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Glibenclamide binding to sulphonylurea receptor subtypes: dependence on adenine nucleotides

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1 ATP-sensitive K^+ channels are composed of pore-forming subunits Kir6.2 and of sulphonylurea receptors (SURs); the latter are the target of the hypoglycaemic sulphonylureas like glibenclamide. Here, we report on the negative allosteric modulation by MgATP and MgADP of glibenclamide binding to SUR1 and to SUR2 mutants with high glibenclamide affinity, SUR2A(Y1206S) and SUR2B(Y1206S).

2 ATP, in the presence of an ATP-regenerating system to oppose hydrolysis during incubation, inhibited glibenclamide binding to SUR1 and SUR2B(Y1206S) by ~60%, to SUR2A(Y1206S) by 21%). Inhibition curves for the SUR2(Y1206S) isoforms were monophasic with IC_{50} values of 5–10 μM; the curve for SUR1 was biphasic (IC_{50} values 4.7 and 1300 μM).

3 Glibenclamide inhibition curves for ADP, performed in the presence of an ATP-consuming system to oppose ATP formation from ADP, were generally shifted rightwards and showed positive cooperativity, in particular with the SUR2(Y1206S) isoforms.

4 In the absence of the coupled enzyme systems, inhibition curves of MgATP or MgADP were generally shifted leftwards. This indicated synergy of MgATP and MgATP in acting together.

5 Coexpression of SUR1 and SUR2B(Y1206S) with Kir6.2 reduced both potency and efficacy of ATP in inhibiting glibenclamide binding; this was particularly marked for Kir6.2/SUR1.

6 The data show (a) that the inhibitory effects of ATP and ADP on glibenclamide binding differ from one another, (b) that they depend on the SUR subtype, and (c) that they are weakened by coexpression with Kir6.2.

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Abbreviations: ACS, ATP-consuming system; AMP-CP, α,β-methyleneadenosine 5′-diphosphate; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; ARS, ATP-regenerating system; GBC, glibenclamide; HEK cells, human embryonic kidney cells; K_{ATP} channels, ATP-sensitive K^+ channels; Kir, inward rectifier potassium channel; NBF, nucleotide binding fold; SUR, sulphonylurea receptor

Introduction

Sulphonylureas like glibenclamide stimulate insulin secretion and are used as treatment in type-2 diabetes (Groop & Neugebauer, 1996). At higher concentrations glibenclamide shows effects also in the ischaemic heart, where it blocks the ischaemia-induced narrowing of the cardiac action potential (Fosset *et al*., 1988) and the dilatation of the coronary vascular bed (Daut *et al*., 1990). Glibenclamide elicits these different effects by blocking ATP-sensitive K^+ channels (K_{ATP} channels) in the cell membrane. K_{ATP} channels are closed by intracellular ATP and opened by MgADP and therefore link membrane potential to the metabolic state of the cell (Ashcroft & Ashcroft, 1990; Nichols & Lederer, 1991).

K_{ATP} channels are composed of two types of subunits, the pore-forming subunits (Kir6.x) and the sulphonylurea receptors (SURs) (reviews: Ashcroft & Gribble, 1998; Aguilar-Bryan & Bryan, 1999; Seino, 1999). SURs are members of the ATP-binding cassette protein superfamily (Aguilar-Bryan *et al*., 1995; Sakura *et al*., 1995; Tusnády *et al*., 1997). They carry binding sites for nucleotides (Ueda *et al*., 1997; 1999; Matsuo *et al*., 1999; 2000) for the sulphonylureas and for the K^+ channel openers, a class of compounds which activate these channels (Aguilar-Bryan *et al*., 1995; Hambrock *et al*., 1998; Schwanstecher *et al*., 1998; Ashcroft & Gribble, 2000). Two main subtypes of SUR have been characterized. SUR1 is the dominant form in pancreatic β-cells and in brain (Aguilar-Bryan *et al*., 1995; Sakura *et al*., 1995) and SUR2 in muscle. SUR2 exists in two isoforms which differ only in the last exon (42 amino acids). One isoform (SUR2A) is found in striated and the other (SUR2B) in smooth muscle (Inagaki *et al*., 1996; Isomoto *et al*., 1996). SUR1 has a higher affinity for the sulphonylureas than the SUR2 isoforms (Aguilar-Bryan *et al*., 1995; Dörschner *et al*., 1999; Russ *et al*., 1999; 2001). This is due, at least in part, to serine in position 1206 in SUR1 (mouse numbering) being exchanged to tyrosine in SUR2 (Ashfield *et al*., 1999). Mutating Ser to Tyr in SUR1 abolishes high affinity glibenclamide binding and high affinity channel inhibition by tolbutamide (Ashfield *et al*., 1999). Conversely, exchan-

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ging Tyr 1206 in SUR2B for Ser increases the affinity of glibenclamide binding 5–10 times (Toman *et al*., 2000; Hambrock *et al*., 2001; Löffler-Walz *et al*., 2002). The pore forming subunit of classical K_{ATP} channels is Kir6.2.

The potency and efficacy of sulphonylureas in closing K_{ATP} channels depend on the nucleotides present with ADP being particularly important (pancreatic β-cells: Zünkler *et al*., 1988; cardiac myocyte: Venkatesh *et al*., 1991; Miyamura *et al*., 2000). [^{3}H]-glibenclamide binding studies in membranes from pancreatic β-cells and brain (i.e. predominantly Kir6.2/SUR1) have shown that ATP reduces glibenclamide binding. The presence of Mg^{2+} is required for this effect and non-hydrolysable ATP analogues like β,γ-methyleneadenosine 5′-triphosphate (AMP–PCP) are inactive (reviewed in Ashcroft & Ashcroft, 1992). Schwanstecher and colleagues showed that this inhibition was due to a reduction in the affinity of SUR for glibenclamide by MgATP binding to a site different from the glibenclamide site (Schwanstecher *et al*., 1991; 1992). MgADP also inhibits glibenclamide binding to SUR in pancreatic islets and brain (review: Ashcroft & Ashcroft, 1992); however, in this case, inhibition was reported to reflect a reduction of the number of binding sites, B_{MAX} (Gopalakrishnan *et al*., 1991).

When assessing these studies one has to consider that during incubation of ATP with membranes a substantial amount is hydrolysed to ADP by various ATPases; conversely, ADP is converted in membranes to ATP (+AMP) by adenylate kinase (Gopalakrishnan *et al*., 1991; Schwanstecher *et al*., 1991; Hambrock *et al*., 1999). Hence, binding studies, in which ATP or ADP are added in the presence of Mg^{2+} without taking precautionary measures, reflect the action of a combination of the nucleotides. However, ATP hydrolysis can be counteracted by coupling of an ATP-regenerating system (ARS, e.g. based on the creatine kinase reaction in which the high phosphate group transfer potential of creatine phosphate is used to resynthesise ATP from ADP (Gopalakrishnan *et al*., 1991; Dickinson *et al*., 1997; Hambrock *et al*., 1999). Similarly, the adenylate kinase reaction can be counteracted by coupling of an ATP-consuming system (ACS), e.g. the hexokinase reaction in which the ATP formed is converted back to ADP by phosphorylation of glucose. Schwanstecher *et al*. (1991) found that MgADP did not affect glibenclamide binding to membranes from pancreatic islets if ATP formation was prevented by the hexokinase reaction.

These complexities are long known. However, we are not aware of studies in which the modulation of glibenclamide binding to K_{ATP} channels (or SUR subtypes) by ATP and ADP was assessed in parallel in the absence and presence of the respective nucleotide-regenerating systems. Here, we present such a study in order to assess the effects of the two nucleotides separately as well as the degree of synergy between them. Another problem concerns potential differences between the different SUR subtypes. In case of SUR2A/B, knowledge about the allosteric coupling between nucleotide and glibenclamide binding is particularly scarce, probably due to the lower affinity of the SUR2 isoforms for glibenclamide. In order to obtain better glibenclamide binding, we have used mutants with increased sulphonylurea affinity (SUR2(Y1206S), see above). For SUR2B(Y1206S) it was shown that the coupling between ATP binding and glibenclamide or opener binding is unperturbed and that upon coexpression with Kir6.2 a typical K_{ATP} channel is formed (Hambrock *et al*., 2001; Löffler-Walz *et al*., 2002). SUR2A(Y1206S) behaves quite similarly; the full characterization of this mutant will be presented elsewhere.

Methods

SUR2 mutants, cell culture, transfection and membrane preparation

The mutants SUR2A(Y1206S) and SUR2B(Y1206S) were constructed from murine SUR2A or SUR2B (GenBank accession numbers D86037 and D86038, respectively; Isomoto *et al*., 1996) using the Site-Directed Mutagenesis System (Stratagene, Amsterdam, The Netherlands) as described for mutant SUR2B (Hambrock *et al*., 2001). The presence of the mutation was confirmed by nucleotide sequencing of the relevant DNA region. Human embryonic kidney (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 pcDNA 3.1 vector (Invitrogen, Karlsruhe, Germany) containing the coding sequence of rat SUR1 (GenBank accession number X97279), SUR2-A(Y1206S) or SUR2B(Y1206S), and cell lines stably expressing these proteins were isolated as described previously (Hambrock *et al*., 1998). For binding studies on Kir6.2/SUR complexes, cells were transfected with Kir6.2 (Genbank accession number D50581, Inagaki *et al*., 1996) and SUR at a molar plasmid ratio of 1 : 1 for transient expression.

For cells stably expressing SUR alone, the antibiotic was withdrawn from the culture medium one week prior to membrane preparation; cells transiently expressing Kir6.2/SUR were harvested 2–3 days after transfection. Membranes were prepared as described (Hambrock *et al*., 1998). In brief, cells were centrifuged for 5 min at 500 *g* and 4°C and lysed by addition of ice-cold hypotonic buffer containing (in mM): HEPES, 10; EGTA, 1 at pH 7.4. The lysate was centrifuged at 10^5 *g* and 4°C for 60 min and the resulting membrane pellet was resuspended in a buffer containing (in mM): HEPES, 5; KCl, 5; NaCl, 139; at pH 7.4 and 4°C at a protein concentration of ~1.5–3.0 mg protein ml^{-1} and frozen at −80°C. Protein concentration was determined according to Lowry *et al*. (1951) using bovine serum albumin as the standard.

[^{3}H]-glibenclamide binding experiments

To study [^{3}H]-glibenclamide binding to SUR, membranes (final protein concentration SUR1: 50–100 μg protein ml^{-1}, SUR2: 200 μg protein ml^{-1}) were added to the incubation buffer (mM) (NaCl 139, KCl 5, HEPES 5 mM) supplemented with [^{3}H]-glibenclamide (~1 nM for SUR1 and 2–2.5 nM for SUR2, respectively) at 37°C. After 15 min at 37°C incubation was stopped by diluting 0.3 ml aliquots in triplicate into 8 ml of quench solution (50 mM tris-(hydroxymethyl)-aminomethane, 154 mM NaCl, pH 7.4) at 0°C and filtration over Whatman GF/B filters. Non-specific binding was determined in the presence of 1 μM glibenclamide (SUR1) or 100 μM unlabelled P1075 (SUR2) and ranged from 6±1% of total

binding (SUR1) to 22–32% (SUR2). Non-transfected HEK 293 cells possess endogenous low affinity glibenclamide sites which are not sensitive to P1075, which, however, completely displaces specific glibenclamide binding (=glibenclamide binding to mutant SURB2 (Hambrock *et al*., 2001) and SUR2A (E. Strauß, personal communication)).

ATP and ADP were added as the sodium salts and $[Mg^{2+}]_{free}$ was kept at $\geqslant 1$ mM. The ATP-regenerating system (ARS) consisted of creatine kinase (5 u ml^{-1}) and creatine phosphate (3 mM) in the presence of 10 mM Mg^{2+} as described (Hambrock *et al*., 1999). In some experiments the system was scaled up to creatine kinase (50 u ml^{-1}) and creatine phosphate (20 mM) in the presence of 25 mM Mg^{2+}. The ATP-consuming system (ACS) consisted of hexokinase (5 u ml^{-1}) and glucose (11 mM). In case of Mg^{2+}-free experiments, Mg^{2+} was omitted from the incubation solution and EDTA (1 mM) was added.

Data analysis

Individual nucleotide inhibition curves were analysed using the logistic form of the Hill equation,

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

to the data. Here A denotes the extent of the effect (amplitude); n ($=n_H$) is the Hill coefficient, x the concentration of the compound under study and K ($=IC_{50}$) the midpoint of the curve with $px = -\log x$ and $pK = -\log IC_{50}$. To analyse biphasic curves, the superposition of two logistic terms with Hill coefficient 1 was used. Saturation experiments were analysed according to the equation.

$$B_{TOT} = B_{MAX} * L * (L + K_D)^{-1} + a * L. \quad (2)$$

Total binding (B_{TOT}) is the sum of specific and non-specific binding. Here, B_{MAX} (fmol mg^{-1} protein) denotes the concentration of specific binding sites in the preparation, K_D is the equilibrium dissociation constant, L the concentration of the radioligand and a the proportionality constant describing nonspecific binding as a linear function of L. Nonspecific binding was determined as described above. Specific binding was also plotted in the Scatchard presentation (Scatchard, 1949).

Curves were analysed according to the method of least squares using the SigmaPlot programme (SPSS, Inc, Chicago, IL, U.S.A.). Errors in the parameters were estimated assuming that amplitudes and pIC_{50} values are normally distributed (Christopoulos, 1998). These were then averaged and pIC_{50} values $\pm$ s.e.mean were converted to IC_{50} values with the 95% confidence interval.

Materials

[^{3}H]-glibenclamide (specific activity 1.85 TBq (52 Ci) $mmol^{-1}$) was purchased from Perkin Elmer Life Sciences (Bad Homburg, Germany). The reagents and media used for cell culture and transfection were from Invitrogen (Karlsruhe, Germany). Na_2ATP and Na_2ADP were from Roche Diagnostics (Mannheim, Germany); creatine kinase, creatine phosphate, hexokinase, adenosine 5′ monophosphate (AMP), α,β-methyleneadenosine 5′-diphosphate (AMP–PCP), β,γ-methyleneadenosine 5′-triphosphate (AMP–CP) and glibenclamide from Sigma (Deisenhofen, Germany). Nucleotides were dissolved in incubation buffer keeping pH at 7.4. P1075 and glibenclamide were dissolved in dimethyl sulphoxide/ethanol (1 : 1) and further diluted in the same solvent or in incubation buffer. In binding studies solvent concentration was always below 0.2%.

Results

[^{3}H]-glibenclamide binding to SUR and adenine nucleotides

In the presence of Mg^{2+}, ATP and ADP inhibited [^{3}H]-glibenclamide binding to the three SUR subtypes (Figure 1A,C) whereas AMP (0.1 and 1 mM) was ineffective (not illustrated). In the absence of Mg^{2+} (no Mg^{2+} added, 1 mM EDTA), neither ATP nor ADP affected [^{3}H]-glibenclamide binding, nor did Mg^{2+} (1–10 mM) in the absence of nucleotides. Further experiments, conducted with SUR2B (Y1206S), showed that the non-hydrolysable analogues of ATP and ADP, AMP–PCP and AMP–CP (both at 0.1 and 1 mM), were ineffective regardless of the presence of Mg^{2+} (not shown). These data with mutant SUR2B extend earlier studies which showed that reduction of glibenclamide binding to SUR1-containing K_{ATP} channels required the presence of Mg^{2+} and of hydrolysable nucleotides (Ashcroft & Ashcroft, 1992). The experiments described below were conducted in the presence of free Mg^{2+} ($\sim$1 mM) and it is understood that the Mg^{2+}-complexes of the adenine nucleotides are the active components.

Inhibition of [^{3}H]-glibenclamide binding to SUR by ATP

Figure 1A shows the concentration-dependent inhibition of [^{3}H]-glibenclamide binding to SUR1 and the two mutant SUR2 subtypes by MgATP. ATP reduced binding to SUR1 and SUR2B(Y1206S) by >70%; in case of SUR2A(Y1206S), the extent of inhibition was slightly less. The inhibition curve of SUR1 exhibited two components of similar amplitudes with IC_{50} values of 3.9 and 224 μM (see Table 1 for parameters). The inhibition curves of the SUR2 mutants were monophasic and had similar IC_{50} values (~ 8 μM); the curve for mutant SUR2B showed slight cooperativity ($n_H = 1.20 \pm 0.02$).

During incubation with membranes in the presence of Mg^{2+}, ATP is hydrolysed partly to ADP, and Figure 1A therefore reflects the combined effect of MgATP in the presence of some MgADP (see Introduction). The amount of ADP formed can be reduced to about 1/10 (i.e. to $\sim$1% of the amount of ATP initially present) by coupling of an ATP-reconstituting system (ARS) based on the creatine phosphate-creatine kinase reaction (Hambrock *et al*., 1999). Figure 1B shows the effect of ATP in the presence of the ARS using 5 u ml^{-1} creatine kinase. Under these conditions, the MgATP inhibition curve of [^{3}H]-glibenclamide binding to SUR1 remained biphasic. The first component showed an unchanged IC_{50} value (3.9 *vs* 4.7 μM) but was smaller in amplitude; the second shifted rightwards by a factor of ~ 5 (from 220 to 1300 μM). Surprisingly, the inhibition curve of SUR2B(Y1206S), which showed positive cooperativity in the absence of the ARS, become also biphasic: a prominent first components with an IC_{50} value similar to that obtained

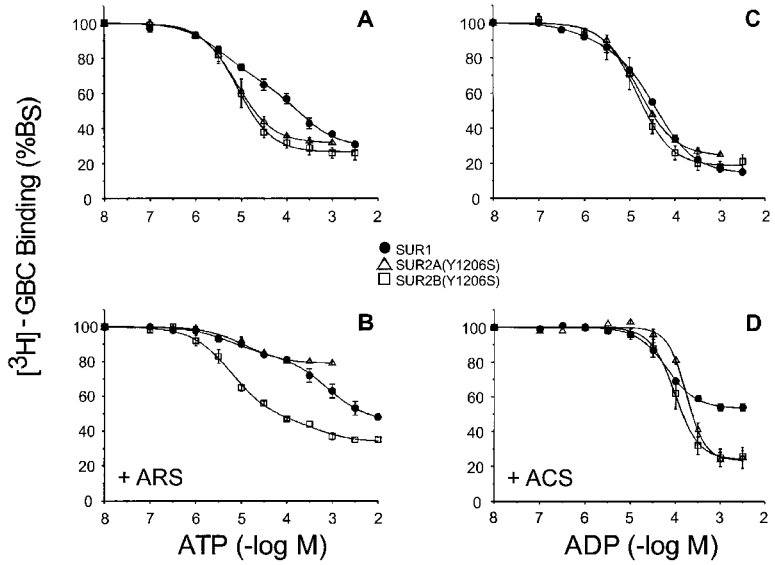

Figure 1 Inhibition of [^{3}H]-glibenclamide binding to SUR subtypes by adenine nucleotides. Left: Dependence on ATP in the absence (A) and in the presence (B) of an ATP-regenerating system (ARS, [creatine kinase] = 5 u ml^{-1}). Right: Dependence on ADP in the absence (C) and presence (D) of an ATP-consuming system (ACS, based on the hexokinase reaction). Free Mg^{2+} was $\geqslant$1 mM. Data points represent means$\pm$s.e.mean from 3–4 independent experiments. The logistic form of the Hill equation was fitted to the individual experiments and the mean values of the parameters are listed in Table 1.

Table 1 Nucleotide dependence of [^{3}H]-glibenclamide binding to SUR subtypes. Parameters listed are IC_{50} values (with confidence interval in brackets), amplitudes (A) and Hill coefficients (n_H, values listed in italics). K_i ($\sim K_D$) value for ATP or ADP binding to NBF_1/NBF_2 of SUR subtypes are from Matsuo *et al.* (2000).

	ATP		*ATP-regenerating system*		*ADP*		*ATP-consuming system*	
		A (%B_s)	*IC_{50}* (μM)			*A* (%B_s)	*IC_{50}* (μM)	*A* (%B_s)
SUR	*IC_{50}* (μM)	n_H[a]	K_i *(ATP)* (μM)[b]	*A (%B_s)*	*IC_{50}* (μM)	n_H[a]	K_i *(ADP)* (μM)[b]	n_H[a]
SUR1	3.9 [2.0, 7.4]	34$\pm$4	4.7 [1.5, 14.8]	20$\pm$4	1.4 [0.9, 2.3]	16$\pm$2	63 [50, 79]	47$\pm$2
	220 [200, 250]	38$\pm$2	1300 [560, 3000]	43$\pm$1	40 [35, 46]	71$\pm$2		*1.30$\pm$0.19*
			4.7$\pm$3.7/60$\pm$26[b]				26$\pm$9/100$\pm$26[b]	
SUR1/Kir6.2	56 [31, 102]	20$\pm$6	n.d.[c]	n.d.[c]	n.d.[c]	25$\pm$1[d]	n.d.[c]	11$\pm$1[d]
SUR2A(Y1206S)	7.6 [5.8, 10.0]	68$\pm$1	10 [4.0, 23]	21$\pm$2	16 [14, 17]	76$\pm$1	170 [150, 200]	78$\pm$4
						1.07$\pm$0.03		*2.03$\pm$0.02*
			110$\pm$41/120$\pm$39[b]				86$\pm$23/170$\pm$70[b]	
SURB(Y1206S)	8.7 [6.3, 12.0]	74$\pm$3	5.6 [3.0, 10.7]	53$\pm$2	14 [9, 24]	82$\pm$4	91 [58, 150]	78$\pm$2
		1.20$\pm$0.02	230 [160, 360]	11$\pm$1		*1.29$\pm$0.28*		*1.75$\pm$0.24*
			51$\pm$13/38$\pm$26[b]				66$\pm$8/67$\pm$40[b]	
SUR2B(Y1206S) /Kir6.2	17 [13, 23]	57$\pm$6 *1.58$\pm$0.06*	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]

Binding experiments were performed as described in Figure 1. [a]Hill coefficients were determined only for monophasic curves and are listed if deviating from 1. [b]K_i ($\sim K_D$) value for ATP or ADP binding to NBF_1/NBF_2 of SUR subtypes from Matsuo *et al.* (2000); values determined by inhibition of 8-azido-[α-^{32}P]ATP binding at 0°C for 10 min in the presence of 1 mM Mg^{2+}. Enzymatic activities were therefore minimized and K_i values are best compared to our IC_{50} values in the presence of the ARS or ACS. [c]n.d. not determined. [d]Value at 1 mM ADP (n = 4).

before ($\sim$6–9 μM) was followed by a small low affinity component (IC_{50} $\sim$230 μM). The inhibition curve of SUR2A(Y1206S) remained monophasic with an IC_{50} value again similar to that in the absence of the ARS; however, the extent of inhibition was now only 21%. This showed that MgADP is a major modulator of [^{3}H]-glibenclamide binding to mutant SUR2A.

The high concentrations of MgATP (>mM) needed to saturate the low affinity components of the SUR1 and mutant SUR2B curves (Figure 1B) raised the suspicion that the ARS may have been insufficient and that contaminating MgADP may have contributed to these components. This possibility was tested by scaling up the ARS 10 fold (creatine kinase 50 u ml^{-1}, creatine phosphate 20 mM, Mg^{2+} 25 mM). Repeating the inhibition curve of SUR1 under these conditions resulted only in a minor change in the amplitudes and left the IC_{50} values unchanged (data not illustrated). This showed that the down-scaled ARS used in Figure 1C was adequate.

In the course of these experiments it was noticed that [^{3}H]-glibenclamide bound to creatine kinase, increasing the amount of binding displaced by 1 μM GBC by 25$\pm$3% at a creatine kinase concentration of $\sim$161 μg protein ml^{-1} in the presence of 50 μg membrane protein ml^{-1} (SUR1). This was not observed with the SUR2 subtypes since the ratio of creatine kinase to membrane protein was smaller (16 : 200 μg

protein ml^{-1}). Binding of GBC to SUR was, however, not affected by the ARS which is noteworthy in view of the fact that creatine kinase (Crawford *et al*., 2002) and adenylate kinase (Carrasco *et al*., 2001; see below) form supramolecular complexes with the K_{ATP} channel in cardiocytes.

Inhibition of glibenclamide binding to SUR by ADP

The results obtained with ADP qualitatively resembled those with ATP (Figure 1C). The SUR1 curve was again biphasic; however, with ADP, the amplitude ratio of the two phases was 20 : 80% and the IC_{50} values were shifted leftwards (1.4 and 40 μM). The inhibition curves of the SUR2 mutants were monophasic with IC_{50} values ($\sim$15 μM) about two times higher than measured with ATP. In all cases, the extent of inhibition by ADP was slightly but significantly larger than observed with ATP.

To counteract synthesis of ATP from ADP by adenylate kinase during incubation, experiments were conducted also in the presence of an ATP-consuming system (ACS) based on the glucose–hexokinase reaction (see Methods). The presence of the ACS did not affect glibenclamide binding to SUR. Figure 1D shows that in the presence of the ACS, the ADP inhibition curves were all monophasic and shifted towards higher concentrations (Table 1). Most importantly, binding to SUR1 was inhibited only by 47% (as compared to an amplitude of 87% in the absence of the ACS, see Table 1); in contrast, the inhibition curves with mutant SUR2 exhibited amplitudes of 78%. This showed that MgADP alone was more effective on the SUR2 subtypes than on SUR1. The Hill coefficients of the inhibition curves were >1 with those of the SUR2 curves approaching 2 (Table 1). AMP, which is formed by hydrolysis of MgADP or by the adenylate kinase reaction, is ineffective in modulating [^{3}H]-glibenclamide binding (see above).

Effects of MgATP on [^{3}H]-glibenclamide binding to Kir6.2/SUR complexes

Experiments were performed also using membranes from cells transiently expressing Kir6.2+SUR1 or SUR2B(Y1206S). The results in Figure 1 had shown that the effects of MgATP were most prominent in the absence of an ARS. In order to pick up eventually small changes induced by coexpression with Kir6.2, the MgATP dependence was measured therefore in the absence of an ARS. Figure 2 illustrates that ATP inhibited glibenclamide binding to Kir6.2/SUR1 in a monophasic manner with an amplitude of 20% and an IC_{50} value of 56 μM (Table 1). ADP (1 mM) inhibited glibenclamide binding to the Kir6.2/SUR1 complex by 25% and the amplitude was reduced further to 11% in the presence of the ACS (Table 1). In case of SUR2B(Y1206S), the effects of coexpression with Kir6.2 were less stringent: the inhibition curve was shifted rightwards by a factor of 2, the amplitude reduced (from 74 to 57%) and the co-operativity increased (from 1.2 to 1.6, Figure 2 and Table 1).

Effects of MgATP on [^{3}H]-glibenclamide saturation experiments

In order to see whether the inhibitory effect of MgATP on [^{3}H]-glibenclamide binding to SUR was due to a reduction in affinity or in the number of high affinity binding sites (B_{MAX}) or in both, saturation binding experiments were performed in the absence and presence of saturating MgATP (Figure 3, Table 2). In the case of SUR1, MgATP increased the K_D value about six times leaving B_{MAX} unchanged. It is easily calculated that at a [^{3}H]-glibenclamide concentration of 1 nM, an increase in K_D from 0.9 to 5.2 nM reduces binding of the radioligand by 70% as is indeed the case (Figure 1). In contrast, for both mutant SUR2 subtypes, MgATP reduced B_{MAX} and left K_D unaltered.

Discussion

Effects of ATP in the presence of an ARS

'Pure' ATP reduced [^{3}H]-glibenclamide binding to all SUR subtypes with a high affinity component (IC_{50} values 5 to 10 μM). In case of SUR1, the IC_{50} value agrees well with the K_D of ATP binding to NBF1 (Matsuo *et al*., 2000; here: Table 1). This could mean that ATP binding to NBF1 and hydrolysis there (Bienengraeber *et al*., 2000) mediates the inhibition of [^{3}H]-glibenclamide binding to SUR1. In case of mutant SUR2A/B, however, such speculation is applied less easily; here, the respective IC_{50} values of the ATP inhibition curves are $\sim$10 times lower than the corresponding K_D values for ATP binding to NBF1 (or NBF2) of wild-type SUR2A/B. It is unlikely that this discrepancy is a consequence of the mutation since there was no difference in the effect of ATP (-ARS) on glibenclamide binding to wild-type and mutant SUR2B (Löffler-Walz *et al*., 2002). Rather, this discrepancy seems to reflect differences in experimental conditions: The photoaffinity labelling experiments were conducted at 0°C, the [^{3}H]-glibenclamide binding studies at 37°C and the temperature dependence of ATP binding and hydrolysis may differ between the SUR subtypes. The difference in amplitude between the SUR2A(Y1206S) curve (21%) and the high affinity component of the SUR2B(Y1206S) curve (53%) is of interest. Since these isoforms differ only in their last 42 carboxyterminal amino acids this suggests that this part of the protein may interact with the NBFs (see below).

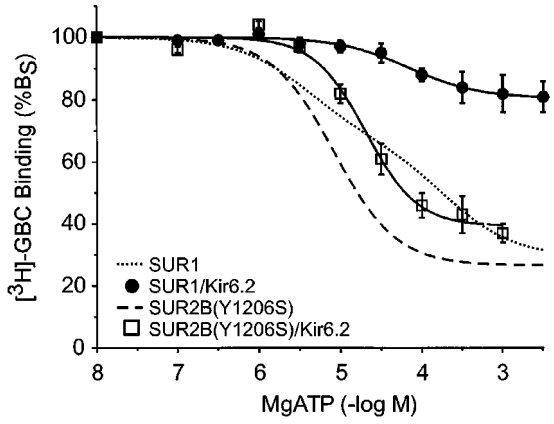

Figure 2 Inhibition of [^{3}H]-glibenclamide binding to Kir6.2/SUR1 and Kir6.2/SUR2B(Y1206S) by MgATP. Experiments (in the absence of an ARS, $n=3-4$) were analysed as described in Figure 1; for parameters see Table 1. The fitting curves for SUR in the absence of Kir6.2 (see Figure 1) are shown for comparison; data points are omitted for clarity.

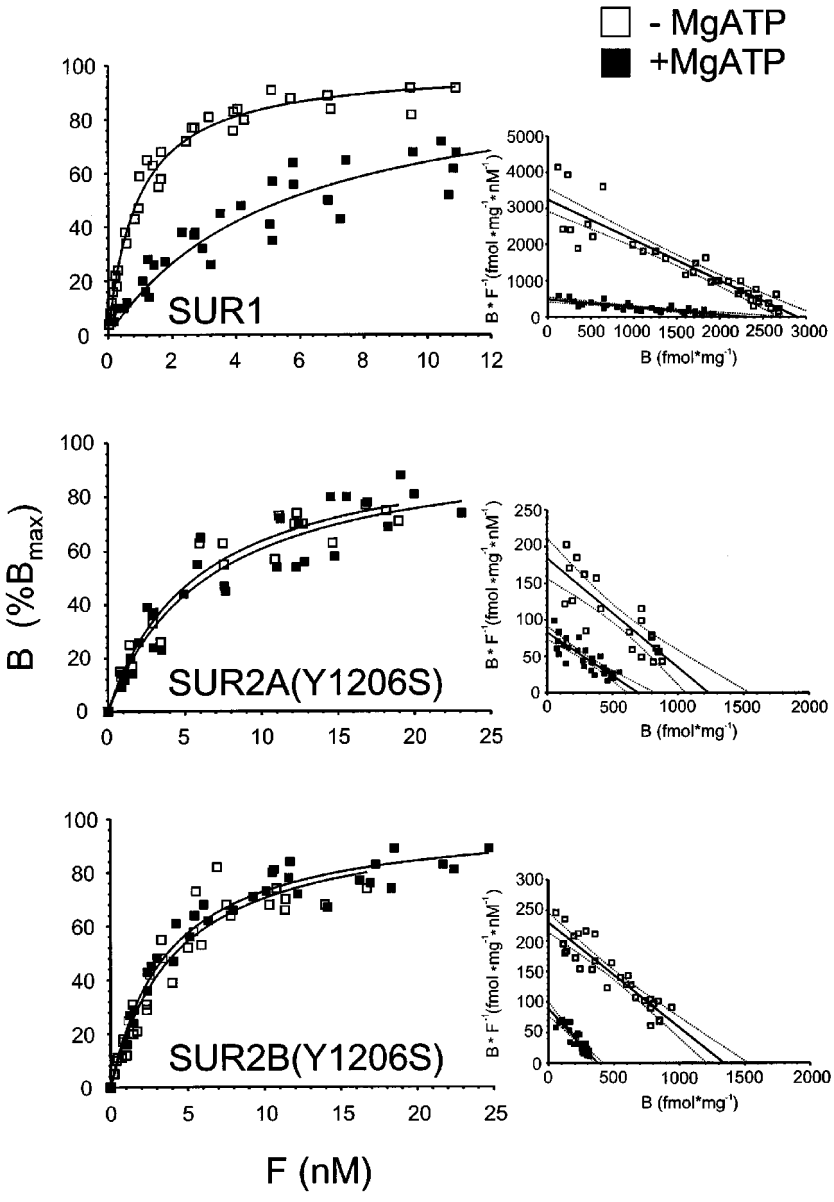

Figure 3 [^{3}H]-glibenclamide binding saturation experiments to SUR subtypes in the absence and presence of MgATP. Left: Original data, right Scatchard representation to directly indicate changes in K_D (slope^{-1}) and B_{MAX} (abscissa intercept); here B denotes the concentration of the radioligand specifically bound and F the free radioligand concentration. Experiments in the absence of MgATP were performed in the presence of EDTA (1 mM), experiments at saturating MgATP in the presence of 1 mM (mutant SUR2A/B) or 3 mM MgATP (SUR1). Individual experiments ($n=3-5$) were evaluated according to equation 2 in Methods (mean parameters listed in Table 2), original data were then normalized to 100% binding at saturation and pooled. 95% confidence bands in the Scatchard representation were calculated according to Draper & Smith (1981). Nonspecific binding was proportional to radioligand concentration with slopes ranging from 34 to 39 fmol mg^{-1} nM^{-1} for the SUR subtypes in the presence of MgATP and 53 to 60 fmol mg^{-1} nM^{-1} in its absence, respectively.

In the case of SUR1 and SUR2B(Y1206S), the MgATP inhibition curve exhibited also a low affinity component. The respective IC_{50} values did not correlate with the K_D values for ATP binding to either NBF as determined by the Ueda group (Table 1). The fact that the low affinity component was seen with SUR2B(Y1206S) but not with SUR2A(Y1206S) highlights again the importance of the carboxyterminus. Interestingly, the curve for SUR1 showed also a second component. This fits with the fact that the amino acids of last carboxyterminal exon of (rat) SUR1 and (mouse) SUR2B show a high degree of identity (74%) whereas that of (mouse) SUR2A is different (33% identity with SUR2B; Isomoto *et al*., 1996). The carboxyterminus of SUR is an important modulator of the effects of nucleotides on channel activation and on the interaction of the channel with openers (Shindo *et al*., 1998; Hambrock *et al*., 1999; Matsuoka *et al*., 2000; Reimann *et al*., 2000). The data presented here show that SUR2A stands out also regarding the modulation of glibenclamide binding by (pure) MgATP; the efficacy of MgATP at this isoform is strikingly poor.

Effects of ADP in the presence of an ACS

The inhibition curves of 'pure' ADP alone for all SURs were monophasic with IC_{50} values ranging from 63 to 170 μM. These values are in reasonable agreement with the K_D values for ADP binding to the NBFs (or with the mean of the two values) determined by Matsuo *et al*. (2000) (Table 1). MgADP displayed a particularly high efficacy at the SUR2(Y1206S) isoforms leading to a marked efficacy difference between ADP and ATP at mutant SUR2A. Conversely, at SUR1, ATP is more efficacious than ADP.

All ADP curves displayed positive cooperativity (Hill coefficients >1) and this was strongest with the SUR2(Y1206S) subtypes. The positive cooperativity of the inhibition curves could reflect positive cooperativity of MgADP binding to the NBFs at 37°C; however, no cooperativity was observed by Matsuo *et al*. (2000) at 0°C. Alternatively, binding of one molecule of MgADP may affect the binding of more than one molecule of glibenclamide. This possibility is most easily explained if SUR expressed alone forms homomultimers (e.g. tetramers), and, recently, we have indeed obtained indirect evidence for tetramerization of (mutant) SUR2B expressed alone (Löffler-Walz *et al*., 2002). In an alternative model the cooperativity could reflect binding of glibenclamide to two states of monomeric SUR which are not at equilibrium (Boeynaems & Dumont, 1980). Without further experiments it is impossible to decide between these models and it remains intriguing that ADP displays strong positive cooperativity in inhibiting glibenclamide binding to the SUR2(Y1206S) subtypes.

Synergy between ATP and ADP

The equilibrium constant of the adenylate kinase reaction is ~ 1 under physiological conditions (Stryer, 1988) and in the absence of hexokinase, ADP and ATP will be present at comparable concentrations. Synergy between the nucleotides, if it occurs, should therefore be apparent if the ADP inhibition curves in presence and absence of the ACS are compared. In the absence of the ACS, the ADP inhibition curves of the SUR2(Y1206S) isoforms were shifted leftwards by factors of 6.5 and 10, indicating synergy between ADP and ATP; the amplitudes were unchanged and the cooperativity was abolished. For SUR1, the curve became biphasic but was again shifted leftwards; the total amplitude was larger than that reached with ADP or ATP alone.

In the case of ATP in the absence of the ARS, the amount of ATP hydrolysis under our experimental conditions ranges from total at low μM ATP to 10% at mM ATP concentrations (Hambrock *et al*., 1999). In the absence of the ARS, the most significant changes in the ATP inhibition curves were the 6 fold leftward shift of the low affinity component of the SUR1 curve, the more than tripling of the amplitude of the SUR2A(Y1206S) curve and the fact that the biphasic SUR2B(Y1206S) curve became monophasic with positive cooperativity. Collectively, the various leftward shifts of the inhibition curves observed in the absence of the respective reconstituting enzyme system are a clear indication that ATP and ADP together are more potent than either one alone in inhibiting glibenclamide binding.

Saturation experiments: differences between SUR1 and SUR2

In case of SUR1, ATP antagonised glibenclamide binding by reducing the affinity, leaving the number of binding sites unchanged. This result is in agreement with earlier experiments in membranes from pancreatic islets (Schwanstecher *et al*., 1991). The data for SUR1 are compatible with a classical two-state scheme in which the receptor exists in two interconvertible states and MgATP shifts the equilibrium towards the state with lower affinity for glibenclamide in a homogeneous manner (Boeynaems & Dumont, 1980).

In case of the SUR2(Y1206S) isoforms, ATP reduced the number of observed binding sites, leaving the K_D value of the remaining sites unchanged. This means that MgATP created an inhomogeneity by shifting a certain proportion of the high affinity sites ($\sim$43% for SUR2A(Y1206S) and $\sim$71% for SUR2B(Y1206S)) towards lower affinity so that they escaped detection in the radioligand binding assay; the other sites remained in the high affinity form (K_D unaffected). There are at least two models which can account for this inhomogeneity (see also above). The first assumes that SUR2, expressed alone, forms tetramers with the degree of negative allosteric coupling between subunits depending on the isoform. In case of $[SUR2B(Y1206S)]_4$, occupation of the nucleotide sites allows high affinity binding of only one glibenclamide molecule per tetramer; in case of $[SUR2A(Y1206S)]_4$, two glibenclamide molecules per tetramer may bind with high affinity. The second model assumes monomeric SUR cycling between two states at steady state but not at equilibrium; the cycle is fuelled by the hydrolysis of ATP (Boeynaems & Dumont, 1980; Löeffler-Walz *et al*., 2002). Whatever the case, these experiments highlight an intriguing mechanistic difference between SUR1 and (mutant) SUR2.

Coexpression

Coexpression with Kir6.2 reduced the efficacy and potency of ATP and ADP in inhibiting glibenclamide binding to SUR1

Table 2 Effects of MgATP on the affinity (K_D) of [^{3}H]-glibenclamide binding to SUR subtypes and on the number of binding sites (B_{MAX})

		−MgATP			*+MgATP*[a]	
SUR	K_D (nM)	B_{max} (pmol mg^{-1})	*(n)*	K_D (nM)	B_{max} (pmol mg^{-1})	*(n)*
SUR1	0.88 [0.74, 1.06]	2.9±0.3	(4)	5.2 [3.6, 7.6][b]	2.5±0.2	(5)
SUR2A(Y1206S)	5.6 [3.9, 8.1]	1.1±0.1	(3)	6.6 [4.5, 10]	0.63±0.11[b]	(4)
SUR2B(Y1206S)	4.1 [3.0, 5.6]	1.2±0.2	(4)	3.9 [3.0, 4.8]	0.35±0.03[b]	(4)

K_D and B_{MAX} values were determined as described in Figure 4; differences between parameters in the presence and absence of MgATP are indicated. [a]3 mM (SUR1) or 1 mM (SUR2). [b]Different from the corresponding values in the absence of MgATP ($P<0.001$).

and SUR2 and SUR2(Y1206S). This was particularly marked for SUR1 where the amplitude of the ATP inhibition curve was decreased from 72% to 20%. A (minor) part of this decrease in amplitude is, however, explained by the experimental conditions since at SUR1, MgATP acts by decreasing the affinity for glibenclamide (see above). Considering that coexpression with Kir6.2 increases the affinity of SUR1 for glibenclamide by 2 times (K_D values in the absence of MgATP: 0.88 and 0.40 nM, respectively), the Law of Mass Action predicts a reduction in amplitude from 72 to ~50% at a radioligand concentration of 1 nM. Hence, a large part of the amplitude decrease is a real consequence of contransfection. In addition, coexpression with Kir6.2 rendered the ATP inhibition curve monophasic with an IC_{50} value (56 μM) in between the IC_{50} values of the biphasic ATP curve for SUR1 alone (3.9 and 220 μM). The data obtained here for Kir6.2/SUR1 at 37°C are at variance with results obtained in membranes from pancreatic islets at 22°C where an ATP inhibition curve with $IC_{50}=11.6$ μM, amplitude ~60% and Hill coefficient 1.5 was reported (Schwanstecher *et al*., 1991). In addition, MgATP was found to be without effect if the hexokinase reaction was coupled whereas we found a residual effect of 11%. These differences may be due to different experimental conditions, in particular temperature.

For SUR2B(Y1206S) too, coexpression with Kir6.2 reduced the negative allosteric effect of ATP on glibenclamide binding in potency and efficacy; however, the changes were less pronounced than with SUR1. Collectively, these results (together with the increase in glibenclamide affinity of SUR1 upon coexpression with Kir6.2, see above) are a further example in point that coexpression with Kir6.x affects the properties of glibenclamide binding to SUR (Hambrock *et al*., 2001).

Limitations of the study

The results presented here have been interpreted assuming that the nucleotide effects on [^{3}H]-glibenclamide binding were mediated by nucleotide binding to the NBFs of SUR. This assumption is based mainly on the analogy with opener binding to SUR. In this case, the presence of Mg^{2+} and of ATP or a hydrolysable analogue is required (Schwanstecher *et al*., 1992; Quast *et al*., 1993). In addition, it has been shown that mutation of the Lys residue in the Walker A motif of either NBF abolishes opener binding to SUR2B and opener-induced activation of the Kir6.2/SUR2B channel (Schwanstecher *et al*., 1998). This indicates that the activating effect of MgATP on opener binding is mediated by nucleotide binding to the NBFs. In case of the adenine nucleotide effects on glibenclamide binding, the presence of Mg^{2+} and of hydrolysable ATP/ADP analogues was also required (see results). In addition, it has been shown that glibenclamide releases bound ATP from SUR1, demonstrating a direct interaction between the NBFs and the glibenclamide site of SUR (Ueda *et al*., 1999). However, one should be aware that at least part of the MgATP effects could be mediated indirectly, e.g. by activation of lipid and/or protein kinases and more direct evidence for the involvement of the NBFs in the effects reported here is required.

In addition, one should note that the results reported here for glibenclamide do not necessarily apply to other sulphonylureas. Substantial differences in the selectivity of different sulphonylureas for the SUR subtypes have been observed (see e.g. Quast, 1996; Gribble *et al*., 1998; Lawrence *et al*., 2001; Song & Ashcroft, 2001), indicating differential interactions of the sulphonylureas with the SUR subtypes and possibly also with the NBFs. However, with the data for the radioligand, [^{3}H]-glibenclamide, available it is now possible to study other sulphonylureas available only in unlabelled form.

Conclusion

In conclusion, we have investigated the negative allosteric modulation of [^{3}H]-glibenclamide binding to the SUR subtypes by ATP and ADP in the presence and absence of the respective reconstituting enzyme systems, i.e. by the 'pure' nucleotides and by their combination. The data show that the effects of ATP and ADP differ from one another, that they depend on the SUR subtype, and that they are weakened by coexpression with Kir6.2.

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