and P1075, [^{3}H]glibenclamide binding is low. The data show that by the completion of the high-affinity component, P1075 has increased the K_D value of [^{3}H]glibenclamide binding 2.4 times, leaving the B_{MAX} value essentially unchanged. An easy calculation based on eq. 1 shows that increasing the K_D value from 3.9 to 9.3 nM at constant B_{MAX} decreases the binding of 2 nM [^{3}H]glibenclamide by ≈50%, thus quantitatively accounting for the amplitude of the high-affinity component.

Discussion

Modeling. This study has produced two major findings. First, MgATP decreased [^{3}H]glibenclamide binding to 25% by reducing the number of binding sites. This means that MgATP induced a heterogeneity in the previously homogeneous and independent glibenclamide sites, leaving one of four sites unchanged and shifting the other three to a low-affinity state so that they were no longer detected by the binding assay. Second, for both mutant and wild-type SUR2B, the class of high-affinity glibenclamide sites remaining in the presence of saturating MgATP was inhibited by P1075 in a biphasic manner.

As an explanation, one might envisage a two-state scheme in which SUR exists in two states, R_1 and R_2. R_1, which predominates in the absence of MgATP, has a high affinity

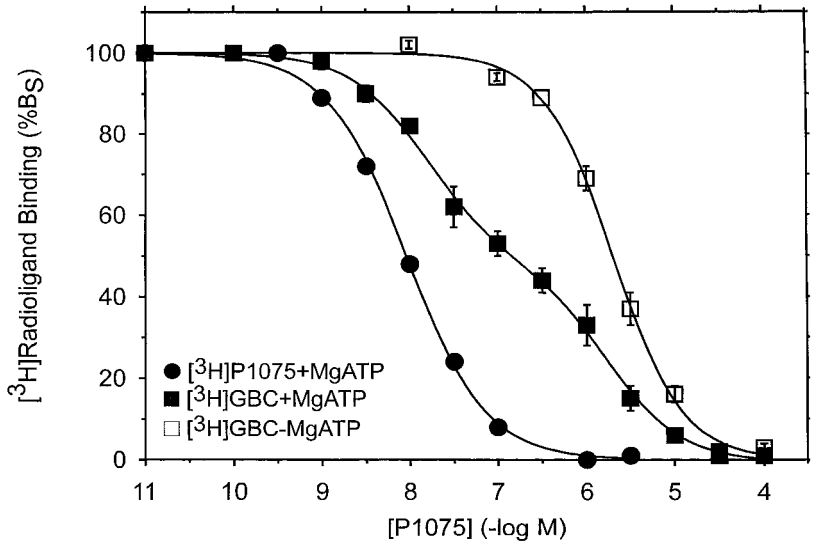

Fig. 3. Binding of P1075 to SUR2B(Y1206S) as seen in competition assays with [^{3}H]P1075 in the presence of 1 mM MgATP (●) and with [^{3}H]glibenclamide in the presence (■) and absence (□) of MgATP (1 mM). Data are expressed as specific binding (% B$_S$) (n = 4–5). Parameters derived from the fit of the Hill equation are listed in Table 1. The [^{3}H]P1075 concentration was 1.5 to 2.0 nM, and [^{3}H]glibenclamide concentration was 2.0 to 3.0 nM.

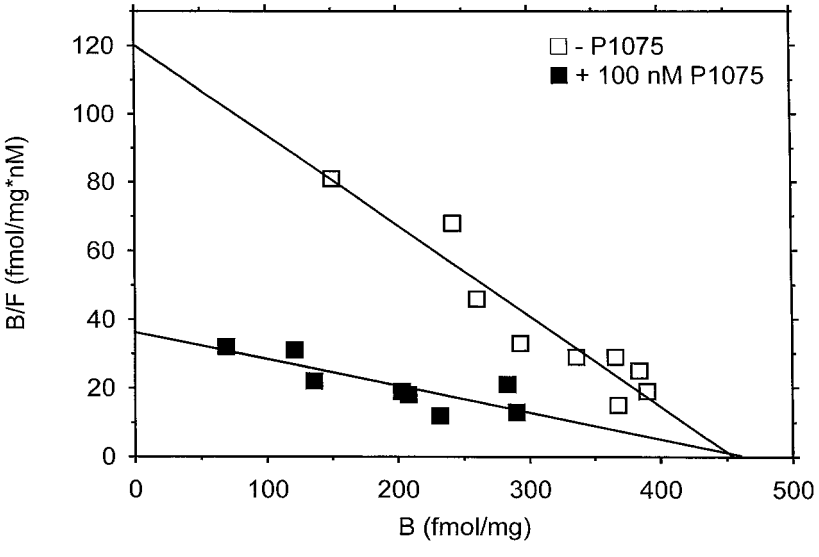

Fig. 4. [^{3}H]glibenclamide binding saturation experiment in the presence and absence of P1075 (100 nM) in the Scatchard representation. The fit of eq. 1 to the untransformed data gave the following parameters (−P1075/+P1075), K_D = 3.5 ± 0.6/11 ± 3 nM and B_{MAX} 444 ± 19/413 ± 69 fmol/mg of protein (difference in B_{MAX} was not significant). The experiment is typical of three (see text for mean parameter values).

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for SUs and a low affinity for openers. MgATP then acts as the allosteric modifier, shifting the equilibrium toward R_2, which has a low affinity for SUs and a high affinity for openers. If each reaction step of the scheme is at equilibrium (principle of detailed balance), the model predicts that MgATP shifts the glibenclamide binding curve in a *homogeneous* manner toward higher concentrations, leaving no fraction with unchanged affinity; in addition, monophasic inhibition curves of glibenclamide binding by openers are predicted (Janin, 1973; Boeynaems and Dumont, 1980). A more realistic model takes into account that the transition $R_1 \rightarrow R_2$ is fueled by the hydrolysis of ATP (Bienengraeber et al., 2000; Zingman et al., 2001) and is irreversible at high MgATP concentrations.[1] Conversely, glibenclamide binding leads to the release of ATP from SUR (Ueda et al., 1999) and the SUR-glibenclamide complex R_2G may return to the ground state: $R_2G \rightarrow R_1G$. In such an open system, in which an influx of energy drives the reaction cycle, a monomeric receptor with a single ligand binding site can display cooperativity in ligand binding at steady state (Boeynaems and Dumont, 1980).[1] This particular model does not fit the data. Ironically, the scheme in which MgATP hydrolysis drives the $R_1 \rightleftharpoons R_2$ transitions in the opposite direction (i.e., glibenclamide binding favors MgATP hydrolysis) and in which the high-affinity state for openers is R_1 (i.e., the state on the absence of MgATP) could be perfectly fitted to the data in Figs. 1 through 3 (U. Quast, unpublished observations). We have not found a modification of such an energy-driven mechanism that is in accordance with both the data and the biochemical knowledge of SUR.

A mechanism meeting these requirements assumes that SUR forms multimers with allosteric coupling of binding sites on different subunits. Taking into account that the completely assembled channel contains four SURs (Clement et al., 1997; Shyng and Nichols, 1997) and that isolated nucleotide binding fold-1 of SUR1 spontaneously forms tetramers (Mikhailov and Ashcroft, 2000), such homomers might be tetramers (Fig. 5). One could speculate that such a tetramer serves as an outer shell in which the four Kir channels assemble to form the complete channel. It should be noted that although the tetramer model explains the data (see below), biochemical evidence for tetramer formation of SUR expressed alone has not yet been presented and that alternative explanations of the data may be possible.

Negative Allosteric Coupling between Nucleotide and Glibenclamide Sites. First, the tetramer model has to explain that MgATP affects three of the four binding sites of tetrameric SUR2B, leaving one site unchanged. One may speculate that the nucleotide induces a strong negative allosteric coupling between the four glibenclamide sites such that binding of a first glibenclamide molecule greatly impedes binding of the other three, and an asymmetry is induced. Alternatively, MgATP could lead to a major symmetrical rearrangement of the tetramer so that only one site remains sterically accessible. In this case, however, it is not easy to explain that the remaining site has the same affinity for glibenclamide as the four sites in the absence of MgATP. Whatever the mechanism, if in the presence of MgATP only one glibenclamide molecule binds with high affinity to tetrameric SUR2B, then this most likely holds true for the complete channel. If so, binding of one glibenclamide molecule per channel should induce high-affinity channel block (in the presence of MgATP). This stoichiometry is in agreement with that found by Dörschner et al. (1999) for glibenclamide block of the Kir6.2/SUR1 channel in the absence of MgATP, but it is in contrast to our earlier proposal that binding of four molecules of glibenclamide is required for block of the Kir6.1/SUR2B channel in intact cells (Russ et al., 1999). Obviously, this proposal, determined from the comparison of binding and channel inhibition curves, is not compatible with the tetramer model.

The negative allosteric coupling between nucleotides and [^{3}H]glibenclamide binding has also been observed with SUR1. At SUR1-containing channels, nucleotides reduced [^{3}H]glibenclamide binding (Niki et al., 1990; Schwanstecher et al., 1991, 1992b); this was recently confirmed using recom-

[1] We thank an anonymous reviewer for indicating this.

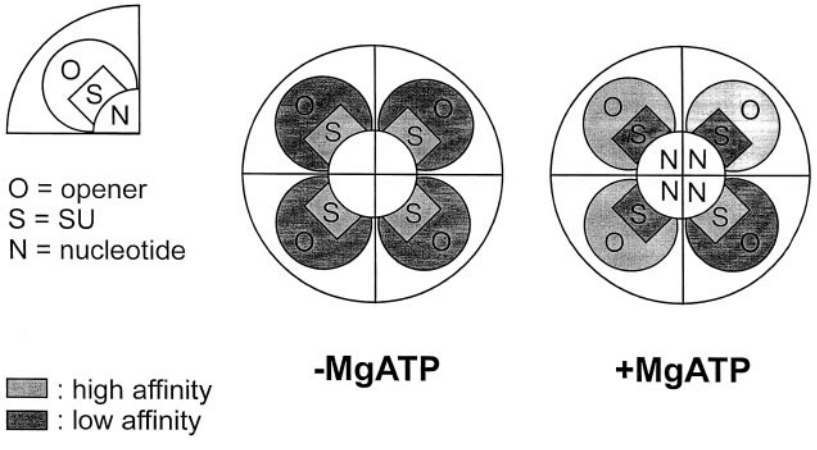

Fig. 5. Proposed model of allosteric interactions between nucleotide (N), opener (O), and sulfonylurea (S) binding to tetrameric SUR2B. Left, monomeric SUR2B has binding sites for N, S, and O, which are linked by positive (N-O) and negative (N-S, O-S) allosteric interactions within the monomer. Middle, in the absence of MgATP, tetrameric SUR has four equal and independent high-affinity binding sites for S; the four opener binding sites are in a low-affinity state. Right, in the presence of MgATP, only one S-site (the site to which the first glibenclamide is bound) remains in the high-affinity state; the three other S-sites are shifted toward low affinity by strong negative allosteric interactions between subunits. The opener sites are switched on (high-affinity state) with the exception of the O-site on the subunit where S is bound. Occupation of the three high-affinity O-sites reduces the affinity of the fourth subunit for S by 2.4-fold, demonstrating weak negative allosteric interactions between the O and S sites on different subunits in the presence of MgATP. The scheme is not meant to represent the stoichiometry of nucleotide binding to $[SUR2B]_4$.

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binant SUR1 expressed alone (A. Hambrock, C. Löffler-Walz, and U. Quast, in preparation). Conversely, glibenclamide induced the release of bound ATP from SUR1 (Ueda et al., 1999). In contrast to our observations with SUR2B, however, nucleotides acted at SUR1 by homogeneously reducing the affinity of all glibenclamide sites, leaving their number unchanged [MgATP (Schwanstecher et al., 1992b; A. Hambrock, C. Löffler-Walz, U. Quast, in preparation) and MgADP (Niki et al., 1990)]. This indicates a profound difference between the SUR subtypes.

Inhibition of [^{3}H]Glibenclamide Binding by P1075. In the presence of MgATP, the inhibition curve of P1075 was biphasic. Within the framework of the tetramer model, one has to assume that binding of one glibenclamide molecule per tetramer not only affects the other three glibenclamide sites (see above), but it creates an asymmetry in the previously homogeneous four opener sites: it leaves some in the original high-affinity state and moves others into a low-affinity state. It seems plausible to assume that all opener sites are in the high-affinity state except for the one at the subunit occupied by glibenclamide (Fig. 5). The high-affinity component of the [^{3}H]glibenclamide-opener inhibition curve in the presence of MgATP then reflects opener binding to the three high-affinity sites. This weakens the affinity of glibenclamide binding by a negative allosteric interaction between subunits of the tetramer, and the increase in K_D causes the reduction in [^{3}H]glibenclamide binding of this component. The low-affinity component reflects opener binding to the last (low-affinity) site and leads to complete displacement of [^{3}H]glibenclamide from SUR. The experiments presented in Fig. 4 quantitatively support this model.

The model described in Fig. 5 predicts that at equilibrium, mixed states of tetrameric SUR2B exist in which P1075 and glibenclamide sites at different subunits are occupied simultaneously. Hence, tetrameric SUR is not described by the concerted transition model of Monod et al. (1965) (Janin, 1973), which predicts that the four subunits are all either in the opener- or the glibenclamide-binding conformation, but by the induced-fit model (sequential model) of Koshland et al. (1966). The negative allosteric interactions between the glibenclamide sites are strong (see above); in contrast, interactions between P1075 and glibenclamide sites on different subunits are subtle: the occupation of the (high-affinity) opener sites by P1075 increases the K_D of glibenclamide binding to an opener-free subunit 2.4 times (Fig. 4). Such allosteric interactions between SUR subunits in the presence of MgATP have also been observed in [^{3}H]glibenclamide-P1075 and [^{3}H]P1075-glibenclamide competition experiments upon coexpression of mutant and wild-type SUR2B with Kir6.x. They depended on the subtype of Kir6.x and were more prominent in whole cells than in membranes (Hambrock et al., 2001). Hence, in the whole channel, allosteric interactions between SUR2B subunits exist and are modulated by the Kir subunit in a subtype-specific way.

MgATP-Free State of SUR. In the absence of MgATP, the binding sites of wild-type and mutant SUR2B for [^{3}H]-glibenclamide were homogenous and independent; in addition, P1075 displaced the radioligand with monophasic inhibition curves. This suggested that in the absence of MgATP, there was no allosteric interaction between the glibenclamide sites neither within the tetramer nor between the glibenclamide and the P1075 sites on different subunits. The K_i value of P1075 against [^{3}H]glibenclamide in the absence of MgATP was similar to that of the low-affinity component of the inhibition curve in the presence of MgATP. In both cases, one monitors opener binding to a SUR subunit occupied by glibenclamide. It seems that the presence of glibenclamide modified the opener site of this subunit profoundly, rendering opener binding difficult. This modification was similar in the presence or absence of MgATP. The lack of subunit interactions in the absence of MgATP also explains that [^{3}H]glibenclamide-P1075 inhibition experiments under these conditions do not provide information on how the opener interacts with the glibenclamide-free subunits of the tetramer.

In conclusion, the negative allosteric coupling between MgATP and glibenclamide binding and the biphasic opener inhibition curve can be explained by a tetrameric model of SUR2B in which MgATP induces allosteric interactions between the subunits. Direct evidence for tetramer formation of SUR2B is desirable.

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