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Coexpression with the Inward Rectifier K^+ Channel Kir6.1 Increases the Affinity of the Vascular Sulfonylurea Receptor SUR2B for Glibenclamide

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ABSTRACT

ATP-sensitive K^+ channels are closed by the hypoglycemic sulfonylureas like glibenclamide (GBC) and activated by a class of vasorelaxant compounds, the K^+ channel openers. These channels are octamers of Kir6.x and sulfonylurea receptor (SUR) subunits with 4:4 stoichiometry. The properties of the opener-sensitive K^+ channel in the vasculature are well matched by the SUR2B/Kir6.1 channel; however, the GBC sensitivity of the recombinant channel is unknown. In binding experiments we have determined the affinity of GBC for SUR2B and the SUR2B/Kir6.1 channel and compared the results with the channel blocking potency of GBC. All experiments were performed in whole transfected human embryonic kidney cells at 37°C. The equilibrium dissociation constants (K_D) of GBC binding to SUR2B and to the SUR2B/Kir6.1 complex were determined to be 32 and 6 nM, respectively; the K_D value of the opener P1075 (*N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*′′-3-pyridylguanidine) (≈5 nM) was, however, not affected by cotransfection. In whole cell voltage-clamp experiments, GBC inhibited the SUR2B/Kir6.1 channel with $IC_{50} \approx 43$ nM. The data show that, in the intact cell: 1) SUR2B, previously considered to be a low-affinity SUR, has a rather high affinity for GBC; 2) coexpression with the inward rectifier Kir6.1 increases the affinity of SUR2B for GBC; 3) the recombinant channel exhibits the same GBC affinity as the opener-sensitive K^+ channel in vascular tissue; and 4) the K_D value of GBC binding to the octameric channel is 7 times lower than the IC_{50} value for channel inhibition. The latter finding suggests that occupation of all four GBC sites per channel is required for channel closure.

ATP-sensitive K^+ channels (K_{ATP} channels) are a family of K^+ channels with weak inward rectification that are gated by intracellular nucleotides and couple cell metabolism to membrane potential. Pharmacologically, they are closed by the hypoglycemic sulfonylureas like glibenclamide (GBC) and activated by the K_{ATP} channel openers, which act preferentially in the vasculature producing hypotension (Ashcroft and Ashcroft, 1990; Quast, 1992; Edwards and Weston, 1993; Quayle et al., 1997). Most studies (for review, see Quayle et al., 1997) agree that the opener-activated channel in the vasculature has a low unitary conductance (15–30 pS, Kajioka et al., 1991; Beech et al., 1993b; Kamouchi and Kitamura, 1994). It is relatively insensitive to inhibition by ATP (Beech et al., 1993a; Xu and Lee, 1994) and is activated by nucleoside diphosphates in the presence of Mg^{2+} (Beech et al., 1993a); therefore, it has also been termed nucleoside diphosphate-dependent K^+ channel (K_{NDP}; Beech et al., 1993a). If no opener is present, the K_{NDP} channel is inhibited by GBC with $IC_{50} \approx 20$ to 40 nM (Beech et al., 1993a; Xu and Lee, 1994; Quast, 1996); in the presence of opener, higher concentrations of GBC are required (e.g., Beech et al., 1993b; Quast, 1996). Using a [^{3}H]GBC binding assay, we have identified a GBC binding site in rat aorta with $K_D = 20$ nM, that matches exactly the pharmacological profile of the vascular K_{NDP} channel (Quast et al., 1993; Löffler and Quast, 1997).

The K_{ATP} channel in several tissues has been shown to be a heteromultimeric complex of sulfonylurea receptor (SUR) and inwardly rectifying K^+ channel (Kir6.x) subunits (reviews: Ashcroft and Gribble, 1998; Babenko et al., 1998). For the pancreatic β-cell K_{ATP} channel, which is composed of SUR1 and Kir6.2, an octameric structure with 4:4 stoichiometry $(SUR1/Kir6.2)_4$ has been proven (Clement et al., 1997;

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ABBREVIATIONS: B_{TOT}, total binding; GBC, glibenclamide; GFP, green fluorescent protein; HEK cells, human embryonic kidney cells; K_{ATP} channel, ATP-sensitive K^+ channel; K_D, equilibrium dissociation constant of the radioligand; P1075, *N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*′′-3-pyridylguanidine); PSS, physiological salt solution; SUR, sulfonylurea receptor; K_{NDP}, nucleoside diphosphate-dependent K^+ channel; $I_{K,\ NDP}$, nucleoside diphosphate-activated K^+ current.

Inagaki et al., 1997; Shyng and Nichols, 1997). The Kir6.x subunits presumably form the pore of the channel and determine the sensitivity of the channel to inhibition by ATP. Electrophysiological experiments (Ashcroft and Gribble, 1998; Babenko et al., 1998) and radioligand binding assays (Hambrock et al., 1998, 1999; Schwanstecher et al., 1998) have shown that SUR is endowed with the binding sites for the openers and the sulfonylureas that mediate the pharmacological effects of these compounds. From the recombinant K_{ATP} channels known to date, the construct SUR2B/Kir6.1 matches best the properties of the K_{NDP} channel (Yamada et al., 1997). The recombinant channel has a unitary conductance of 33 pS in high-K^+ solution and is activated by low (μM), but inhibited by high (mM) concentrations of ATP. It is inhibited by 10 μM GBC (Yamada et al., 1997; Satoh et al., 1998); its exact GBC sensitivity, however, is not yet known.

If one wants to ascertain that the SUR2B/Kir6.1 channel represents indeed the vascular K_{NDP} channel, the precise determination of its GBC sensitivity is of importance. Binding experiments using the radiolabeled opener, [^{3}H]P1075 [[^{3}H]*N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*′′-3-pyridylguanidine; Bray and Quast, 1992; Manley et al., 1993] showed that in membranes from cells expressing SUR2B alone, GBC inhibited opener binding with K_i values ranging from 0.3 (Schwanstecher et al., 1998) to 2.4 μM (Hambrock et al., 1998). This is difficult to reconcile with the fact that GBC blocks the native K_{NDP} channels with IC_{50} values between 20 and 40 nM (see above). To resolve this question we have performed binding studies in human embryonic kidney (HEK) cells transfected with SUR2B or SUR2B + Kir6.1 using [^{3}H]GBC as the radiolabel; the results were compared with binding studies using [^{3}H]P1075. In addition, the GBC sensitivity of the current through SUR2B/Kir6.1 channels was measured. It is known that the binding affinity of GBC to the vascular K_{ATP} channel (Löffler-Walz and Quast, 1998) and the potency of GBC in blocking the cardiac K_{ATP} channel (Brady et al., 1996; Yokoshiki et al., 1997) depend on the presence of an intact actin cytoskeleton. Therefore, all experiments were performed in whole cells and at 37°C. The results show that coexpression with Kir6.1 increases the affinity of SUR2B for GBC and that the GBC sensitivity of the recombinant channel is similar to that reported for the native K_{NDP} channel. When this manuscript was being submitted, a paper by Dörschner et al. (1999) appeared that addresses similar questions. The results of their study, which was conducted under quite different conditions, differ in central aspects from ours (see *Discussion*).

Experimental Procedures

Cell Culture and Transfection. HEK 293 cells were cultured in plastic dishes with a diameter of 9.4 cm at 37°C in a humidified atmosphere with 95% air and 5% CO_2 in Minimum Essential Medium containing glutamine and supplemented with 10% fetal bovine serum and 20 μg/ml gentamycin. Cells were transfected with the pcDNA 3.1 vector (Invitrogen, San Diego, CA) containing the coding sequence of murine SUR2B and murine Kir6.1 (GenBank accession numbers D86038 and D88159, respectively; Isomoto et al., 1996; Yamada et al., 1997). Cell lines stably transfected with SUR2B were isolated as described previously (Hambrock et al., 1998). Transient transfections were performed using lipofectAMINE and OPTIMEM (Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. HEK cells were cotransfected with SUR2B + Kir6.1 at a molar plasmid ratio of 1:1 or 1:4. In general, pEGFP-C1 vector (Clontech, Palo Alto, CA), encoding for green fluorescent protein (GFP), was added for easy identification of transfected cells; this did not affect the results of the binding studies. Cells were allowed to express transfected DNA for 48 h and were then used for binding studies and electrophysiological experiments.

Equilibrium Competition Experiments. Transiently transfected HEK 293 cells were harvested 48 h after transfection at 60 to 80% confluence, nontransfected cells or permanently transfected cells at a similar density. Cells were suspended by rinsing with a HEPES-buffered physiological salt solution (PSS) containing 139 mM NaCl; 5 mM KCl; 1.2 mM $MgCl_2$; 1.25 mM $CaCl_2$; 11 mM D(+)-glucose; 5 mM HEPES; gassed with 95% O_2/5% CO_2; and titrated to pH 7.4 with NaOH at 37°C. After two centrifugations at 500*g* for 5 min, cells were resuspended in PSS. Incubation was started by the addition of cells (final concentrations 2×10^6 and 3×10^6 cells/ml for SUR2B and SUR2B/Kir6.1, respectively, corresponding to 0.5 and 0.7 mg protein/ml) to PSS containing 2.0 to 4.1 nM [^{3}H]GBC or 1.0 to 1.5 nM [^{3}H]P1075 and the inhibitor of interest in a total volume of 1 ml at pH 7.4 and 37°C. 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 by rapidly filtrating under a 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 Instruments, Meriden, CT). In case of [^{3}H]P1075 experiments, nonspecific binding was determined in the presence of 10 μM unlabeled P1075; in the [^{3}H]GBC studies, nonspecific binding could not be determined because at the highest concentration of GBC soluble (200 μM), low-affinity binding was not yet saturated.

Patch-Clamp Experiments. The patch-clamp technique was used in the whole-cell configuration as described by Hamill et al. (1981) with an extracellular buffer containing 142 mM NaCl; 2.8 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 11 mM D(+)-glucose; 10 mM HEPES; titrated to pH 7.4 with NaOH. Temperature in the bath was 37°C. Patch pipettes were drawn from filament borosilicate glass capillaries (GC 150F-15; Clark Electromedical Instruments, Pangbourne, UK) and heat polished using a horizontal microelectrode puller (Zeitz, Augsburg, Germany). After filling with 132 mM K-glutamate; 10 mM NaCl; 2 mM $MgCl_2$; 10 mM HEPES; 1 mM ethylene glycol-bis-(2-aminoethyl ether)-*N*, *N*,*N*′,*N*′-tetraacetic acid; 1 mM Li_2GDP; 0.3 mM Na_2ATP; titrated to pH 7.2 with NaOH, pipettes had a resistance of 3 to 5 MΩ. Data were recorded with an EPC 9 (HEKA, Lambrecht, Germany) amplifier using Pulse software (HEKA). Cell capacitance (6–22 pF) and series resistance (5–14 MΩ) were measured before the start of each pulse train with the EPC9 amplifier-system. Series resistance was compensated by 70% with the compensation circuit of the EPC9 patch-clamp amplifier. The voltage potentials were corrected for a liquid junction potential of +10 mV. Two to three days after transfection, isolated cells showing GFP fluorescence were clamped to −60 mV and every 12 s seven square pulses ranging from −110 to 10 mV (0.5 s each) were applied. Signals were sampled with 1 kHz and filtered at 200 Hz using the four-pole Bessel filter of the EPC9 amplifier. For evaluation of the GBC-block, the current at −60 mV was used.

Data Analysis and Modeling. Equilibrium inhibition curves were analyzed according to the logistic equation for up to three components:

$$y = 100 - \sum_{i=1}^{3} A_i(1 + 10^{n_i(px - pIC_{50,i})})^{-1}; \quad i = 1 - 3 \qquad (1)$$

Here A_i denotes the amplitude of component i, n_i (= $n_{H,\,i}$) the Hill coefficient, and $IC_{50,i}$ the midpoint of component i with $pIC_{50,i} = -\log IC_{50,i}$; x is the concentration of the compound under study with $px = -\log x$. Because IC_{50} values are log-normally distributed (Christopoulos, 1998), IC_{50} values followed by the 95% confidence interval

in parentheses are given in the text. IC_{50} values were converted into K_i by correcting for the presence of the radioligand, L, according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50}(1 + L/K_D)^{-1} \quad (2)$$

where K_D is the equilibrium dissociation constant of the radioligand. In case of homologous competition experiments, K_i is identical with the K_D value.

Fits of the equations to the data were performed according to the method of least-squares using the FigP program (Biosoft, Cambridge, UK). Errors in the parameters derived from the fit to a single curve were estimated using the univariate approximation (Draper and Smith, 1981).

Materials. [^{3}H]P1075 [specific activity = 4.5 TBq (121 Ci)/mmol] was purchased from Amersham Buchler (Braunschweig, Germany) and [^{3}H]GBC [specific activity = 1.85 TBq (50 Ci)/mmol] from DuPont-NEN (Bad Homburg, Germany). The reagents and media used for cell culture and transfection were obtained from Life Technologies. Na_2ATP and Li_2GDP were purchased from Boehringer Mannheim (Mannheim, Germany) and GBC from Sigma (Deisenhofen, Germany). P1075 was a gift from 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 ≤0.1‰.

Results

[^{3}H]P1075 Binding Experiments. Specific binding of [^{3}H]P1075 was only found in HEK cells transfected with SUR2B or SUR2B/Kir6.1; in nontransfected cells and in cells expressing Kir6.1 alone, no specific binding was detected. Figure 1 shows the inhibition of specific [^{3}H]P1075 binding by P1075 and GBC in HEK cells transfected with SUR2B and with SUR2B + Kir6.1. The inhibition curves of P1075 were closely together with K_D values of 4.3 (SUR2B) and 5.8 nM (cotransfection, see Table 1). For GBC, however, there was a significant difference; in cells transfected with SUR2B, GBC inhibited [^{3}H]P1075 binding with a K_i value of 1 μM; coexpression with Kir6.1 reduced the K_i value to 280 nM. In these experiments, cotransfection was performed at a molar plasmid ratio of 1:1 and it was uncertain whether all SUR2B was complexed with Kir6.1; therefore, cells were also transfected with a SUR2B to Kir6.1 plasmid ratio of 1:4. Figure 1 shows that this did not change the K_i value of GBC more, indicating that at a molar ratio of 1:1, most SUR2B existed as the complex with Kir6.1. For additional experiments, cotransfections were therefore performed at the ratio of 1:1. In the course of these experiments we noted that total [^{3}H]P1075 binding was considerably reduced when SUR2B was coexpressed with Kir6.1 at a ratio of 1:1 and even more at a ratio of 1:4 [see total binding (B_{TOT}) values in the legend to Fig. 1]. This may in part reflect differences between permanently (SUR2B) and transiently (cotransfection) transfected cells; in

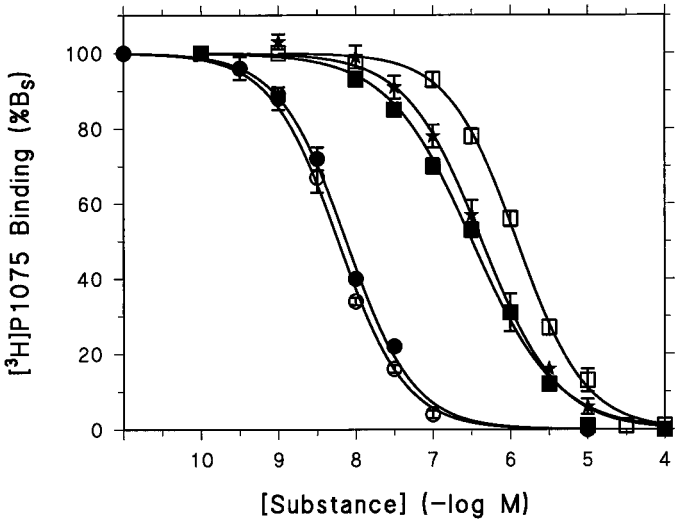

Fig. 1. Inhibition of specific [^{3}H]P1075 binding by P1075 and GBC in HEK cells transfected with SUR2B and SUR2B + Kir6.1 at 37°C. SUR2B: P1075, ○; GBC, □; cotransfection with SUR2B + Kir6.1 at a ratio of 1:1: P1075, ●; GBC, ■; cotransfection with a ratio 1:4: GBC, ⋆. Data are means ± S.E. from three to five experiments, and fits of the logistic equation with one component to the data are shown. IC_{50} values were corrected for the presence of the radiolabel according to eq. 2 and are listed in Table 1; Hill coefficients were close to 1. [^{3}H]P1075 concentration was 1.0 (SUR2B) or 1.5 nM (cotransfection); B_{TOT} (fmol/mg) was 248 ± 24 (SUR2B), 61 ± 6 (cotransfection 1:1), and 15 ± 8 (cotransfection 1:4); nonspecific binding was 8 ± 1, 15 ± 3, and 35 ± 15% of B_{TOT}, respectively.

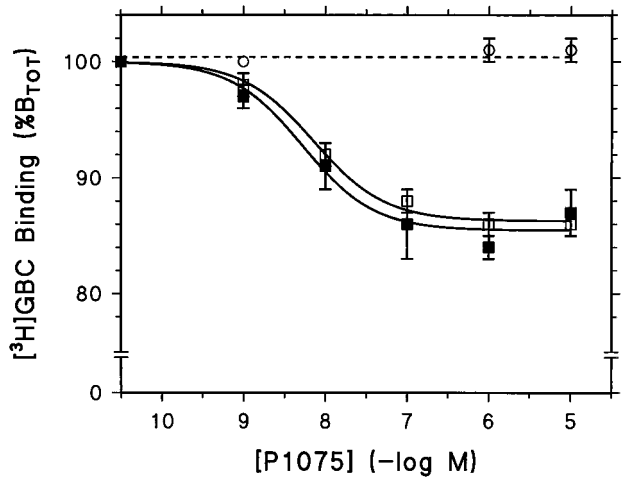

Fig. 2. Inhibition of total [^{3}H]GBC binding to HEK cells by P1075. Control cells, ○; cells transfected with SUR2B, □; and with SUR2B + Kir6.1, ■. The fit of the logistic equation with one component and Hill coefficient 1 to the data gave for SUR2B- and SUR2B + Kir6.1-transfected cells amplitudes of 14 ± 1 and 15 ± 1% B_{TOT}. The K_i values derived from the fits are listed in Table 1. [^{3}H]GBC concentrations and B_{TOT} values were 3.4 nM/163 ± 3 fmol mg^{-1} (SUR2B) and 4.1 nM/160 ± 9 fmol mg^{-1} (cotransfection); data are means from three to four experiments.

TABLE 1

[^{3}H]P1075 and [^{3}H]GBC binding to HEK cells transfected with SUR2B and SUR2B + Kir6.1

Competition experiments were performed as described in Figs. 1 to 3. K_D and K_i values (with 95% confidence intervals in parentheses) were calculated from the corresponding IC_{50} values according to eq. 2 and are given in nanomolar concentrations.

	[^{3}H]P1075		[^{3}H]GBC	
	K_D (P1075)	K_i (GBC)	K_D (GBC)	K_i (P1075)
SUR2B	4.3 (3.5, 5.1)	1000 (830, 1200)	32 (16, 65)	6.6 (5.0, 8.7)
SUR2B + Kir6.1	5.8 (5.2, 6.3)	280 (260, 310)[a]	6.3 (3.5, 11.5)	3.2 (1.6, 6.4)

[a] Average of the values from cells cotransfected with SUR2B + Kir6.1 at molar plasmid ratios of 1:1 (269 nM) and 1:4 (282 nM).

addition, the protein synthesizing machinery of the cell may become limiting so that less SUR2B is expressed at higher Kir/SUR ratios.

[^{3}H]GBC Binding Experiments. [^{3}H]GBC binding was found in control and transfected HEK cells and it was first examined whether openers interfered with this binding. P1075 (10 μM) did not displace any radioactivity in control cells and cells transfected with Kir6.1. However, in cells transfected with SUR2B or SUR2B + Kir6.1, the opener reduced total [^{3}H]GBC binding by 15 ± 2% (n = 13, SUR2B) and 15 ± 1% (n = 10, cotransfection); levcromakalim and diazoxide produced similar inhibitions (≈13%). The effect of P1075 was examined in detail. Figure 2 shows inhibition curves with P1075, giving K_i values of 6.6 and 3.2 nM in cells expressing SUR2B and SUR2B/Kir6.1, respectively. These values are in good agreement with those obtained in the homologous competition studies using [^{3}H]P1075 (Fig. 1). This suggested that the P1075-sensitive fraction of total [^{3}H]GBC binding in transfected cells represents GBC binding to SUR2B (or to the SUR2B-Kir6.1 complex).

Figure 3 illustrates homologous competition experiments of [^{3}H]GBC with GBC in control and cells transfected with

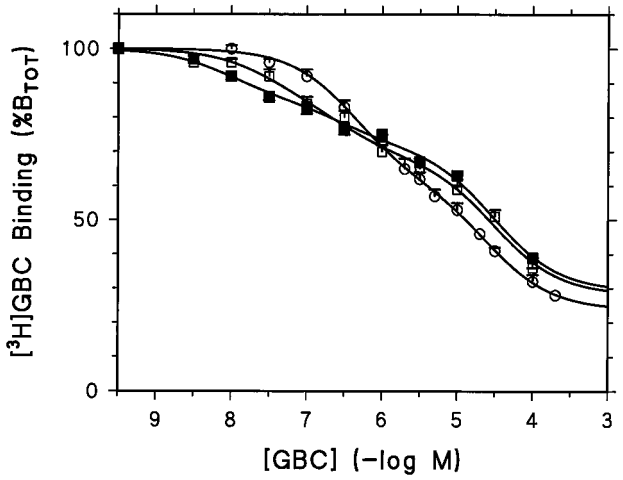

Fig. 3. Homologous competition of total [^{3}H]GBC binding with GBC in HEK cells. Control cells, ○; SUR2B-, □, and SUR2B + Kir6.1-transfected, ■ HEK cells. [^{3}H]GBC was 3 nM and samples were incubated for 30 min at 37°C. Data are means ± S.E. from four to six experiments. Control cells: the logistic equation with two components (i.e., A_1 = 0) was fitted to the data giving for the amplitudes (A_2/A_3, % B_{TOT}) 39 ± 2/37 ± 1%; the K_D values were (K_{D2}/K_{D3}, confidence interval in parentheses) 305 (220,420) nM/31 (15,63) μM. The data from SUR2B- and from cotransfected cells were analyzed according to eq. 1 setting A_1 = 15% and inserting for $pIC_{50,2}$ and $pIC_{50,3}$ the values determined for control cells (see text). From the fit the following parameter values were obtained (SUR2B/cotransfection): K_{D1}: 32 (16,65)/6.3 (3.5,11.5); A_2 (%): 17 ± 1/14 ± 1; A_3 (%): 40 ± 3/42 ± 2; Hill coefficients were set to 1. B_{TOT} (fmol/mg) was 109 ± 9, 134 ± 6, and 164 ± 13 for control, SUR2B-, and cotransfected cells, respectively.

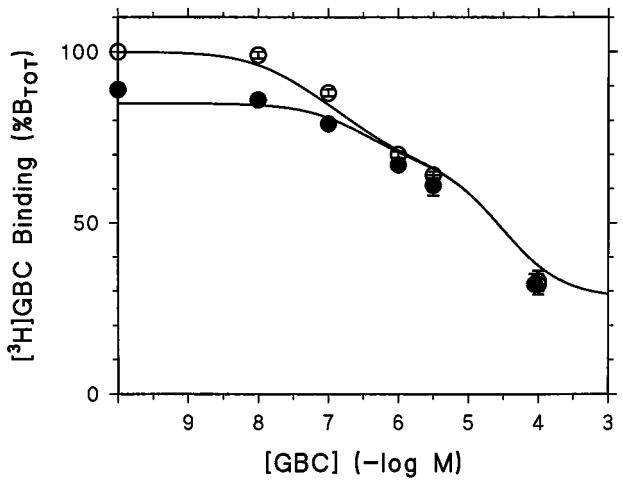

Fig. 4. Inhibition of total [^{3}H]GBC binding to SUR2B-transfected HEK cells by GBC in the absence (○) and presence (●) of P1075 (10 μM). Experiments ± P1075 were done side by side; means of five experiments are shown. The curve relating to the data in the absence of P1075 was calculated using the parameters determined in Fig. 3; the lower curve (+ P1075) was calculated setting A_1 = 0. The curves do not represent an attempt to fit the equations to the data; they merely illustrate the hypothesis that P1075 exclusively interferes with high-affinity GBC binding.

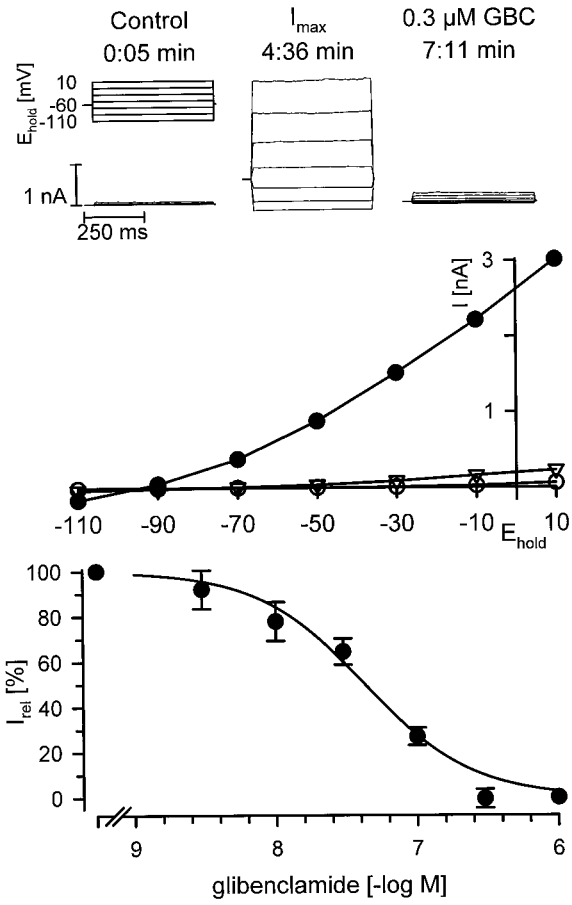

Fig. 5. Activation of the SUR2B/Kir6.1 current and inhibition with GBC. Recordings are from a green fluorescent HEK cell transfected with SUR2B + Kir6.1 + GFP; experiments were performed at 37°C and under a physiological K^+ gradient. A, current traces. After establishing the whole cell configuration, currents were initially small (right). Dialysis of the cell with GDP (1 mM) in the presence of ATP (0.3 mM) activated a hyperpolarizing current, $I_{K,\ NDP}$ (middle). GBC (0.3 μM) blocked this current by ≈95% (right). B, current-voltage relationships obtained from the traces in (A). Initial current, ○. The calculated resting potential of the HEK cells was positive to −50 mV. Current after cell dialysis with GDP and ATP, $I_{K,\ NDP}$, ●. The current reversed around the Nernst potential for potassium (≈ −100 mV). Residual current after block by 0.3 μM GBC, ▽. C, concentration-dependent inhibition of $I_{K,\ NDP}$ by GBC. From the fit of eq. 1 with one component to the data, an IC_{50} value of 43 (33,55) nM and a Hill coefficient of 1.1 ± 0.2 were obtained. Each data point represents the mean of five to eight experiments.

SUR2B or SUR2B + Kir6.1. In all cases, the competition curves were multiphasic. The competition curve in control cells was analyzed assuming two binding components with Hill coefficient 1 and gave K_D values of 0.31 and 31 μM with the first component contributing ≈39% to B_{TOT}. In membranes from control cells, no specific [^{3}H]GBC binding was found, suggesting that the binding sites labeled in whole cells were lost during membrane preparation and were most probably of cytosolic origin.

In cells transfected with Kir6.1, the GBC inhibition curve did not differ significantly from that in control cells (n = 4; not shown). In contrast, the [^{3}H]GBC-GBC competition curves in cells transfected with SUR2B and SUR2B + Kir6.1 were shifted leftward, reflecting the presence of an additional high-affinity binding component (Fig. 3). When these curves were analyzed assuming two independent classes of binding sites, K_i values of 100 and 20 nM were obtained for the high-affinity component in cells transfected with SUR2B and SUR2B + Kir6.1, respectively. However, the analysis of these curves has to accommodate one component for binding to SUR2B and the two binding components found also in control cells, giving a total of three components. Quantitatively, it was assumed that the binding to SUR2B contributed 15% to overall binding (see above), and that the endogenous binding components had the same affinities as determined in control cells; in addition, Hill coefficients were set to 1. The fit of this model to the data gave K_D values of 32 nM for GBC binding to SUR2B and 6.3 nM for the SUR2B-Kir6.1 complex (Table 1). This indicated that coexpression with Kir6.1 increased the affinity of SUR2B for GBC about 5-fold.

The three-component analysis was tested in computer simulations that showed that the estimated K_D values for the SUR2B binding component were remarkably stable against large variations of the K_D values used to account for endogenous GBC binding. To further prove that the high-affinity component indeed represented binding to SUR2B we measured GBC inhibition curves in SUR2B-transfected cells in the absence and presence of P1075 (10 μM) in parallel. Figure 4 shows the experiments together with the theoretical inhibition curves calculated using the parameters determined from Fig. 3. The agreement is good, showing that the opener interfered solely with the high-affinity component of [^{3}H]GBC binding; hence this component represents binding to SUR2B.

Electrophysiological Experiments. After establishing the whole cell configuration, currents were small and the calculated resting potential of the HEK cells was positive to −50 mV. In HEK cells transiently transfected with SUR2B + Kir6.1 or with SUR2B + Kir6.1 + GFP, dialysis of the cell with GDP (1 mM) in the presence of ATP (0.3 mM) activated a hyperpolarizing current ($I_{K,\ NDP}$) within 3 to 5 min (Fig. 5A). $I_{K,\ NDP}$ reversed around the Nernst potential for potassium (≈ −100 mV, Fig. 5B) and was blocked to ≈95% by 0.3 μM GBC in the experiment shown in Fig. 5A. The concentration dependence of this block was measured applying GBC cumulatively to the bath and correcting for the slow fading of the current in the absence of GBC (3%/min). The resulting curve, shown in Fig. 5C, gave an IC_{50} value of 43 nM and a Hill coefficient close to 1 (1.1 ± 0.2).

Discussion

[^{3}H]GBC versus [^{3}H]P1075 Competition Experiments in Cells and Membranes. A major goal of this study was to determine the affinity of SUR2B for GBC. To this end we performed homologous competition studies of [^{3}H]GBC binding to whole cells yielding a K_D value of 32 nM; in contrast, experiments using the opener [^{3}H]P1075 gave about 30 to 45 times higher K_i values. This is in contrast to results published recently by Dörschner et al. (1999), who reported identical values using the two radiolabels (≈300 nM). However, in agreement with the observations presented here, we had found earlier in rat aorta that binding studies using [^{3}H]GBC gave a K_D value for GBC of 20 nM (Löffler and Quast, 1997) whereas the K_i value, determined in heterologous [^{3}H]P1075-GBC inhibition experiments, was 20 times higher (400 nM; Bray and Quast, 1992). Potential explanations for the different findings of the two groups will be discussed below; here we concentrate first on the question of why homologous and heterologous competition experiments can give different results.

It is well established that the binding sites for GBC and the openers are negatively allosterically coupled (Bray and Quast, 1992; Schwanstecher et al., 1992; Hambrock et al., 1998). Hence, K_i values determined in heterologous competition experiments need not give the true affinity of the ligand, and the discrepancies listed above indicate that the affinity of GBC for SUR2B should be determined using [^{3}H]GBC as the radiolabel. In contrast, heterologous competition studies of [^{3}H]GBC binding by P1075 give K_i values that are close to the affinity of opener binding to SUR2B (cf. Figs. 1 and 2). Similarly, in rat aorta, the K_i values for openers determined in [^{3}H]GBC experiments agree within a factor of 2 with those measured with [^{3}H]P1075 (Löffler and Quast, 1997). The relative insensitivity of [^{3}H]P1075 binding to GBC and other sulfonylureas reflects an asymmetry in the negative allosteric coupling of opener and sulfonylurea sites at the channel, which requires additional study.

Binding experiments were performed with whole cells rather than with membranes. This approach takes into account the coupling of SUR2-based K_{ATP} channels to the actin cytoskeleton (Brady et al., 1996; Furukawa et al., 1996; Terzic and Kurachi, 1996; Yokoshiki et al., 1997); in membrane preparations, however, the actin cytoskeleton is destroyed. An intact actin cytoskeleton does not seem to be of importance in [^{3}H]P1075 assays, which gave similar results in cells (Table 1) and membranes (Hambrock et al., 1998; see also Dörschner et al., 1999). In contrast, high-affinity binding of [^{3}H]GBC in membranes containing SUR2B ± Kir6.1 is shifted to much higher concentrations than in cells and is barely sensitive to inhibition by openers (<5%, F. A., unpublished observation); in rat aorta, it is completely abolished by metabolic inhibition (Löffler and Quast, 1997) and by agents that disrupt the actin cytoskeleton (Löffler-Walz and Quast, 1998). This shows the necessity of working in whole, metabolically intact cells. The major disadvantage of the [^{3}H]GBC binding experiments in whole cells is that control cells too showed GBC binding, thus complicating analysis of the competition curves in transfected cells.

Affinity of GBC for SUR2B and the SUR2B-Kir6.1 Complex. In this study, the K_D value for GBC binding to SUR2B has been determined to ≈30 nM. This represents an

appreciable affinity; however, this value is about 60 times higher than that for binding to SUR1, the high-affinity SUR typical of the K_{ATP} channels in pancreatic β-cells and in neurons (0.55 nM; Schwanstecher et al., 1998). On the other hand, early work has suggested that the SUR2 subtypes had a low affinity for GBC with K_D values in the μM range (review: Babenko et al., 1998); in their recent publication, Dörschner et al. (1999) reported a value of ≈0.3 μM.

Using [^{3}H]GBC as radiolabel we determined the K_D value of GBC binding to the SUR2B/Kir6.1 complex to 6 nM. First, this value is still in good agreement with the K_D value of 20 nM found for high-affinity GBC binding in rat aorta (Löffler and Quast, 1997). More importantly, it is 5 times lower than that for binding to SUR2B alone; in contrast, cotransfection left the affinity of P1075 binding unchanged. Similar findings were obtained with [^{3}H]P1075 as the radiolabel [shift in K_i (GBC) ≈4]. Hence the SUR2B/Kir6.1 complex has a higher affinity for GBC than SUR2B alone. One could speculate that Kir6.2 contributes (to a small degree) to the formation of the GBC binding pocket on SUR2B, either directly or indirectly, by inducing a conformational change in SUR2B. Using [^{125}I]azido-GBC, a close physical association between the GBC binding pocket on SUR1 and the Kir6.x subunit has indeed been demonstrated since both proteins were cophotolabeled (Schwanstecher et al., 1994; Clement et al., 1997). It is also possible that differences in the glycosylation of SUR2B and the SUR2B/Kir6.1 complex, as demonstrated for the glycosylation of SUR1 versus SUR1/Kir6.x complexes (Clement et al., 1997), could affect GBC affinity; alternatively, interactions with additional cell components, e.g., the actin cytoskeleton, could play a role. This effect of coexpression on GBC affinity is reminiscent of the 3-fold increase in potency of ATP in blocking the Kir6.2Δ26 channel after coexpression with SUR1 (Tucker et al., 1997).

The affinity shift for GBC binding upon coexpression was not observed by Dörschner et al. (1999); in addition, they found no difference between [^{3}H]P1075 and [^{3}H]GBC experiments and determined the affinity of GBC for SUR2B to be ≈0.3 μM (see above). How can these discrepancies with our results be explained? The experimental conditions under which the two studies were performed are very different. Dörschner and colleagues used SUR2B clones from rat and human expressed in COS cells and performed the experiments at room temperature; in addition, glucose seems to have been absent in the binding experiments to intact cells. Taking only the latter point, it is possible that glucose deprivation led to a depletion of the ATP level in the cells which, in turn, may have affected the cytoskeleton (Bacallao et al., 1994). Indeed, we had found that in rat aorta, metabolic inhibition abolishes high-affinity [^{3}H]GBC binding (Löffler and Quast, 1997). Additional experiments are required to resolve the discrepancies between the two studies.

Stoichiometry of GBC Binding and Channel Closure. GBC inhibited the current through the SUR2B/Kir6.1 channel with IC_{50} = 43 (33,55) nM and Hill coefficient = 1.1 ± 0.2. This is in excellent agreement with the value of Dörschner et al. (1999) for the recombinant channels formed from SUR2B or 2A with Kir6.2 (42 nM), and it agrees very well with the IC_{50} value of GBC inhibiting the vascular K_{NDP} channel in the absence of opener [rabbit portal vein: 25 nM, Beech et al., 1993a; canine coronary artery: 20 nM, Xu and Lee, 1994; rat aorta: 20–40 nM (using $^{86}Rb^+$ efflux), Quast, 1996]. Collectively, this is strong pharmacological evidence for the view first expressed by Yamada et al. (1997) that the SUR2B/Kir6.1 channel is indeed the opener-sensitive K_{NDP} channel in the vasculature.

Comparing binding of GBC to the channel (K_D = 6 nM) with the potency of GBC for channel inhibition (IC_{50} = 43 nM), one notes that the channel inhibition curve is shifted to the right of the binding curve by a factor of ≈7. Taking into account the four SUR subunits of the channel (Clement et al., 1997; Inagaki et al., 1997; Shyng and Nichols, 1997), this is essentially what one expects if binding of four molecules of GBC to identical independent GBC sites per channel is required for channel closure. In this case, the channel inhibition curve is shifted to the right of the binding curve by a factor of $(2^{1/4} - 1)^{-1} = 5.3$ and has a Hill coefficient of 16 $(2^{1/4} - 1)/2^{5/4} = 1.3$ (U.Q., unpublished results). Approximately, our data meet these requirements. In contrast, Dörschner et al. (1999), having determined a K_D value for the binding of ≈300 nM, found a leftward shift of the channel inhibition curve and conclude that occupation of a single GBC site is sufficient to close the channel. The different stoichiometries proposed in the two studies are a direct consequence of the different K_D determinations for GBC binding discussed above. Interestingly, four molecules of openers are required to activate the K_{ATP} channel (Schwanstecher et al., 1998). Together, these observations show a certain symmetry between the four opener and the four sulfonylurea sites at the channel $(SUR2B/Kir6.1)_4$. The sites of either class are identical and independent, and occupation of all four sites within one class is required to gate the channel. Opener sites and sulfonylurea sites on SUR2B are linked by negative allosteric coupling which, however, shows an asymmetry in that GBC binding recognizes opener binding with the approximately correct affinities whereas P1075 binding is relatively insensitive to sulfonylurea binding. The reason for this asymmetry remains to be elucidated.

In conclusion, we have shown here that the vascular SUR has a relatively high affinity for GBC (32 nM), which is increased upon complex formation with Kir6.1. The data suggest that occupation of all four GBC sites per channel is required for channel block.

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