British Journal of Pharmacology (2004) 141, 1098–1105
www.nature.com/bjp

Interaction of a novel dihydropyridine K^+ channel opener, A-312110, with recombinant sulphonylurea receptors and K_{ATP} channels: comparison with the cyanoguanidine P1075

[1]Holger Felsch, [1]Ulf Lange, [1]Annette Hambrock, [1]Cornelia Löffler-Walz, [1]Ulrich Russ, [2]William A Carroll, [2]Murali Gopalakrishnan & *,[1]Ulrich Quast

[1]Department of Pharmacology and Toxicology, Medical Faculty, University of Tübingen, Wilhelmstr. 56, D-72074 Tübingen, Germany and [2]Neuroscience Research, Abbott Laboratories, Abbott Park, IL, U.S.A.

1 ATP-sensitive K^+ channels (K_{ATP} channels) are composed of pore-forming subunits (Kir6.x) and of regulatory subunits, the sulphonylurea receptors (SURx). Synthetic openers of K_{ATP} channels form a chemically heterogeneous class of compounds that are of interest in several therapeutic areas. We have investigated the interaction of a novel dihydropyridine opener, A-312110 ((9*R*)-9-(4-fluoro-3-iodophenyl)-2,3,5,9-tetrahydro-4*H*-pyrano[3,4-b]thieno [2,3-e]pyridin-8(7*H*)-one-1,1-dioxide), with SURs and Kir6/SUR channels in comparison to the cyanoguanidine opener P1075.

2 In the presence of 1 mM MgATP, A-312110 bound to SUR2A (the SUR in cardiac and skeletal muscle) and to SUR2B (smooth muscle) with K_i values of 14 and 18 nM; the corresponding values for P1075 were 16 and 9 nM, respectively. Decreasing the MgATP concentration reduced the affinity of A312110 binding to SUR2A significantly more than that to SUR2B; for P1075, the converse was true. At SUR1 (pancreatic β-cell), both openers showed little binding up to 100 μM.

3 In the presence of MgATP, both openers inhibited [^{3}H]glibenclamide binding to the SUR2 subtypes in a biphasic manner. In the absence of MgATP, the high-affinity component of the inhibition curves was absent.

4 In inside-out patches, the two openers activated the Kir6.2/SUR2A and Kir6.2/SUR2B channels with similar potency ($\sim$50 nM). Both were almost 2 $\times$ more efficacious in opening the Kir6.2/SUR2B than the Kir6.2/SUR2A channel.

5 The results show that the novel dihydropyridine A-312110 is a potent K_{ATP} channel opener with binding and channel-opening properties similar to those of P1075.
British Journal of Pharmacology (2004) **141,** 1098–1105. doi:10.1038/sj.bjp.0705718

Keywords: K_{ATP} channel openers; dihydropyridine A-312110; cyanoguanidine P1075; binding to SUR and to Kir6.2/SUR; opener binding and MgATP; MgATP shift; opener affinity; opener potency; opener efficacy

Abbreviations: A-312110, (9*R*)-9-(4-fluoro-3-iodophenyl)-2,3,5,9-tetrahydro-4*H*-pyrano[3,4-b]thieno [2,3-e]pyridin-8(7*H*)-one-1,1-dioxide; GBC, glibenclamide; HEK cells, human embryonic kidney 293 cells; K_{ATP} channel, ATP-sensitive K^+ channel; KCO, K_{ATP} channel opener; Kir, inwardly rectifying K^+ channel; P1075, *N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*″-3-pyridylguanidine; SUR, sulphonylurea receptor; SUR2(YS), SUR2(Y1236S)

Introduction

ATP-sensitive K^+ (K_{ATP}) channels are composed of pore-forming subunits (Kir6.x) and of sulphonylurea receptors (SURx), which serve as regulatory subunits (Aguilar-Bryan *et al*., 1995; Sakura *et al*., 1995). K_{ATP} channels have a tetradimeric architecture, $(Kir6.x)_4(SURx)_4$ (Clement IV *et al*., 1997; Shyng & Nichols, 1997). Kir6.x and SURx each are encoded by two genes, giving rise to different subtypes. Additional complexity arises from alternative splicing of the SUR genes (Hambrock *et al*. (2002b) and references therein). A prominent example for this is given by the SUR2 isoforms, SUR2A and SUR2B, which differ in the use of the last exon (Inagaki *et al*., 1996; Isomoto *et al*., 1996). Different Kir 6.x and SURx subtypes combine to form the K_{ATP} channels in the various tissues; the resulting channels differ in their pharmacological and biophysical properties (review: Babenko *et al*., 1998; Gonoi & Seino, 2000; Seino & Miki, 2003).

K_{ATP} channels are gated by nucleotides; in particular, Kir6.2 containing K_{ATP} channels are closed by ATP and opened by MgADP. Hence, these channels link the metabolic state of the cell to membrane potential and cellular excitability (Ashcroft & Ashcroft, 1990; Seino & Miki, 2003). In the pancreatic β-cell, Kir6.2/SUR1 channels close when plasma glucose increases and thereby couple the early phase of insulin secretion to the plasma glucose level (Cook & Hales, 1984; Seghers *et al*., 2000; Seino & Miki, 2003). In glucose-responsive neurons in the ventromedial nucleus of the hypothalamus, the channels close when glucose increases (Ashford *et al*., 1990) and affect glucose homeostasis (Liss & Roeper, 2001). In some vascular beds, Kir6.1/SUR2B channels are activated by cAMP-dependent phosphorylation and contribute to the

*Author for correspondence; E-mail: ulrich.quast@uni-tuebingen.de
Advance online publication: 15 March 2004

control vascular tone (Quayle *et al*., 1997). In the urinary bladder and other smooth muscle, opening of Kir6.x/SUR2B channels exerts a spasmolytic effect (Coghlan *et al*., 2001). In neurons (mostly Kir6.2/SUR1) and in skeletal and cardiac myocytes (Kir6.2/SUR2A), K_{ATP} channels open in response to ischaemia and hypoxia and help to preserve organ function (Fujita & Kurachi, 2000; Seino & Miki, 2003).

The important role of K_{ATP} channels in various tissues makes these channels an attractive drug target, and, according to the medical need, either opening or closing of the channel may be required (Lawson, 1996; Coghlan *et al*., 2001). The best known K_{ATP} channel modulators are the antidiabetic sulphonylureas and glinides that promote insulin secretion by binding to SUR1 and closing the channel in the pancreatic β-cell (Sturgess *et al*., 1985; Proks *et al*., 2002; Gribble & Reimann, 2003). K_{ATP} channel openers (KCOs) also bind to SUR and activate the channel closed by ATP. KCOs generally prefer Kir6.1 SUR2B, thereby inducing vasodilatation and hypotension as the predominant effects (Lawson, 1996; Quast, 1996). They belong to several chemically distinct families including benzopyrans, cyanoguanidines, thioformamides and dihydropyridines (Edwards & Weston, 1990; Coghlan *et al*., 2001).

Very recently, the synthesis of a dihydropyridine, A-312110, was reported, which has almost no affinity ($>100\,\mu$M) for L-type Ca^{2+} channels and which relaxes electrically stimulated bladder strips from guinea-pig with $EC_{50} = 18.5$ nM (Davis-Taber *et al*., 2003). A-312110 carries an iodine and, after radioiodination, was shown to specifically bind to K_{ATP} channels in membranes from guinea-pig heart and bladder, with K_D values of 5.8 and 4.9 nM, respectively. The high affinity and high specific activity (2000 Ci mmol^{-1}) make [^{125}I]A-312110 a very valuable addition to the other well-characterised radiolabelled openers, which are both tritiated, for example, the cyanoguanidine [^{3}H] P1075 (Bray & Quast, 1992; Manley *et al*., 1993) and the benzopyran [^{3}H]217-774 (Manley *et al*., 2001). It was the aim of this study to compare the binding and the channel-opening properties of A-312110 to those of the standard cyanoguanidine opener P1075 in human embryonic kidney (HEK) 293 cells expressing recombinant SURx and Kir6.2.

Methods

Cell culture and transfection

The mutant SUR2 subtypes SUR2A(Y1206S) and 2B(Y1206S) were constructed from the respective murine SUR2 clones (SUR2A: GenBank D86037, and SUR2B: GenBank D86038; Isomoto *et al*., 1996), using the QuikChange Site-Directed Mutagenesis System (Stratagene, Amsterdam, The Netherlands) as described (Hambrock *et al*., 2001). HEK 293 cells were cultured in Minimum Essential Medium containing glutamine and supplemented with 10% foetal bovine serum and 20 μg ml^{-1} gentamycin (Hambrock *et al*., 1998). Cells were transfected with the mammalian expression vector pcDNA 3.1 (Invitrogen, Karlsruhe, Germany) containing the coding sequence of rat SUR1 (GenBank X97279) or the murine SUR2 clones mentioned above using lipofectAMINE and OPTIMEM (Invitrogen), and cell lines stably expressing these proteins were generated as described (Hambrock *et al*., 1998). Cells transiently coexpressing SUR2 and murine Kir6.2 (D50581; Inagaki *et al*., 1996) were transfected with the plasmids at a molar ratio of 1 : 1. In cotransfections prepared for electrophysiological experiments, the pEGFP-C1 vector (Clontech, Palo Alto, CA, U.S.A.), encoding for green fluorescent protein, was added for identification of transfected cells. At 2–4 days after transfection, cells were used for binding studies and electrophysiological experiments.

Membrane preparation and radioligand-binding competition experiments

For cells stably expressing SUR alone, the antibiotic was withdrawn from the culture medium 3 days prior to membrane preparation. Membranes were prepared as described (Hambrock *et al*., 1998). Protein concentration was determined according to Lowry *et al*. (1951) using bovine serum albumin as the standard.

For radioligand-binding competition experiments in the absence/presence of MgATP, membranes were added to an incubation buffer containing (in mM): HEPES 5; NaCl 139; KCl 5; $MgCl_2$ 0/2.2; EDTA 1/0 and Na_2ATP 0/1, and supplemented with the radioligand ([^{3}H]P1075 $\sim$3 nM or [^{3}H]GBC $\sim$2.2 nM) and (unlabelled) A-312110 or P1075. In case of 3 μM MgATP in the incubation medium, $MgCl_2$ was reduced to 1 mM. After equilibrium was reached (25 min for [^{3}H]P1075 and 15 min for [^{3}H]GBC), incubation was stopped by diluting 0.3 ml aliquots (in triplicate) in 8 ml of ice-cold quench solution (50 mM Tris-(hydroxymethyl)-aminomethane, 154 mM NaCl, pH 7.4). The bound and free ligand were separated by rapid filtration over Whatman GF/B filters, washed twice with quench solution and counted for [^{3}H] in the presence of 6 ml of scintillant (Ultima Gold: Packard, Meriden, CT, U.S.A.). Nonspecific binding (B_{NS}) of [^{3}H]P1075/[^{3}H]GBC was determined in presence of 10/100 μM P1075 (Hambrock *et al*., 2001) and was $\sim$10/25% of total binding.

Electrophysiological experiments

The patch-clamp technique was used in the inside-out configuration, as described by Hamill *et al*. (1981). Patch pipettes were drawn from borosilicate glass capillaries (GC 150T, Harvard Apparatus, Edenbridge, U.K.) and heat polished using a horizontal microelectrode puller (Zeitz, Augsburg, Germany). Bath and pipette were filled with a high K^+-Ringer solution containing (in mM) KCl 142; NaCl 2.8; $MgCl_2$ 1; $CaCl_2$ 1; D(+)-glucose 11; HEPES 10; titrated to pH 7.4 with NaOH at 22°C. Filled pipettes had a resistance of 1.0–1.5 MΩ. After excision of the patch, the pipette was moved in front of a pipe with a high K^+-Ringer solution containing (in mM) KCl, 143; $CaCl_2$, 1; D(+)-glucose, 11; HEPES, 10; EGTA, 5, titrated to pH 7.2 with NaOH at 22°C. $MgCl_2$ was added such that $[Mg^{2+}]_{free}$ was 0.7 mM. Openers were dissolved as described below and added to the pipe solution. Patches were clamped to -50 mV. Data were recorded with an EPC 9 amplifier (HEKA, Lambrecht, Germany) using the 'Pulse' software (HEKA). Signals were filtered at 200 Hz using the four-pole Bessel filter of the EPC9 amplifier and sampled with 1 kHz.

Data analysis

Individual binding inhibition curves were analysed using the logarithmic form of the Hill equation

$$y = 100 - A(1 + 10^{n(px - pIC_{50})})^{-1}, \quad (1)$$

here, A denotes the extent (amplitude) of inhibition, n ($=n_H$) the Hill coefficient and IC_{50} the midpoint of the curve with $pIC_{50} = -\log IC_{50}$; x is the concentration of the compound under study with $px = -\log x$. If necessary, two-component analysis was used with $n_H = 1$ and $A_2 = 100 - A_1$.

IC_{50} values were converted into inhibition constants, K_i, by correcting for the presence of the radioligand, L, according to the Cheng & Prusoff (1973) equation

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

where K_D is the equilibrium dissociation constant of the radioligand. In case of homologous competition experiments, the inhibition constant K_i is identical to the K_D value. The correction was always <2.0.

The concentration dependence of channel opening was analysed using the ascending form of the Hill equation and taking all individual data points into account.

Data are shown as means$\pm$s.e.m. Fits of the equations to the data were performed according to the method of least squares using the programme SigmaPlot 6.1 (SPSS Science, Chicago, IL, U.S.A.). Individual binding experiments were analysed and the parameters averaged assuming that amplitudes and pIC_{50} values are normally distributed (Christopoulos, 1998). In the text, K_D/K_i values are given, followed by the 95% confidence interval in parentheses. When the decision for the two-sites model was not obvious and the Hill coefficient was between 0.7 and 0.85, the fits to the one- and the two-sites model were compared by Fisher's F-test and the 'Akaike minimum information criterion', as described in Quast & Mählmann (1982). In calculations involving two mean values with standard errors, propagation of errors was taken into account according to Bevington (1969). Significance of differences between two (normally distributed) parameters was assessed using the two-tailed unpaired Student's t-test.

Materials and solutions

[^{3}H]P1075 (specific activity 4.5 TBq (117 Ci) mmol^{-1}) was purchased from Amersham Buchler (Braunschweig, Germany) and [^{3}H]glibenclamide ([^{3}H]GBC, specific activity 1.85 TBq (50 Ci) mmol^{-1}) from Perkin-Elmer Life Sciences (Bad Homburg, Germany). The reagents and media used for cell culture and transfection were from Invitrogen. A-312110 was synthesised as described by Carroll *et al*. (2001). P1075 was a kind gift from Leo Pharmaceuticals, Ballerup, Denmark. GBC was purchased from Sigma (Deisenhofen, Germany) and Na_2ATP was from Roche Diagnostics (Mannheim, Germany). K_{ATP} channel modulators were dissolved in dimethyl sulphoxide/ethanol (50%/50%, v v^{-1}) to give stock solutions of 0.1 M. These were further diluted with the same solvent or with incubation buffer to give final solvent concentrations $<0.3\%$. Mg^{2+}-free solutions (no Mg^{2+} added, contaminating $Mg^{2+} \leqslant 10\,\mu$M (Forestier & Vivaudou, 1993; EDTA = 1 or 5 mM) contained $\leqslant 10$ nM $[Mg^{2+}]_{free}$ (Hambrock *et al*., 1998).

Results

Binding of A-312110 and P1075 to SUR2 subtypes: [^{3}H]P1075 competition experiments

The interaction of A-312110 with SUR2A was first investigated using the tritiated cyanoguanidine opener [^{3}H]P1075. High-affinity binding of openers to SUR requires the presence of MgATP (Schwanstecher *et al*., 1992; Quast *et al*., 1993; Dickinson *et al*., 1997; Hambrock *et al*., 1998; Schwanstecher *et al*., 1998) and experiments were first conducted in the presence of 1 mM MgATP, a saturating nucleotide concentration. Figure 1a illustrates the inhibition of [^{3}H]P1075 binding to SUR2A by A-312110. The inhibition curve was regular ($n_H = 1$), reached completion and gave a K_i value of 14 nM (Table 1). MgATP was then reduced to 3 μM, a manoeuvre that decreased [^{3}H]P1075 binding to 21$\pm$1% of that obtained with 1 mM MgATP (see legend to Figure 1). The A-312110 inhibition curve was shifted to the right by a factor $f = 2.2$ (Figure 1a, Table 1). Analogous experiments were performed with P1075 and the results are summarised in Table 1. At 1 mM MgATP, a K_D value of 16 nM was determined and this was not affected by the reduction in MgATP (comparison of pK_D values by t-test).

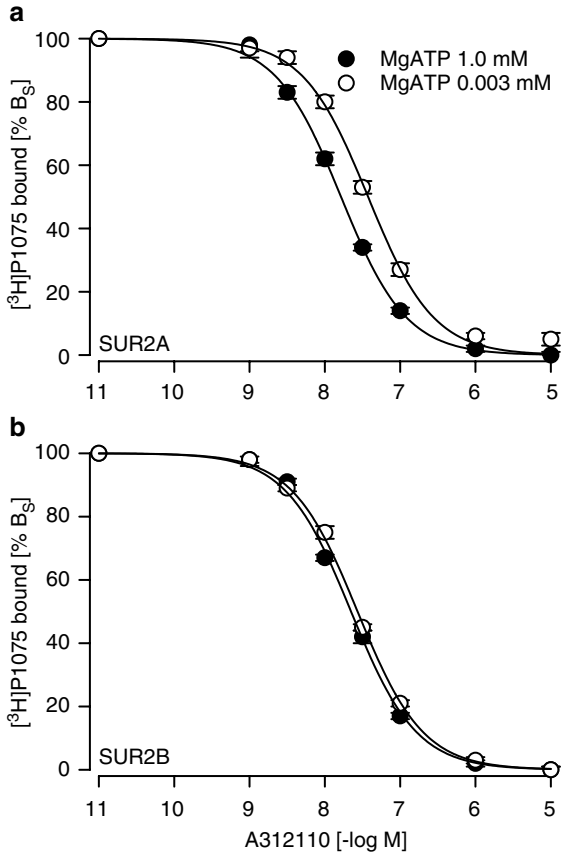

Figure 1 Inhibition of [^{3}H]P1075 binding to SUR2A and 2B by A-312110 at 1 and 0.003 mM MgATP. (a) SUR2A, (b) SUR2B. Data were normalised with respect to % specific binding (% B_S) and are means from 4–5 experiments. [^{3}H]P1075 concentration was 3 nM and B_S at 1 mM/0.003 mM MgATP was 120$\pm$13/25$\pm$3 fmol mg^{-1} for SUR2A and 230$\pm$12/60$\pm$7 fmol mg^{-1} for SUR2B, respectively. Individual binding curves were evaluated according to eqn (1), the parameters averaged as described in Methods and the mean values listed in Table 1.

Table 1 Binding of A-312110 and P1075 to SUR subtypes

			A-312110			*P1075*		
SUR	*Radiolig.*	*MgATP* (μM)	K_i (nM)	A (%)[a]	f[b]	K_D/K_i (nM)	A (%)[a]	f[b]
SUR2A	^{3}H-P1075	1000	14 (12, 15)	100	2.2 (1.9, 2.6)*	16 (14, 18)	100	1.3 (1.0, 1.7)[c]
		3	30 (28, 33)*	100		20 (16, 25)[c]	100	
SUR2B	^{3}H-P1075	1000	18 (16, 20)*	100	1.3 (1.1, 1.4)*	9.1 (7.1, 11)	100	2.0 (1.6, 2.5)
		3	22 (20, 25)	100		18 (16, 20)	100	
SUR2A(YS)	^{3}H-GBC	1000	8.0 (7.0, 10)*	69±2	140 (100, 190)	19 (14, 26)	63±2	110 (76, 170)
			3000 (1800, 3900)	31±2		1500 (800, 3000)	37±2	
		0	1100 (880, 1400)[d]	91±6		2200 (1700, 2800)	100	
			($n_H = 0.74 \pm 0.05$)[d]			($n_H = 0.89 \pm 0.05$)		
Kir6.2/ SUR2A(YS)	^{3}H-GBC	1000	30 (24, 37)	71±6	50 (38, 66)	Not done		
			3500 (1400, 5600)	29±6				
		0	1500 (1300, 1700)	100				
SUR2B(YS)	^{3}H-GBC	1000	12 (9, 15)*	42±5	71 (54, 93)*	5.9 (3.5, 9.8)	46±4	200 (120, 350)
			850 (600, 1200)	58±5		710 (490, 1000)	54±4	
		0	830 (690, 1000)	100		1200 (1100, 1300)	100	
SUR1	^{3}H-GBC	1000	Inhibition at 100 μM by 4±3%			Inhibition at 100 μM by 28±6%		
		0	Inhibition at 300 μM by 2±2%			Inhibition at 100 μM: 0%		

Data for A-312110 were obtained as described in Figures 1 and 2; data for P1075 are from Stauss *et al.* (in preparation). For K_D/K_i values, the 95% confidence intervals are given.
[a]Amplitude (extent) of inhibition in % specific binding (% B_S); in case of biphasic competition curves, $A_2 = 100 - A_1$.
[b]f denotes the MgATP shift calculated as $f = 10 \exp[pK_i (1\,mM\ MgATP) - pK_i (3\,\mu M\ MgATP)]$. In case of a biphasic curve at 1 mM MgATP, the pK_i of the high-affinity component was used. f values are log normally distributed and were compared on the log scale.
[c]K_D value not different from that at 1 mM MgATP; f value not different from 1.0.
[d]The fit to the two-sites model with $n_H = 1$ gave K_i values of 360 (190, 680) and 6200 (3400, 11,000) nM and amplitudes of 58±5 and 42±5% for the high- and low-affinity component, respectively. The two analyses were statistically equivalent as assessed by the F-test and the Akaike criterion.
*Indicates a significant difference ($P < 0.05$) between the respective parameters for A-312110 and P1075.

The interaction of A-312110 with SUR2B is shown in Figure 1b and Table 1. The K_i values obtained in the presence of 1 and 0.003 mM MgATP differed slightly from those determined for SUR2A. Reduction of MgATP reduced again the specific binding of the radioligand and induced a small but still significant rightward shift of the inhibition curve ($f = 1.3$). This shift was significantly smaller than that observed for binding to SUR2A ($f = 2.2$; $P < 0.05$; comparison of log$f$ values), whereas the opposite was true for P1075 (Table 1).

Binding of A-312110 and P1075 to SUR subtypes: [^{3}H]GBC inhibition experiments

The interaction of openers with SUR can also be studied using [^{3}H]GBC as the radioligand; this gives additional information since openers interfere with GBC binding by allosteric inhibition (Bray & Quast, 1992; Schwanstecher *et al.*, 1992; Hambrock *et al.*, 2002a). The low affinity of the SUR2 subtypes for GBC ($K_D \sim 25$–30 nM; Hambrock *et al.*, 2001; Stauß *et al.*, unpublished results) is, however, insufficient for high-precision binding studies with this lipophilic radioligand. We have therefore prepared SUR2 mutants in which Tyr in position 1206 is replaced by Ser, which is the corresponding amino acid in SUR1. This mutation increases the affinity of the SUR2 subtypes for GBC by $\sim 25\times$ (Toman *et al.*, 2000) or $\sim 10\times$ (Hambrock *et al.*, 2001; Stauß *et al.*, unpublished results), while decreasing the opener affinity by less than $2\times$ (Hambrock *et al.*, 2001; Russ *et al.*, 2003; Stauß *et al.*, unpublished results).

Figure 2a illustrates the inhibition of [^{3}H]GBC binding to SUR2A(YS) by A-312110. In the presence of 1 mM MgATP, the inhibition curve was strongly biphasic. The high-affinity component comprised 69% of the total inhibition with a K_i value of 8 nM; the low-affinity component (31%) had a K_i value of 3000 nM (Table 1). In the absence of MgATP (no ATP, presence of 1 mM EDTA), the inhibition curve was shifted rightwards and looked essentially homogeneous with $K_i = 1.1\,\mu$M; however, the Hill coefficient of 0.74±0.05 (< 1.0; $P < 0.001$) suggested slight heterogeneity of the binding sites. The fit to a two-sites model gave two components with K_i values of 360 and 6200 nM, respectively, and was statistically equivalent to the Hill fit (see Table 1 for details). Inspection of Table 1 shows that similar results were obtained with P1075: In the presence of 1 mM MgATP, the inhibition curve was strongly biphasic; in its absence, the curve was essentially monophasic ($n_H = 0.89 \pm 0.05 < 1.0$; $P < 0.05$) and could not be broken down into two components.

It was also of interest to examine whether coexpression of SUR2A(YS) with Kir6.2 affected the allosteric interactions between [^{3}H]GBC and A-312110 binding. In the presence of MgATP, the A-312110 inhibition curve was again biphasic (Table 1). Coexpression shifted the high-affinity component from 8 to 30 nM, whereas the low-affinity component and the ratio of the amplitudes remained unchanged. In the absence of MgATP, the A-312110 inhibition curve was now strictly monophasic ($n_H = 1.0$ compared to 0.74 with SUR2A(YS) expressed alone) with a K_D of 1.5 μM. This value was similar to that found when the inhibition curve for SUR2A(YS) alone was analysed with a one-component Hill model (Table 1). Hence, both in the presence and absence of MgATP, coexpression with Kir6.2 reduced the heterogeneity in the interaction of A-312110 with SUR2A(YS), apparent in the [^{3}H]GBC-binding studies.

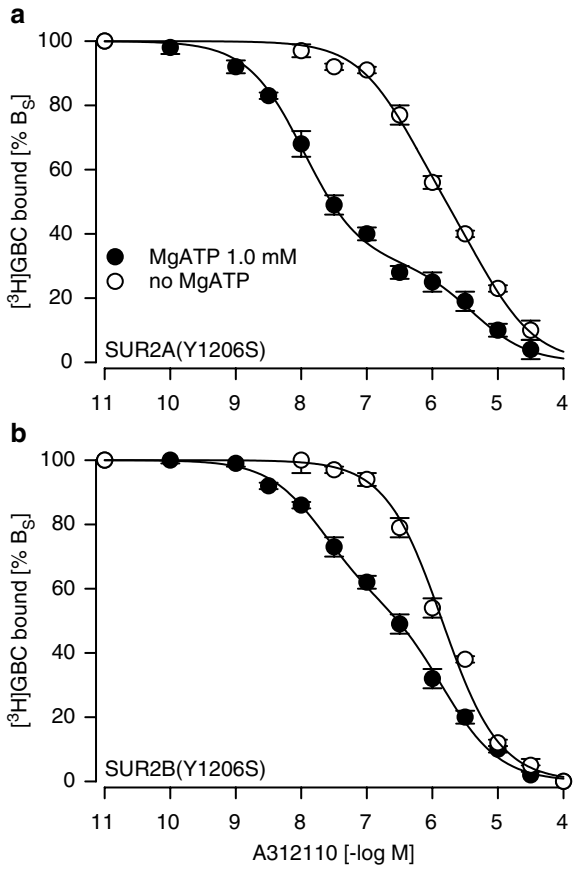

Figure 2 Inhibition of [^{3}H]GBC binding to SUR2(YS) by A-312110 in the presence of 1 and 0 mM MgATP. (a) SUR2A(YS), (b) SUR2B(YS). Data are means from four experiments and are presented as % B_S. At the [^{3}H]GBC concentration of 2.2 nM, B_S in the absence of MgATP was 204 ± 8 and 370 ± 15 fmol mg^{-1} for mutant SUR2A and SUR2B, respectively; MgATP (1 mM) reduced B_S to $67 \pm 4/57 \pm 3$ for mutant SUR2A/2B, respectively, without affecting K_D (Hambrock *et al*., 2002a). For parameters, see Table 1.

The analogous experiments using SUR2B(YS) are shown in Figure 2b and the results are summarised in Table 1. As with SUR2A(YS), the A-312110 and P1075 inhibition curves were biphasic in the presence of 1 mM MgATP; in the absence of MgATP, they were strictly monophasic. The respective K_i values were similar to those determined with SUR2A(YS) (Table 1). A clear difference between SUR2 subtypes was the amplitude ratio of the biphasic inhibition curves: for both openers, the high-affinity component was significantly smaller for SUR2B(YS) than for SUR2A(YS).

In [^{3}H]GBC-binding experiments using SUR1 and in the presence of MgATP, A-312110 did not show a significant interaction with SUR1; P1075 was very week.

Electrophysiological experiments

The channel-opening ability of the KCOs was examined in inside-out patches from HEK cells expressing Kir6.2/SUR2x channels. Experiments were performed in the presence of 1 mM MgATP to keep the channel closed. Figure 3 shows a recording of A-312110 opening the Kir6.2/SUR2A channel. In this experiment, A-312110 was ineffective at 0.01 μM, but elicited currents at 0.1 and 1 μM. The currents faded slowly upon washout of the drug; in the absence of MgATP, fading was accelerated (cf. Gribble *et al*., 2000; Reimann *et al*., 2000).

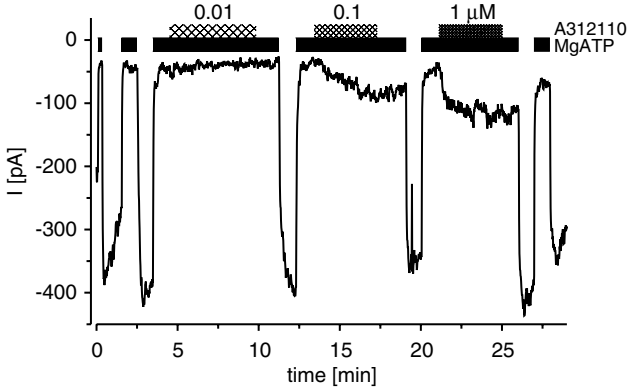

Figure 3 Recording from an inside-out patch showing activation of the Kir6.2/SUR2A channel by A-312110. After excision of the patch into nucleotide-free solution, a current was present which was essentially abolished by superfusion with MgATP (1 mM). This showed that the current was indeed flowing through K_{ATP} channels. A-312110 was applied in the presence of MgATP as indicated by the hatched bars. Holding potential was -50 mV; experiments were performed in symmetrical high K^+ buffer at 22°C.

In the course of these experiments, it was observed that MgATP (1 mM) inhibited Kir6.2/SUR2A channels more strongly thanKir6.2/SUR2B channels (see the legend of Figure 4 for details). Figure 4 illustrates the concentration-dependent activation of the Kir6.2/SUR2x channels by the openers; the parameters of the activation curves are listed in Table 2. A-312110 and P1075 were of similar potency ($\sim$50 nM) in activating the two channels. The maximum effects (amplitudes) obtained from the Hill fit showed that at both channels P1075 was $\sim$20–30% more efficacious than A312110 (Table 2); however, if only the data in the saturation region were considered (Figure 4), this difference was not maintained. It is also seen that both openers were $\sim$75% more efficacious in activating the Kir6.2/SUR2B than the Kir6.2/SUR2A channel (efficacy ratios of 170 ± 21 and 184 ± 12% for A-312110 and P1075, respectively; propagation of errors taken into account).

Discussion

Affinity to SUR subtypes

At saturating MgATP, the [^{3}H]P1075–A-312110 inhibition curves for SUR2A and 2B were monophasic and gave the same K_i value ($\sim$15 nM); hence, in the binding step, the new opener showed no selectivity for one of the SUR2 subtypes. This conclusion agrees with Davis-Taber *et al*. (2003), who measured [^{125}I]A-312110 binding in membranes from guinea-pig heart (Kir6.2/SUR2A) and bladder (Kir6.x/SUR2B) and obtained K_D values of 5.8 and 4.9 nM, respectively. The three-fold difference in the K_D (K_i) values from the two studies probably reflects differences in experimental conditions (e.g. presence or absence of an ATP-regenerating system and/or native vs recombinant system, etc.). In contrast to A-312110, P1075 showed a slight ($\sim$two-fold) selectivity for SUR2B over SUR2A. This agrees with results obtained in the recombinant

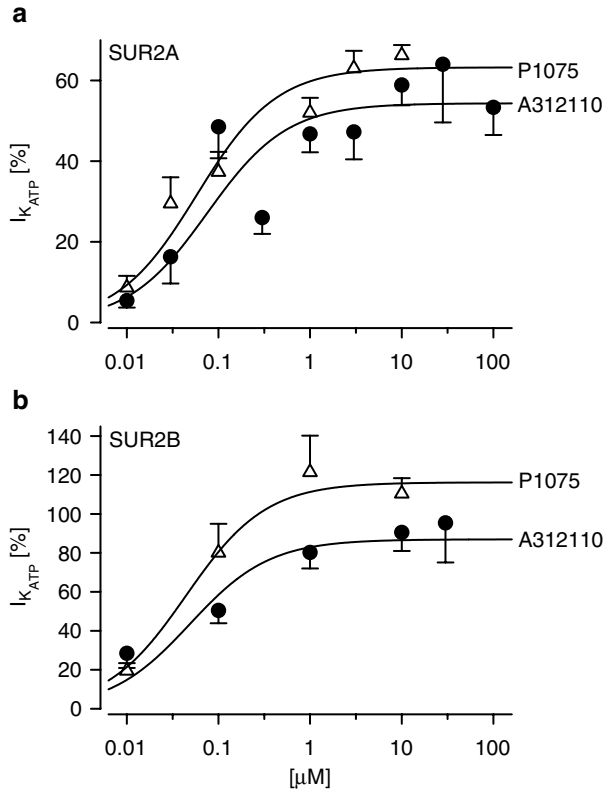

Figure 4 Concentration-dependent activation of Kir6.2/SUR2x channels by A-312110 and P1075. (a) Kir6.2/SUR2A, (b) Kir6.2/SUR2B channels. Data were obtained from experiments as shown in Figure 3. The KCO-induced current (I_{KCO}) was normalised with respect to the peak current in the absence of MgATP before run-down, since run-down is reversed by MgATP (Ribalet *et al*., 2000). Concentration dependencies were analysed using the logarithmic form of the Hill equation with $n_H = 1$; $n = 3$–12 per data point. Control currents in the presence of MgATP (1 mM) and absence of opener were 3.5 (0, 29)% of the current in the absence of ATP for Kir6.2/SUR2A ($n = 174$) and 6.5 (0, 33) for Kir6.2/SUR2B ($n = 74$; median and 95% confidence interval; $P = 0.027$ by Mann–Whitney rank sum test).

Table 2 Activation of K_{ATP} channels by A-312110 and P1075

	A-312110		*P1075*	
Channel	*EC_{50}* (nM)	*A (%)*	*EC_{50}* (nM)	*A (%)*
Kir6.2/SUR2A	58 (25,137)	51±5	58 (35,96)	63±2[a]
Kir6.2/SUR2B	49 (19,123)	87±7[b]	44 (17,116)	116±9[a,b]

Parameters are from fits of the logistic equation with $n_H = 1$ to the individual data points obtained as shown in Figure 3; mean values are presented in Figure 4. A = amplitude.
[a]Significantly different from the value with A-312110 ($P < 0.05$).
[b]Significantly different from the value with Kir6.2/SUR2A ($P < 0.01$).

system (Schwanstecher *et al*., 1998; Hambrock *et al*., 1999) and in membranes from native tissues (Davis-Taber *et al*., 2003).

With [^{3}H]GBC as the radioligand and in the presence of MgATP, the inhibition curves of the two openers to the SUR2(YS) subtypes were biphasic. The K_i values of the high-affinity components were close to the K_i values obtained with [^{3}H]P1075, and thus reflected the true affinity of the openers for the SUR2 subtypes in the presence of MgATP. The K_i values of the low-affinity components agreed well with those using [^{3}H]GBC in the absence of MgATP. Comparing the inhibition of [^{3}H]GBC binding to SUR2B(YS) by a large number of KCOs, we recently found that cyanoguanidines, benzopyrans and aprikalim showed this biphasic behaviour and these openers were classified as 'typical' KCOs; in contrast, nicorandil, minoxidil sulphate and diazoxide were monophasic (Russ *et al*., 2003). This study shows that the dihydropyridine A-312110 also belongs to the group of 'typical' KCOs and that the biphasic [^{3}H]GBC-opener inhibition curves, first observed with SUR2B(YS) (Hambrock *et al*., 2001; Hambrock *et al*., 2002a; Russ *et al*., 2003), occur also with SUR2A(YS).

MgATP dependence

Turning first to the [^{3}H]P1075 experiments, we recall that high-affinity opener binding to SUR requires the presence of MgATP. Binding of MgATP converts SUR into a high-affinity state for openers (Schwanstecher *et al*., 1998), in which the SUR-opener complex is stabilised by a decreased dissociation rate (Gribble *et al*., 2000; Reimann *et al*., 2000). SUR exhibits ATPase activity and the two nucleotide-binding domains of SUR are linked by positive cooperativity and act in concert (Ueda *et al*., 1999; Bienengraeber *et al*., 2000). Reducing MgATP from 1 mM to 3 μM decreased the binding of [^{3}H]P1075 (3 nM) to SUR2A and 2B by 75–80%. This loss reflects conversion of SUR to the low-affinity state for openers due to the lack of MgATP. The fraction of SUR which can still be labelled by [^{3}H]P1075 is in a state different from that at high MgATP, probably due to differences in occupation of the nucleotide-binding domains by MgATP and its hydrolysis products. Considering SUR2B, we have found that in this low MgATP state the affinity of SUR for openers is generally reduced and that for blockers like GBC and phloxin B is increased (Hambrock *et al*., 2000). Here we found that for SUR2B reduction of MgATP gave a smaller shift for A-312110 than for P1075 (1.3 vs 2.0), whereas for SUR2A the opposite was true (2.2 vs 1.3).

Use of [^{3}H]GBC as the radioligand offers the advantage that opener binding can be monitored in the presence and the complete absence of MgATP; the K_i values under the two conditions reflect the opener affinity of SUR in these two states. For SUR2B(YS), we found for A-312110 a shift of 70 which is significantly lower than for P1075 (200; this study) and that for the other 'typical openers' (~200; Russ *et al*., 2003). For SUR2A(YS), the absence of MgATP increased the K_i values of A-312110 and P075 to a similar degree (140 vs 110, respectively). Taken together, the results show that reduction of MgATP decreased the affinity of SUR2B for A-312110 significantly less than for P1075 (and other typical KCOs); for SUR2A, the [^{3}H]P1075 experiments showed the converse.

Channel opening

The two openers activated the Kir6.2/SUR2A and /SUR2B channels with the same potency (EC_{50} values ~50 nM at 22°C). This is lower than the K_D/K_i values for binding to SUR2 (without coexpression of Kir6.2) determined here (~10–20 nM) and the K_D values for binding to native channels in

membranes from smooth muscle and heart (5–6 nM; Bray & Quast, 1992; Löffler-Walz & Quast, 1998; Davis-Taber *et al*., 2003; all values at 37°C). This discrepancy has generally been observed with KCOs (see, for example, Schwanstecher *et al*., 1998) and is aggravated if both binding and channel opening are assessed at the same temperature (37°C; Quast *et al*., 1993). It probably reflects the complex signal transduction within the channel in which KCO binding affects the ATPase activity of SUR, thereby determining the channel activity (Bienengraeber *et al*., 2000; Zingman *et al*., 2001). Regarding the efficacies in channel opening, the two openers were similar as well. Interestingly, both openers exhibited an almost two times higher efficacy in activating the Kir6.2/SUR2B than the SUR2A channel, and this may be related to the stronger inhibition of the Kir6.2/SUR2A channels by MgATP (see also Reimann *et al*., 2000).

In conclusion, we have characterised here the binding and channel-opening properties of a novel dihydropyridine KCO, A-312110, in comparison to the standard opener P1075, in recombinant systems. The results show that A-312110 is a potent KCO with properties similar to those of P1075. The new opener, which is available in radioiodinated form (Davis-Taber *et al*., 2003), makes a welcome addition to the chemically diverse group of K_{ATP} channel openers.

This study was supported by the Deutsche Forschungsgemeinschaft (Qu 100/3-1, U.Q.), the Federal Ministry of Education, Science, Research and Technology (Fö 01KS9602) and the Interdisciplinary Center of Clinical Research (IZKF), Tübingen. We thank Drs Y. Kurachi and Y. Horio (Osaka) for the generous gift of the murine clones of SUR2A, 2B and Kir6.2, and Dr C. Derst, Marburg for the rat clone of SUR1.

References

AGUILAR-BRYAN, L., NICHOLS, C.G., WECHSLER, S.W., CLEMENT IV, J.P., BOYD III, A.E., GONZÁLES, G., HERRERA-SOZA, H., NGUY, K., BRYAN, J. & NELSON, D.A. (1995). Cloning of the β cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science,* **268,** 423–426.

ASHCROFT, S.J.H. & ASHCROFT, F.M. (1990). Properties and functions of ATP-sensitive K-channels. *Cell. Signal.,* **2,** 197–214.

ASHFORD, M.L., BODEN, P.R. & TREHERNE, J.M. (1990). Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K^+ channels. *Pflügers Arch. Eur. J. Physiol.,* **415,** 479–483.

BABENKO, A.P., AGUILAR-BRYAN, L. & BRYAN, J. (1998). A view of SUR/K_{IR}6.X, KATP channels. *Annu. Rev. Physiol.,* **60,** 667–687.

BEVINGTON, P.R. (1969). *Data Reduction and Error Analysis for the Physical Sciences,* pp. 55–65 and 92–118. New York: McGraw-Hill.

BIENENGRAEBER, M., ALEKSEEV, A.E., ABRAHAM, M.R., CARRASCO, A.J., MOREAU, C., VIVAUDOU, M., DZEJA, P.P. & TERZIC, A. (2000). ATPase activity of the sulfonylurea receptor: a catalytic function for the K_{ATP} channel complex. *FASEB J.,* **14,** 1943–1952.

BRAY, K.M. & QUAST, U. (1992). A specific binding site for K^+ channel openers in rat aorta. *J. Biol. Chem.,* **267,** 11689–11692.

CARROLL, W.A., AGRIOS, K.A., ALTENBACH, R.J., DRIZIN, I. & KORT, M.E. (2001). Inventors, Abbot Laboratories, assignee. Preparation of 4-arylbisannelated-1, 4-dihydropyridines as potassium channel openers. U.S. Patent 6,191,140B1.2001 February 20.

CHENG, Y. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099–3108.

CHRISTOPOULOS, A. (1998). Assessing the distribution of parameters in models of ligand–receptor interaction: to log or not to log. *Trends Pharmacol. Sci.,* **19,** 351–357.

CLEMENT IV, J.P., KUNJILWAR, K., GONZALEZ, G., SCHWANSTECHER, M., PANTEN, U., AGUILAR-BRYAN, L. & BRYAN, J. (1997). Association and stoichiometry of K_{ATP} channel subunits. *Neuron,* **18,** 827–838.

COGHLAN, M.J., CARROLL, W.A. & GOPALAKRISHNAN, M. (2001). Recent developments in the biology and medicinal chemistry of potassium channel modulators: update from a decade of progress. *J. Med. Chem.,* **44,** 1627–1653.

COOK, D.L. & HALES, C.N. (1984). Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature,* **311,** 271–273.

DAVIS-TABER, R., MOLINARI, E.J., ALTENBACH, R.J., WHITEAKER, K.L., SHIEH, C.-C., ROTERT, G., BUCKNER, S.A., MALYSZ, J., MILICIC, I., MCDERMOTT, J.S., GINTANT, G.A., COGHLAN, M.J., CARROLL, W.A., SCOTT, V.E. & GOPALAKRISHNAN, M. (2003). ^{125}I]A-312110, a novel high-affinity 1,4-dihydropyridine ATP-sensitive K^+ channel opener: characterization and pharmacology of binding. *Mol. Pharmacol.,* **64,** 143–153.

DICKINSON, K.E.J., BRYSON, C.C., COHEN, R.B., ROGERS, L., GREEN, D.W. & ATWAL, K.S. (1997). Nucleotide regulation and characteristics of potassium channel opener binding to skeletal muscle membranes. *Mol. Pharmacol.,* **52,** 473–481.

EDWARDS, G. & WESTON, A.H. (1990). Structure activity relationships of K+ channel openers. *Trends Pharmacol. Sci.,* **11,** 417–422.

FORESTIER, C. & VIVAUDOU, M. (1993). Modulation by Mg^{2+} and ADP of ATP-sensitive potassium channels in frog skeletal muscle. *J. Membr. Biol.,* **132,** 87–94.

FUJITA, A. & KURACHI, Y. (2000). Molecular aspects of ATP-sensitive K^+ channels in the cardiovascular system and K^+ channel openers. *Pharmacol. Ther.,* **85,** 39–53.

GONOI, T. & SEINO, S. (2000). Structure and function of ATP-sensitive K^+ channels. In: *Pharmacology of Ionic Channel Function: Activators and Inhibitors,* ed. Endo, M., Kurachi, Y. & Mishina, M. pp. 271–295. New York: Springer Verlag.

GRIBBLE, F.M. & REIMANN, F. (2003). Sulphonylurea action revisited: the post-cloning era. *Diabetologia,* **46,** 875–891.

GRIBBLE, F.M., REIMANN, F., ASHFIELD, R. & ASHCROFT, F.M. (2000). Nucleotide modulation of pinacidil stimulation of the cloned K_{ATP} channel Kir6.2/SUR2A. *Mol. Pharmacol.,* **57,** 1256–1261.

HAMBROCK, A., LÖFFLER-WALZ, C., KLOOR, D., DELABAR, U., HORIO, Y., KURACHI, Y. & QUAST, U. (1999). ATP-sensitive K^+ channel modulator binding to sulfonylurea receptors SUR2A and SUR2B: opposite effects of MgADP. *Mol. Pharmacol.,* **55,** 832–840.

HAMBROCK, A., LÖFFLER-WALZ, C., KURACHI, Y. & QUAST, U. (1998). Mg^{2+} and ATP dependence of K_{ATP} channel modulator binding to the recombinant sulphonylurea receptor, SUR2B. *Br. J. Pharmacol.,* **125,** 577–583.

HAMBROCK, A., LÖFFLER-WALZ, C. & QUAST, U. (2002a). Glibenclamide binding to sulphonylurea receptor subtypes: dependence on adenine nucleotides. *Br. J. Pharmacol.,* **136,** 995–1004.

HAMBROCK, A., LÖFFLER-WALZ, C., RUSS, U. & QUAST, U. (2000). Allosteric coupling between the binding sites for sulphonylureas and ATP on the sulphonylurea receptor SUR2B. *Naunyn-Schmiedeberg's Arch. Pharmacol. (Suppl.),* **361,** R76 (abstract).

HAMBROCK, A., LÖFFLER-WALZ, C., RUSS, U., LANGE, U. & QUAST, U. (2001). Characterization of a mutant sulfonylurea receptor SUR2B with high affinity for sulfonylureas and openers: differences in the coupling to Kir6.x subtypes. *Mol. Pharmacol.,* **60,** 190–199.

HAMBROCK, A., PREISIG-MÜLLER, R., RUSS, U., PIEHL, A., HANLEY, P.J., RAY, J., DAUT, J., QUAST, U. & DERST, C. (2002b). Four novel splice variants of sulfonylurea receptor 1. *Am. J. Physiol. (Cell Physiol.),* **283,** C587–C598.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch. – Eur. J. Physiol.,* **391,** 85–100.

INAGAKI, N., GONOI, T., CLEMENT IV, J.P., WANG, C.Z., AGUILAR-BRYAN, L., BRYAN, J. & SEINO, S. (1996). A family of suphonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. *Neuron,* **16,** 1011–1017.

ISOMOTO, S., KONDO, C., YAMADA, M., MATSUMOTO, S., HIGASHIGUCHI, O., HORIO, Y., MATSUZAWA, Y. & KURACHI, Y. (1996). A novel sulfonylurea receptor forms with BIR (KIR6.2) a smooth muscle type ATP-sensitive K+ channel. *J. Biol. Chem.,* **271,** 24321–24324.

LAWSON, K. (1996). Potassium channel activation: a potential therapeutic approach? *Pharmacol. Ther.,* **70,** 39–63.

LISS, B. & ROEPER, J. (2001). Molecular physiology of neuronal K-ATP channels. *Mol. Membr. Biol.,* **18,** 117–127.

LÖFFLER-WALZ, C. & QUAST, U. (1998). Binding of K_{ATP} channel modulators in rat cardiac membranes. *Br. J. Pharmacol.,* **123,** 1395–1402.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MANLEY, P.W., LÖFFLER-WALZ, C., RUSS, U., HAMBROCK, A., MOENIUS, T. & QUAST, U. (2001). Synthesis and characterization of a novel tritiated K_{ATP} channel opener with a benzopyran structure. *Br. J. Pharmacol.,* **133,** 275–285.

MANLEY, P.W., QUAST, U., ANDRES, H. & BRAY, K.M. (1993). Synthesis of and radioligand binding studies with a tritiated pinacidil analogue: receptor interactions of structurally different classes of potassium channel openers and blockers. *J. Med. Chem.,* **36,** 2004–2010.

PROKS, P., REIMANN, F., GREEN, N., GRIBBLE, F. & ASHCROFT, F. (2002). Sulfonylurea stimulation of insulin secretion. *Diabetes,* **51** (Suppl 3), S368–S376.

QUAST, U. (1996). Effects of potassium channel activators in isolated blood vessels. In: *Potassium Channels and their Modulators: From Synthesis to Clinical Experience,* ed. Evans, J.M., Hamilton, T.C., Longman, S.D. & Stemp, G. pp. 173–195. London: Taylor & Francis.

QUAST, U., BRAY, K.M., ANDRES, H., MANLEY, P.W., BAUMLIN, Y. & DOSOGNE, J. (1993). Binding of the K^+ channel opener [^{3}H]P1075 in rat isolated aorta: relationship to functional effects of openers and blockers. *Mol. Pharmacol.,* **43,** 474–481.

QUAST, U. & MÄHLMANN, H. (1982). Interaction of [^{3}H]flunitrazepam with the benzodiazepine receptor: evidence for a ligand-induced conformation change. *Biochem. Pharmacol.,* **31,** 2761–2768.

QUAYLE, J.M., NELSON, M.T. & STANDEN, N.B. (1997). ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol. Rev.,* **77,** 1165–1232.

REIMANN, F., GRIBBLE, F.M. & ASHCROFT, F.M. (2000). Differential response of K_{ATP} channels containing SUR2A or SUR2B subunits to nucleotides and pinacidil. *Mol. Pharmacol.,* **58,** 1318–1325.

RIBALET, B., JOHN, S.A. & WEISS, J.N. (2000). Regulation of cloned ATP-sensitive K channels by phosphorylation, MgADP, and phosphatidylinositol bisphosphate (PIP_2) – A study of channel rundown and reactivation. *J. Gen. Physiol.,* **116,** 391–409.

RUSS, U., LANGE, U., LÖFFLER-WALZ, C., HAMBROCK, A. & QUAST, U. (2003). Binding and effect of K_{ATP} channel openers in the absence of Mg^{2+}. *Br. J. Pharmacol.,* **139,** 368–380.

SAKURA, H., ÄMMÄLÄ, C., SMITH, P.A., GRIBBLE, F.M. & ASHCROFT, F.M. (1995). Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic ß-cells, brain, heart and skeletal muscle. *FEBS Lett.,* **377,** 338–344.

SCHWANSTECHER, M., BRANDT, C., BEHRENDS, S., SCHAUPP, U. & PANTEN, U. (1992). Effect of MgATP on pinacidil-induced displacement of glibenclamide from the sulphonylurea receptor in a pancreatic ß-cell line and rat cerebral cortex. *Br. J. Pharmacol.,* **106,** 295–301.

SCHWANSTECHER, M., SIEVERDING, C., DÖRSCHNER, H., GROSS, I., AGUILAR-BRYAN, L., SCHWANSTECHER, C. & BRYAN, J. (1998). Potassium channel openers require ATP to bind to and act through sulfonylurea receptors. *EMBO J.,* **17,** 5529–5535.

SEGHERS, V., NAKAZAKI, M., DEMAYO, F., AGUILAR-BRYAN, L. & BRYAN, J. (2000). *Sur1* knockout mice – a model for K_{ATP} channel-independent regulation of insulin secretion. *J. Biol. Chem.,* **275,** 9270–9277.

SEINO, S. & MIKI, T. (2003). Physiological and pathophysiological roles of ATP-sensitive K^+ channels. *Prog. Biophys. Mol. Biol.,* **81,** 133–176.

SHYNG, S.-L. & NICHOLS, C.G. (1997). Octameric stoichiometry of the K_{ATP} channel complex. *J. Gen. Physiol.,* **110,** 655–664.

STURGESS, N.C., ASHFORD, M.L., COOK, D.L. & HALES, C.N. (1985). The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet,* **2,** 474–475.

TOMAN, A., UHDE, I. & SCHWANSTECHER, M. (2000). A single residue in SURs is essential for sulphonylurea binding. *Naunyn-Schmiedeberg's Arch.Pharmacol. (Suppl.),* **361,** R75 (abstract).

UEDA, K., KOMINE, J., MATSUO, M., SEINO, S. & AMACHI, T. (1999). Cooperative binding of ATP and MgADP in the sulfonylurea receptor is modulated by glibenclamide. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 1268–1272.

ZINGMAN, L.V., ALEKSEEV, A.E., BIENENGRAEBER, M., HODGSON, D., KARGER, A.B., DZEJA, P.P. & TERZIC, A. (2001). Signaling in channel/enzyme multimers: ATPase transitions in SUR module gate ATP-sensitive K^+ conductance. *Neuron,* **31,** 233–245.

(Received November 22, 2003
Accepted January 29, 2004)