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Structural requirements of sulphonylureas and analogues for interaction with sulphonylurea receptor subtypes

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1 The structure-activity relationship for hypoglycaemic sulphonylureas and analogues was examined. Binding affinities were compared using membranes from HIT-T15 cells (β-cell line) and from COS-7 cells transiently expressing sulphonylurea receptor subtypes (SUR1, SUR2A and SUR2B). Inhibition of adenosine-triphosphate-sensitive K^+ channels (K_{ATP}-channels) was measured in mouse pancreatic β-cells.

2 The tested compounds displayed similar binding affinities for SUR2A and SUR2B.

3 Meglitinide (benzoic acid derivative) bound to SUR1 and the SUR2 isoforms with similar affinities. Replacement of the carboxyl group of meglitinide by a methyl group significantly decreased the binding affinities for SUR1 and the SUR2 isoforms (>4 fold) and the potency to inhibit K_{ATP}-channel activity of β-cells (24 fold). Replacement of the carboxyl group of meglitinide by a sulphonylurea group significantly increased the affinities for SUR1 (5 fold) and the SUR2 isoforms (13–16 fold).

4 Glibenclamide bound to the SUR2 isoforms with 300–500 fold lower affinity than to SUR1. Exchanging the cyclohexyl ring of glibenclamide by a methyl group or removal of the lipophilic side chain of glibenclamide (5-chloro-2-methoxy-benzamidoethyl chain) markedly reduced but did not abolish the selectivity for SUR1.

5 In conclusion, interaction of sulphonylureas and acidic analogues with SUR1, SUR2A and SUR2B is favoured by the anionic group of these drugs. Hypoglycaemic sulphonylureas (e.g. glibenclamide) owe selectivity for SUR1 to lipophilic substitution on their urea group. Sulphonylureas without lipophilic substitution on the urea group could represent lead compounds for the development of SUR2-selective drugs.

Keywords: Sulphonylureas; sulphonylurea analogues; glibenclamide; meglitinide; sulphonylurea receptor subtypes; K_{ATP}-channel; pancreatic β-cell; structure activity relationships

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DMEM-HG, Dulbecco's modified Eagle's medium with 10 mM glucose; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid; FCS, foetal calf serum; GDP, guanosine diphosphate; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid); K_{ATP}-channel, adenosine-triphosphate-sensitive K^+ channel; K_{IR}, inwardly-rectifying K_{ATP}-channel subunit; SUR, sulphonylurea receptor

Introduction

Hypoglycaemic sulphonylureas inhibit adenosine-triphosphate-sensitive K^+ channels (K_{ATP}-channels) in the pancreatic β-cell plasma membrane and thereby initiate insulin release (Sturgess *et al*., 1985; for a review see Edwards & Weston, 1993). These channels have also been identified in many neurons, cardiac myocytes, skeletal muscle and vascular and non-vascular smooth muscle. K_{ATP}-channels are composed of two proteins, an inwardly rectifying K_{ATP}-channel subunit ($K_{IR}6.1$ or $K_{IR}6.2$) and the sulphonylurea receptor (SUR), a member of the adenosine-triphosphate-binding cassette protein superfamily (Aguilar-Bryan *et al*., 1995; Inagaki *et al*., 1995; 1996; Isomoto *et al*., 1996; Yamada *et al*., 1997). Three sulphonylurea receptor isoforms have been cloned, SUR1, and two splice products of a single gene differing only in their carboxyterminal 42–45 amino acids, SUR2A and SUR2B (for a review see Aguilar-Bryan *et al*., 1998). While SUR1 acts as the regulatory subunit of the K_{ATP}-channels in β-cells and many neurons, SUR2A has been suggested to represent the sulphonylurea receptor in heart and skeletal muscle and SUR2B in smooth muscle (Inagaki *et al*., 1995; 1996; Isomoto *et al*., 1996; Yamada *et al*., 1997; Okuyama *et al*., 1998; Schwanstecher *et al*., 1998b). Glibenclamide has been reported to bind with high affinity (K value of about 2 nM) to SUR1 but with more than 100 fold lower affinity to SUR2A and SUR2B (Aguilar-Bryan *et al*., 1995; Inagaki *et al*., 1996; Hambrock *et al*., 1998).

Several derivatives of 3-phenylpropionic acid or benzoic acid (e.g. meglitinide, compound X in Figure 1) inhibit K_{ATP}-channels by binding to the same receptor site that mediates the response to sulphonylureas (for a review see Edwards & Weston, 1993). This receptor site is located at the cytoplasmic face of the plasma membrane and interacts with the anionic (dissociated) forms of sulphonylureas and analogues (Schwanstecher *et al*., 1994). In pancreatic islets incubated in media supplemented with albumin, a meglitinide derivative devoid of the anionic group (compound XI, Figure 1) released less insulin within one hour than meglitinide at equimolar concentrations (Henquin *et al*., 1987). It is unclear whether this observation reflected a difference in potency of the two compounds. Firstly, the secretory responses of islets were rapid at all meglitinide concentrations but slow at submaximally

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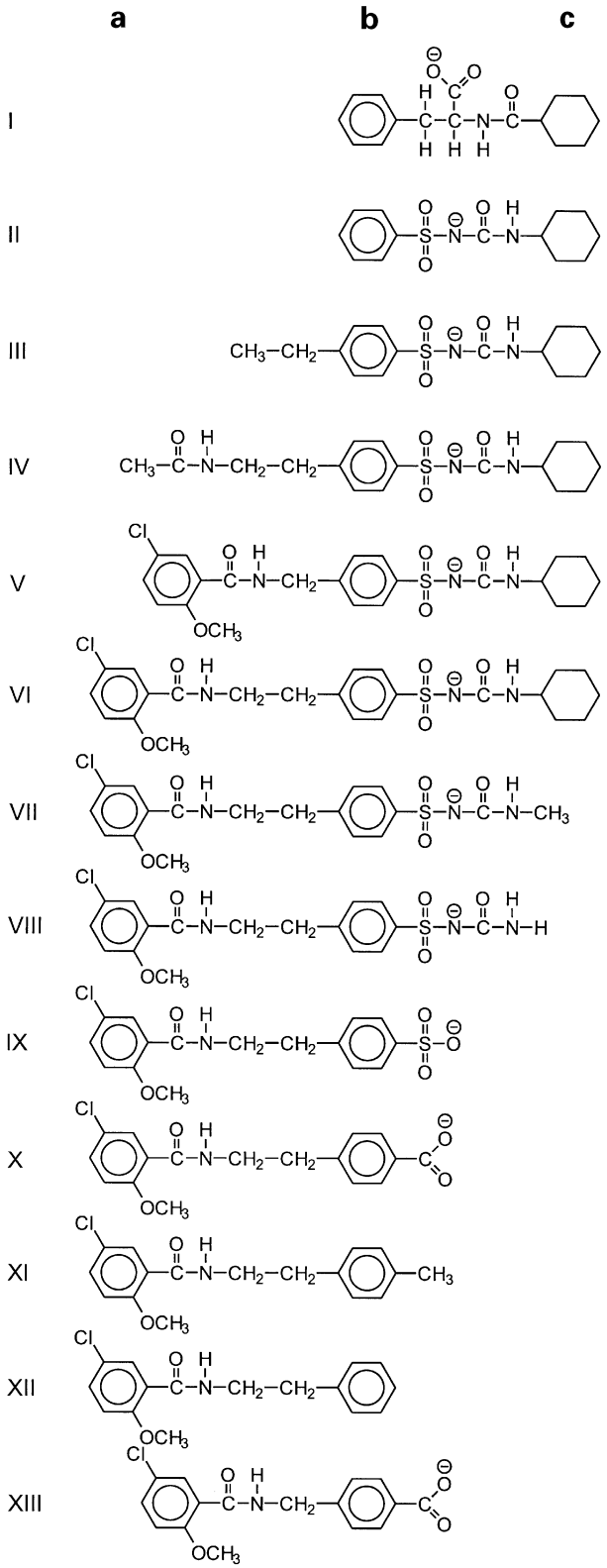

Figure 1 Structures of N-cyclohexylcarbonyl-D-phenylalanine (I), N-benzenesulphonyl-N′-cyclohexylurea (II), N-[4-(ethyl)benzenesulphonyl]-N′-cyclohexylurea (III) N-[4-(2-acetamidoethyl)benzenesulphonyl]-N′-cyclohexylurea (IV), N-[4-({5-chloro-2-methoxy-benzamido}methyl)benzenesulphonyl]-N′-cyclohexylurea (V), glibenclamide (VI), N-[4-(2-{5-chloro-2-methoxybenzamido}ethyl)benzenesulphonyl]-N′-methylurea (VII), 4-[2-(5-chloro-2-methoxybenzamido)ethyl]-benzenesulphonylurea (VIII), 4-[2-(5-chloro-2-methoxy-benzamido)ethyl]-benzenesulphonic acid (IX), meglitinide (X), 4-[2-(5-chloro-2-methoxy-benzamido)ethyl]-toluene (XI), 2-(5-chloro-2-methoxybenzamido)ethyl-benzene (XII) and 4-[(5-chloro-2-methoxybenzamido)-methyl]-benzoic acid (XIII). The anionic forms of acids are shown. a, b and c label the lipophilic areas in the molecules.

effective concentrations of compound XI (Panten *et al*., 1989; Moshiri & Panten, unpublished observations). Secondly, reduction of the free concentrations by albumin binding might have been more pronounced in the case of the more lipophilic compound XI. In cerebral cortex membranes similar binding affinities were observed for meglitinide and another non-acidic derivative of meglitinide (compound XII;. K_I values of 0.6 μM and 1 μM, respectively, Bernardi *et al*., 1988; Challinor-Rogers *et al*., 1995), arguing against involvement of the anionic group in ligand recognition at the receptor site. Compound XII inhibited the K_{ATP}-channel currents in skeletal muscle cells with much lower potency (EC_{50} value of 125 μM; Barrett-Jolley & McPherson, 1998), suggesting that selectivity of glibenclamide for SUR1 versus SUR2 is due to that part of the glibenclamide molecule which is represented by compound XII. In contrast, meglitinide did not display a difference in potency to inhibit cloned β-cell and cardiac K_{ATP}-channels (Gribble *et al*., 1998).

In view of these inconsistent data we aimed to further elucidate which structural features of sulphonylureas and analogues determine their recognition at the receptor sites of SUR1, SUR2A and SUR2B. This information should contribute to characterize the biochemical properties of the receptor sites and to develop ligands selective for SUR2A and SUR2B.

Methods

Binding experiments

Culture of HIT-T15 cells (SV-40 transformed hamster β-cells) and culture and transient transfections of COS-7 cells were performed as previously described (Schwanstecher *et al*., 1992; 1998b; Clement *et al*., 1997). HIT-T15 cells were grown in RPMI 1640 medium containing 10% foetal calf serum (FCS), 100 nM sodium selenite, 100 μg ml^{-1} streptomycin and 100 u ml^{-1} penicillin G. The cells were fed twice-weekly and subcultured once per 10 days. COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 10 mM glucose (DMEM-HG), supplemented with 10% FCS, were plated at a density of 5×10^5 cells per dish (94 mm) and allowed to attach overnight. pECE-SUR (200 μg) complementary DNA was used to transfect 10 plates. For transfection the cells were incubated for 4 h in a Tris-buffered salt solution containing SUR plasmid (5–10 μg ml^{-1}) plus diethylaminoethyl-dextran (1 mg ml^{-1}), shocked for 2 min in *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulphonic acid)-buffered (HEPES-buffered) salt solution plus dimethyl sulphoxide (10%) and incubated for 4 h in DMEM-HG plus chloroquine (100 μM). The cells were then returned to DMEM-HG plus 10% FCS.

Membranes were prepared from HIT-T15 cells and COS-7 cells (60–72 h after transfection) as described (Schwanstecher *et al*., 1992). The protein content of the microsomal suspensions was determined according to Smith *et al*. (1985) with bovine serum albumin as standard. For measurement of [^{3}H]-glibenclamide binding to SUR1, resuspended membranes (final protein concentration 4–10 μg ml^{-1}) were incubated in 1 ml Tris-buffer pH 7.4 (185 mM) containing (final concentrations) [^{3}H]-glibenclamide (0.1 nM as indicated in the Results section, 0.3 nM in all other experiments) and test substances (final concentrations indicated in the Results section). Nonspecific binding was defined by 100 nM glibenclamide. For measurement of [^{3}H]-P1075 binding to SUR2A and SUR2B, resuspended membranes (final protein concentration

10–50 μg ml^{-1}) were incubated in 0.5 ml Tris-buffer pH 7.4 (140 mM) containing (final concentrations) [^{3}H]-P1075 (3 nM), $MgCl_2$ (1 mM), ATP (0.1 mM) and test substances (final concentrations indicated in the Results section); specific binding of [^{3}H]-P1075 required MgATP (Schwanstecher *et al*., 1998b). Nonspecific binding was defined by 100 μM pinacidil. Incubations were carried out for 1 h at room temperature and were terminated by rapid filtration of aliquots through Whatman GF/B filters. The filters were immediately washed three times with 4 ml of ice-cold Tris-buffer (50 mM, pH 7.4), and their ^{3}H content was determined by liquid scintillation counting. Membranes from COS-7 cells not transfected with SUR plasmid did not show specific binding of [^{3}H]-glibenclamide or [^{3}H]-P1075.

Electrophysiological recording and analysis

Isolation and culture of mouse β-cells and patch-clamp experiments using inside-out patches of these cells were performed as previously described (Schwanstecher *et al*., 1994). The membrane potential was clamped at -50 mV, and inward membrane currents flowing through K_{ATP}-channels were measured. All experiments were carried out at room temperature (20–22°C). Current signals were low-pass filtered at 2 kHz with a 4-pole Bessel filter (AF 173, Thomatronik, Rosenheim, Germany), digitized with an A/D converter (Instrutech, New York, U.S.A.) and stored on video tape. For Figure 2a, taped data were replayed into a chart recorder (WR7400, Graphtec, Solingen, Germany). For analysis, taped data were replayed, digitized at 10 kHz and stored in a microcomputer using an Axolab 1200 computer interface and the programme pCLAMP 5.5.1 (Axon Instruments, Foster City, CA, U.S.A.). With this programme data samples of at least 10 s duration were analysed. Data sampling during control and test periods started 5 s after medium change and ended 5 s before medium change. The single-channel current amplitudes of the K_{ATP}-channels were not changed by the applied concentrations of nucleotides, compound XI and dimethyl sulphoxide.

The solution at the cytoplasmic side of the inside-out membrane (intracellular solution) contained (in mM): KCl 140, $CaCl_2$ 2, $MgCl_2$ 1, ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) 10 and HEPES 5 (titrated to pH 7.15 with KOH) (free $[Ca^{2+}]=0.05$ μM). The free Mg^{2+} concentration was held close to 0.7 mM by adding appropriate amounts of $MgCl_2$ to nucleotide-containing solutions. The required amounts of $MgCl_2$ were calculated as described in Schwanstecher *et al*. (1994). After addition of 1 mM Na_2-ATP, the intracellular solution was also used for filling the U-shaped polythene capillary of the microflow system used in our experimental set-up. The pipette solution contained (in mM): KCl 146, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10 (titrated to pH 7.40 with KOH).

Chemicals and solutions

Chemicals for organic syntheses were purchased from Aldrich Chemical Co. (Steinheim, Germany) and Fluka Chemie AG (Buchs, Switzerland). Na_2ATP and KADP were from Boehringer (Mannheim, Germany). Glibenclamide (compound VI, Figure 1), compound VII (known as HB 985; Figure 1), compound VIII (known as HOE 36320; Figure 1) and meglitinide (compound X; Figure 1) were from Hoechst-Marion-Roussel (Frankfurt, Germany). [^{3}H]-Glibenclamide (51 Ci mmol^{-1}, >99% radiochemical purity) and [^{3}H]-P1075 (116 Ci mmol^{-1}, >99% radiochemical purity) were purchased from NEN (Dreieich, Germany) and Amersham Buchler (Braunschweig, Germany), respectively. All other chemicals were obtained from sources described elsewhere (Panten *et al*., 1989; Schwanstecher *et al*., 1992).

Stock solutions (20–50 mM) of the tested compounds (except compounds XI and XII) were prepared daily in dimethyl sulphoxide (DMSO) or in NaOH (50 mM). The pH of all test solutions was determined after addition of test substances and was readjusted if necessary. Stock solutions of compounds XI and XII (2–10 mM) were prepared daily in DMSO, and appropriate amounts of stock solution were added slowly, while stirring, to Tris buffer (binding experiments) or intracellular solution supplemented with 1 mM ADP (patch-clamp experiments). Control experiments verified that ligand binding and K_{ATP}-channel activity were unaffected by the final concentrations of DMSO (0.3% maximum concentra-

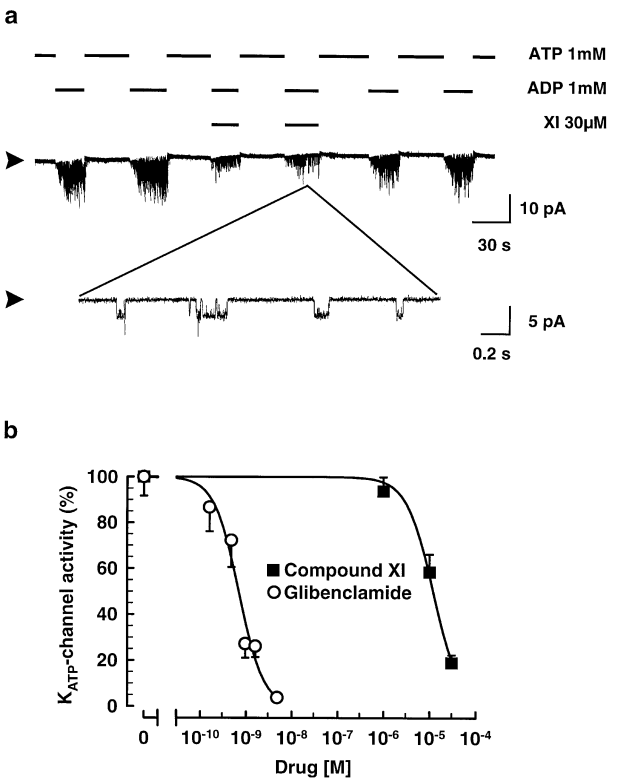

Figure 2 Effect of compound XI on K_{ATP}-channel activity in inside-out patches of mouse pancreatic β-cells. (a) Current trace obtained from an inside-out patch. Free Mg^{2+} (0.7 mM) was always present in the solutions applied at the cytoplasmic membrane side. The uppermost horizontal bars indicate application of intracellular solution containing 1 mM ATP by a microflow system (switched by hand). The other horizontal bars above the current trace indicate application of intracellular solution containing 1 mM ADP (with or without 30 μM compound XI) by the bath. The arrowheads denote the zero-current level (all K_{ATP}-channels closed by ATP). During application of compound XI (presence of 0.3% of DMSO) the current trace is also shown at an extended time-scale. (b) Relationship between normalized K_{ATP}-channel activity and concentration of compound XI in the presence of ADP. By use of the experimental design shown in (a), K_{ATP}-channel activity was normalized to K_{ATP}-channel activity during the control periods (presence of ADP, absence of drug) before and after drug application in each single experiment. Symbols are means (with s.e.mean shown when larger than symbols) from 4–6 experiments. Analysis revealed an EC_{50} value of 12.2 μM and a Hill coefficient of -1.5. For comparison, data for glibenclamide are included (taken form Zünkler *et al*., 1988a; free glibenclamide concentrations were calculated according to Panten *et al*., 1989). Analysis revealed an EC_{50} value of 0.7 nM and a Hill coefficient of -1.6.

tion). Compounds I-XIII were completely dissolved at the applied concentrations.

Syntheses

Unless stated otherwise, chemicals (pure grade) were used as received. Solvents were dried and distilled before use. Compound I was prepared by the addition of cyclohexanecarboxylic acid chloride to D-phenylalanine in the presence of NaOH (Shinkai *et al*., 1988). Sulphonylureas were prepared by the addition of cyclohexyl isocyanate to appropriate sulphonamides in the presence of NaOH (Francia *et al*., 1975; Vicentini & Guarneri, 1983). The sodium salt of compound XI was synthesized and purified as previously described with minor modifications (Schwanstecher *et al*., 1994). Compounds XI, XII and XIII were prepared by the addition of 5-chloro-2-methoxybenzoyl chloride to 2-(p-tolyl)ethylamine, 2-phenylethylamine or 4-(aminomethyl)benzoic acid, respectively, using minor modifications of the method described by Brown & Foubister (1984). The synthesized compounds were purified by recrystallization from ethanol (compound IX) or ethanol-water (all other compounds). All melting points were determined on a Linström melting point apparatus (Bühler, Tübingen, Germany), and the following uncorrected values were obtained (°C, compound number in parentheses): 148 (I), 189 (II), 162 (III), 176-178 (IV), 166 (V), >400 (IX), 78 (XI), 64 (XII), 213 (XIII). Measurement of optical rotation of compound I gave $[\alpha]^{20}_{D} - 43.9°C$ (c 1.0, 1N NaOH, Perkin Elmer 241 polarimeter). Elementary analyses of the synthesized compounds were performed by means of a Carlo Erba 1106 analyser and were within $\pm 0.2\%$, $\pm 2\%$ and -2% of the theoretical values (100%) for C, H and N, respectively. The structures of the synthesized compounds (Figure 1) were confirmed by ^{1}H-nmr (DMSO, 400 MHz) and ^{13}C-nmr (DMSO, 100 MHz) spectroscopy on a model AM400 spectrometer (Bruker, Karlsruhe, Germany) and infra-red spectroscopy on a model FTIR spectrometer (ATI Unicam, Kassel, Germany), supported by electron-impact mass spectrometry on a Finnigan MAT 8430 spectrometer.

Treatment of results

Values are presented as mean ± s.e.mean. Significances were calculated by the two-tailed U-test of Wilcoxon and of Mann & Whitney with the Bonferroni correction for multiple comparisons. $P<0.05$ was considered significant. Relations between drug concentration and specific binding or channel activity were analysed by fitting the function

$$y = \frac{a-b}{1+(K_{50}/x)^{n_H}} + b$$

to the experimental data by a non-linear least-squares method where y = specific binding or K_{ATP}-channel activity, K_{50} = midpoint parameter (EC_{50} or IC_{50}), x = drug concentration, n_H = slope parameter (Hill coefficient), a = upper plateau (maximum value for specific binding or K_{ATP}-channel activity) and b = lower plateau. IC_{50} is the drug concentration that inhibits the binding of radioligand by 50%. For each individual inhibition curve the pIC_{50} ($-\log IC_{50}$) value was determined and used to calculate the pK_I ($-\log K_I$) value according to the Cheng & Prusoff (1973) equation

$$IC_{50} = K_I * (1 + R/K_D).$$

This correction needs to be made because the IC_{50} value depends on the concentration of the radioligand R. K_I is the inhibition constant, K_D is the equilibrium dissociation constant of the radioligand. The used K_D values were 0.22 nM (SUR1 in HIT-cells) or 0.55 nM (cloned SUR1) for glibenclamide and 46 nM (cloned SUR2A) or 12 nM (cloned SUR2B) for P1075 (Schwanstecher *et al*., 1992; 1998b).

Results

The anionic group of sulphonylureas and analogues

Figure 2a shows the typical design of our inside-out patch experiments. All experiments were carried out in the presence of 0.7 mM free Mg^{2+} at the cytoplasmic membrane side. To slow the run-down of channel activity, the cytoplasmic face of the patch was exposed for 35–40 s periods to an intracellular solution containing 1 mM ATP, alternating with ATP-free 20–30 s periods serving as test or control (absence of drug) periods. ADP (1 mM) was applied to the cytoplasmic membrane side during the test and control periods since complete inhibition of K_{ATP}-channel activity by maximally-effective concentrations of sulphonylureas and analogues was always observed in the presence of 1 mM of cytosolic ADP (Zünkler *et al*., 1998b; Schwanstecher *et al*., 1994; 1998a). The example in Figure 2a shows that 30 μM compound XI reduced K_{ATP}-channel activity to 21% of control. Compound XI was half-maximally inhibitory at 12.2 μM (Figure 2b). This EC_{50} value was obtained assuming complete inhibition of K_{ATP}-channel activity by maximally-effective concentrations of compound XI (see above). Compound XI was completely soluble at 30 μM but incompletely soluble at concentrations >70 μM (see also Henquin *et al*., 1987). The EC_{50} value for compound XI was more than 17,000 fold higher than that for glibenclamide (Figure 2b).

In binding assays with membranes from HIT-cells (expressing SUR1), inhibition of specific [^{3}H]-glibenclamide binding by compound XI (70 μM) and compound XII (50 μM) produced K_I values >30 μM and >21 μM, respectively (Figure 3c, Table 1). Using membranes from COS-7 cells transiently expressing hamster SUR1, inhibition of specific [^{3}H]-glibenclamide binding by compound XI (46 μM, incubation medium contained 0.1 nM [^{3}H]-glibenclamide) and compound XII (50 μM) produced K_I values >50 μM and >32 μM, respectively (Table 1).

In binding assays with membranes from COS-7 cells transiently expressing rat SUR2A or SUR2B, inhibition of specific [^{3}H]-P1075 binding by compound XI (30 μM) and compound XII (30 μM) produced K_I values >28 μM for SUR2A and >23 μM for SUR2B (Figure 3c, Table 1). A negative allosteric coupling of the binding site for sulphonylureas to the site for P1075 was assumed (Hambrock *et al*., 1998; Schwanstecher *et al*., 1998b).

Binding affinities of meglitinide (compound X) for the three sulphonylurea receptor subtypes were very similar (Figure 3b, Table 1). These affinities did not differ significantly from the corresponding binding affinities of the sulphonic acid analogue of meglitinide (compound IX) (Table 1), implying that the COO^- group of meglitinide and the SO_3^- group of compound IX are isosteres during interaction with SUR1, SUR2A and SUR2B. Affinities of meglitinide and compound IX for all three SUR subtypes were significantly higher than the corresponding binding affinities of compounds XI and XII.

Linking a urea group with the sulphonic group of compound IX caused a 5 fold increase in affinity for

membranes from HIT-T15 cells and COS-7 cells containing SUR1 and a 10 fold increase in affinity for membranes from COS-7 cells containing SUR2A or SUR2B (compound VIII, Table 1).

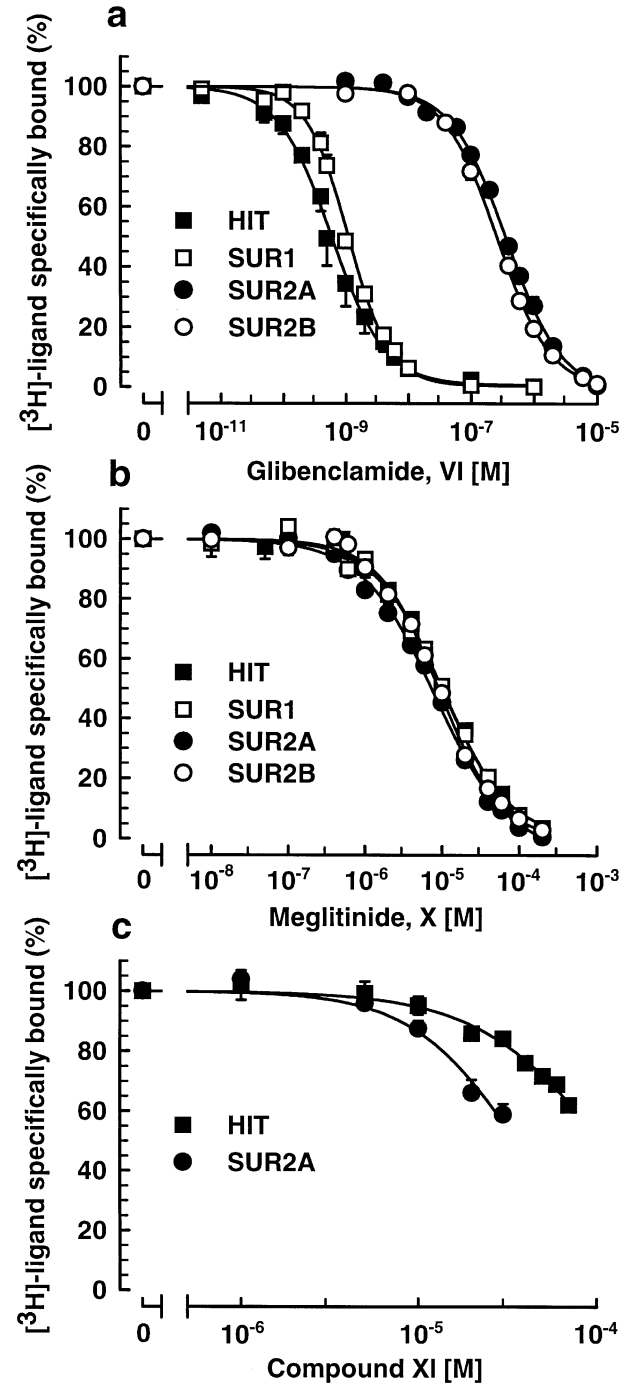

Figure 3 Effects of compounds VI (glibenclamide) (a), X (meglitinide) (b) and XI (c) on specific [^{3}H]-ligand binding to membranes form HIT-cells and from COS-7 cells transiently expressing hamster SUR1, rat SUR2A or rat SUR2B. For binding to HIT-cell membranes and recombinant SUR1 the [^{3}H]-ligand was [^{3}H]-glibenclamide, for binding to recombinant SUR2 isoforms the [^{3}H]-ligand was [^{3}H]-P1075. Non-specific binding was defined by incubations in the additional presence of 100 nM glibenclamide or 100 μM pinacidil, respectively. Results are expressed as percentage of control (absence of displacing drugs). Symbols are means (with s.e.mean shown when larger than symbols) from 4–8 separate binding experiments. The means of pK_I values and Hill coefficients obtained from curve analysis are given Table 1.

Table 1 Receptor binding of sulphonylureas and analogues

Compound	*HIT-SUR1* pK_I ($-\log$ M)	*SUR1* pK_I ($-\log$ M)	*SUR2A* pK_I ($-\log$ M)	*SUR2B* pK_I ($-\log$ M)	*SUR2A/SUR1* K_I/K_I	*SUR1/HIT-SUR1* K_I/K_I
I	5.04 ± 0.03 (−1.0)	4.91 ± 0.02 (−1.0)	3.33 ± 0.02 (−1.0)[a]	3.34 ± 0.04 (−1.1)[a]	38	1.35
II	4.97 ± 0.01 (−1.0)	4.82 ± 0.02 (−1.0)	3.98 ± 0.04 (−1.1)[e]	3.85 ± 0.01 (−1.1)[e]	6.9	1.41
III	6.37 ± 0.01 (−1.0)[a]	6.20 ± 0.02 (−1.0)[a]	3.89 ± 0.02 (−1.0)	3.88 ± 0.03 (−1.1)	204	1.48
IV	6.95 ± 0.03 (−1.0)	6.98 ± 0.01 (−1.0)	4.03 ± 0.02 (−1.0)	4.09 ± 0.07 (−1.0)	897	0.93
V	7.69 ± 0.08 (−1.0)	7.37 ± 0.01 (−1.0)	4.78 ± 0.02 (−1.4)	4.79 ± 0.03 (−1.3)	395	2.10
Glibenclamide (VI)	9.50 ± 0.08 (−1.0)	9.17 ± 0.03 (−1.3)	6.46 ± 0.02 (−1.0)	6.68 ± 0.03 (−1.0)	510	2.14
VII	6.54 ± 0.04 (−1.0)[b,c]	6.24 ± 0.01 (−1.0)[b,c]	5.65 ± 0.01 (−1.0)[b,c]	5.75 ± 0.02 (−1.0)[b,c]	3.9	2.00
VIII	6.17 ± 0.01 (−1.0)	5.82 ± 0.04 (−1.0)	6.25 ± 0.02 (−1.0)[e]	6.37 ± 0.03 (−1.0)[e]	0.4	2.23
IX	5.47 ± 0.02 (−1.0)[c]	5.13 ± 0.02 (−1.0)[c]	5.21 ± 0.04 (−1.0)[e]	5.35 ± 0.04 (−1.0)[c]	0.8	2.22
Meglitinide (X)	5.36 ± 0.03 (−1.0)	5.17 ± 0.03 (−1.0)	5.13 ± 0.04 (−0.9)	5.15 ± 0.02 (−1.1)	1.1	1.55
XI	<4.52[d]	<4.30[d]	<4.55[d]	<4.64[d]		
XII	<4.68[d]	<4.49[d]	<4.55[d]	<4.64[d]		
XIII	3.89 ± 0.02 (−1.0)	3.74 ± 0.01 (−1.0)	3.67 ± 0.02 (−1.0)	3.49 ± 0.03 (−1.3)	1.2	1.42

Competition assays were performed with membranes from HIT-T15 cells expressing SUR1 (HIT-SUR1) or COS-7 cells transiently expressing hamster SUR1 (SUR1), rat SUR2A (SUR2A) or rat SUR2B (SUR2B). For HIT and SUR1 the tracer was [^{3}H]-glibenclamide (0.1 nM in the assays with SUR1 in the presence compound XI, 0.3 nM in all other assays); for SUR2A and SUR2B the tracer was [^{3}H]-P1075 (3 nM). The listed pK_I values ($-\log$ M; pIC_{50} values corrected according to Cheng & Prusoff (1973)) are means ± s.e.mean obtained from 4–8 independent competition curves. The means of the Hill coefficients are given in parenthesis. [a] $P<0.05$ for comparison with the corresponding value for compound II. [b] $P<0.05$ for comparison with the corresponding value for glibenclamide. [c] $P<0.05$ for comparison with the corresponding value for compound VIII. [d] $P<0.05$ for comparison with the corresponding value for meglitinide. [e] $P<0.05$ for comparison with the corresponding value for SUR1.

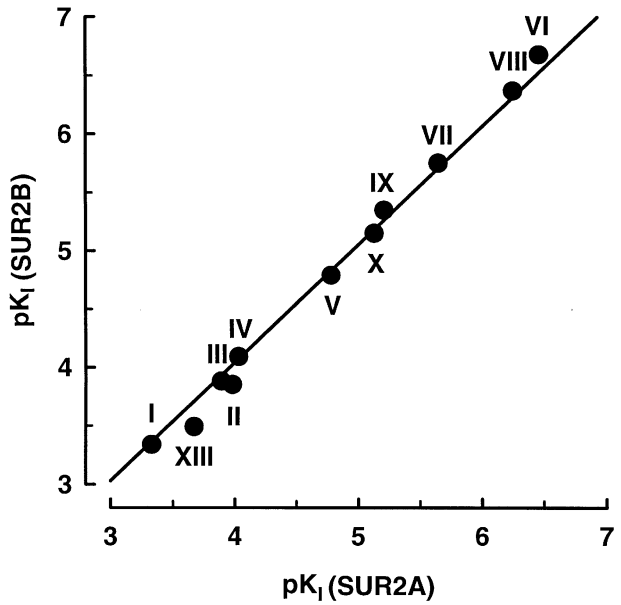

Figure 4 Correlation curve of pK_1 values (taken from Table 1) for binding to SUR2A or SUR2B. The numbers indicate the tested compounds. A slope of 1.01 was obtained ($r = 0.99$).

The lipophilic groups of sulphonylureas

In membranes from HIT-cells and COS-7 cells transiently expressing SUR1, similar sub-nanomolar binding affinities of glibenclamide (compound VI) were observed (Figure 3a, Table 1). Glibenclamide displayed 300–500 fold lower binding affinities for SUR2A and SUR2B. Exchanging the lipophilic cyclohexyl ring (region c in Figure 1) of glibenclamide by a methyl group (compound VII) reduced the affinities for the SUR2 isoforms by 6–9 fold (Table 1), but the affinities for HIT-cell SUR1 and transiently-expressed SUR1 by about 900 fold. In contrast, exchanging the lipophilic chloro-methoxy-benzene ring (region a in Figure 1) of glibenclamide by a methyl group (compound IV) reduced the affinities for all sulphonylurea receptor subtypes by 150–400 fold (Table 1).

Compound II does not bear a lipophilic group on its benzene ring (region b in Figure 1). This molecule exhibited a binding affinity for SUR1-containing membranes which was more than 20,000 fold lower than that of glibenclamide (Table 1). The binding affinities of compound II for the SUR2 isoforms were 7–9 fold lower than that for SUR1. Introduction of a *p*-ethyl group into the benzene ring (compound III) enhanced SUR1 binding affinity by about 25 fold but did not influence the binding affinities for the SUR2 isoforms (Table 1).

The chains linking the lipophilic groups of sulphonylureas and analogues

Replacing the sulphonylurea chain linking the two lipophilic rings of compound II by the chain characteristic of hypoglycaemic N-acyl-D-phenylalanines (compound I) did not affect SUR1 binding affinity but resulted in a 3–5 fold decrease in binding affinities for SUR2A and SUR2B (Table 1).

We also studied the carboxamido-ethyl chain linking the two lipophilic rings (regions a and b in Figure 1) of meglitinide (compound X) and the structurally corresponding rings of glibenclamide (compound VI). Shortening of this chain by a methylene group reduced the affinities of meglitinide and glibenclamide for SUR1 and the SUR2 isoforms by 25–45 fold (compound XIII) and 50–80 fold (compound V), respectively (Table 1).

Discussion

Previous experiments with N-acylphenylalanines (phenylpropionic acid derivatives, e.g. compound I in Figure 1) presented evidence that the position of the anionic group relative to the lipophilic regions of the ligand molecule is critical for interaction with SUR1 (Schwanstecher *et al*., 1998a). The present study indicates favourable involvement of the anionic group in the ligand recognition process at the receptor site. Replacement of the methyl group of compound XI (Figure 1) by a SO_3^- group (compound IX) or a COO^- group (meglitinide, compound X) increased the potency to inhibit K_{ATP}-channels of β-cells (inside-out configuration) by 15–24 fold (EC_{50} values of 12 μM, 0.8 μM and 0.5 μM for compound XI, compound IX and meglitinide, respectively; Figure 2, Schwanstecher *et al*., 1994). Moreover, contrary to expectation from previous reports (Bernardi *et al*., 1988; Challinor-Rogers *et al*., 1995), the affinities of meglitinide to all three SUR subtypes were significantly higher than the corresponding affinities of compounds XI and XII (Table 1).

In the present study, we confirm that glibenclamide (compound VI) binds to SUR1 with more than 100 fold higher affinity than to the SUR2 isoforms (Aguilar-Bryan *et al*., 1995; Inagaki *et al*., 1996; Hambrock *et al*., 1998). This high selectivity for SUR1 cannot be attributed to that moiety which is represented by compound XII since this does not confer selectivity for SUR1 on compound IX or meglitinide (compound X) (Table 1). In support of this view, exchanging the lipophilic chloro-methoxy-benzene ring of glibenclamide (a in Figure 1) by a methyl group strongly decreases the affinities for all sulphonylurea receptor subtypes, but does not reduce the selectivity for SUR1 (compare compound IV and glibenclamide, Table 1). The carboxamido-ethyl chain linking the chloro-methoxy-benzene ring with the benzene ring (b in Figure 1) might serve as a flexible spacer allowing spacial configurations of the lipophilic rings favourable for interaction with the sulphonylurea receptor subtypes. Firstly, shortening of the chain by a methylene group causes a marked reduction in binding affinity for all receptor subtypes (compare meglitinide with compound XIII and glibenclamide with compound V, Table 1). Secondly, shortening of the ethyl group in compound III by a methylene group produces only a small decrease in binding affinity for cerebral membranes (Challinor-Rogers *et al*., 1995). Thirdly, the carboxamido group in the chain seems to contribute only slightly to the binding affinity (compare compounds III and IV, Table 1). This interpretation does not, however, preclude additional functions of the carboxamido-ethyl chain (see below).

Linking a urea group with the sulphonic group of compound IX enhances the affinity for all sulphonylurea receptor subtypes without inducing selectivity for SUR1 (compare compounds VIII and IX, Table 1). However, introduction of a methyl group into the sulphonylurea moiety of compound VIII decreases the affinity for the SUR2 isoforms by 4 fold while increasing the affinity for SUR1 by 3 fold (compound VII, Table 1). When introducing the large lipophilic cyclohexyl ring instead of the small lipophilic methyl group the affinity for the SUR2 isoforms increases by 2 fold, but the affinity for SUR1 by >2000 fold (glibenclamide, compound VI, Table 1). From these findings it is concluded that selectivity of sulphonylureas for SUR1 is achieved by

introduction of a lipophilic substituent (c in Figure 1) into the sulphonylurea moiety. Binding of this substituent to the receptor sites of SUR2A and SUR2B seems to be weak and might impair binding of the sulphonylurea moiety. In contrast, the SUR1 receptor site seems to represent a close fit for both a large lipophilic substituent and the sulphonylurea moiety.

Selectivity for SUR1 does not require lipophilic substituents on the benzene ring of sulphonylureas (b in Figure 1) as indicated by compound II (Table 1). However, introduction of such substituents enhances the selectivity for SUR1 (compare compounds II-VI, Table 1). A gain in selectivity for SUR1 is also achieved by replacing the sulphonylurea moiety with the anionic segment connecting the lipophilic components of hypoglycaemic N-acyl-D-phenylalanines (compare compounds I and II, Table 1). This finding might indicate that differences in the recognition of anionic moieties exist between the SUR1 receptor site on the one hand and the receptor sites of SUR2A and SUR2B on the other hand. In support of this view, linking a urea group with the sulphonic group of the non-selective compound IX produces a derivative (compound VIII) displaying some selectivity for the SUR2 isoforms (Table 1).

Sulphonylureas and analogues displayed slightly higher affinity to SUR1 of HIT-cells than to cloned SUR1 (Table 1). This difference might be due to differences in the membrane environment. We also found that the tested compounds did not differentiate between SUR2A and SUR2B (Table 1, Figure 4), supporting the conclusion that the C-terminal 42 amino acids of SUR2A and SUR2B are not essential for binding of sulphonylureas (Dörschner *et al*., 1999). Sulphonylurea sensitivities in skeletal muscle (expressing SUR2A) differed from those in smooth muscle (expressing SUR2B) (Challinor-Rogers *et al*., 1995; Barrett-Jolley & McPherson, 1998). These findings might reflect the presence of SUR subtypes in skeletal muscle and/or smooth muscle which have not been identified up to now. In skeletal muscle cells compound XII inhibited the K_{ATP}-channel currents with very low potency (EC_{50} value of 125 μM; Barrett-Jolley & McPherson, 1998). It would be interesting to know the affinity for binding of compound XII to the sulphonylurea receptors expressed in these cells.

The affinities for glibenclamide binding to SUR1 on the one hand or SUR2A and SUR2B on the other hand (Table 1) were very similar to the affinities for glibenclamide binding to membranes from β-cells or heart and vascular smooth muscle, respectively (Schmid-Antomarchi *et al*., 1987; Gaines *et al*., 1988; Panten *et al*., 1989; Quast *et al*., 1993; Atwal *et al*., 1998; Löffler-Walz & Quast, 1998). This contrasts with the results from a recent study (Gribble *et al*., 1998) in which glibenclamide inhibited cloned β-cell and cardiac K_{ATP}-channels with EC_{50} values of 4.2 nM and 27 nM, respectively. This finding led to the interpretation of poor selectivity of glibenclamide for SUR1 and to the suggestion that *in vivo* the effect of glibenclamide on cardiac K_{ATP}-channels might be markedly reduced by cytosolic substances. However, the potency of glibenclamide to inhibit the cloned β-cell channel might have been underestimated since at low concentrations of glibenclamide a steady state of inhibitory response is not reached within 30 s (Panten *et al*., 1989; Gromada *et al*., 1995). In β-cells, EC_{50} values for glibenclamide of 0.05–0.7 nM (free concentrations) have been observed (Zünkler *et al*., 1998a; Gromada *et al*., 1995; Figure 2). Moreover, glibenclamide is predicted to inhibit the β-cell K_{ATP}-channel with an EC_{50} value of <0.1 nM when using the corresponding K_I value (Table 1) and a K_I/EC_{50} ratio of >3 (calculated from the data for compound XI; Table 1; Figure 1). This K_I/EC_{50} ratio is in accordance with the ratios (2–10) which have previously been obtained for several sulphonylureas and analogues and suggests that more receptor sites are present in the plasma membrane than required for K_{ATP}-channel inhibition (Panten *et al*., 1989; Schwanstecher *et al*., 1994; 1998a; Dörschner *et al*., 1999).

In conclusion, the anionic group of hypoglycaemic sulphonylureas and acidic analogues considerably contributes to the binding affinity for SUR1, SUR2A and SUR2B. Selectivity for SUR1 is not induced by the sulphonylurea moiety but results from lipophilic substitution on the urea group. Sulphonylureas without lipophilic substitution on the urea group could represent lead compounds for the development of SUR2-selective drugs.

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