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Interaction of N-benzoyl-D-phenylalanine and related compounds with the sulphonylurea receptor of β-cells

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1 The structure activity relationships for the insulin secretagogues N-benzoyl-D-phenylalanine (NBDP) and related compounds were examined at the sulphonylurea receptor level by use of cultured HIT-T15 and mouse pancreatic β-cells. The affinities of these compounds for the sulphonylurea receptor were compared with their potencies for K_{ATP}-channel inhibition. In addition, the effects of cytosolic nucleotides on K_{ATP}-channel inhibition by NBDP were investigated.

2 NBDP displayed a dissociation constant for binding to the sulphonylurea receptor (K_D value) of 11 μM and half-maximally effective concentrations of K_{ATP}-channel inhibition (EC_{50} values) between 2 and 4 μM (in the absence of cytosolic nucleotides or presence of 0.1 mM GDP or 1 mM ADP).

3 In the absence of cytosolic nucleotides or presence of GDP (0.1 mM) maximally effective concentrations of NBDP (0.1–1 mM) reduced K_{ATP}-channel activity to 47% and 44% of control, respectively. In the presence of ADP (1 mM), K_{ATP}-channel activity was completely suppressed by 0.1 mM NBDP.

4 The L-isomer of N-benzoyl-phenylalanine displayed a 20 fold lower affinity and an 80 fold lower potency than the D-isomer.

5 Introduction of a p-nitro substituent in the D-phenylalanine moiety of NBDP did not decrease lipophilicity but lowered affinity and potency by more than 30 fold.

6 Introduction of a p-amino substituent in the D-phenylalanine moiety of NBDP (N-benzoyl-p-amino-D-phenylalanine, NBADP) reduced lipophilicity and lowered affinity and potency by about 10 fold. This loss of affinity and potency was compensated for by formation of the phenylpropionic acid derivative of NBADP. A similar difference in affinity was observed for the sulphonylurea carbutamide and its phenylpropionic acid derivative.

7 Replacing the benzene ring in the D-phenylalanine moiety of NBDP by a cyclohexyl ring increased lipophilicity, and the K_D and EC_{50} values were slightly lower than for NBDP. Exchange of both benzene rings in NBDP by cyclohexyl rings further increased lipophilicity without altering affinity and potency.

8 This study shows that N-acylphenylalanines interact with the sulphonylurea receptor of pancreatic β-cells in a stereospecific manner. Their potency depends on lipophilic but not aromatic properties of their benzene rings. As observed for sulphonylureas, interaction of N-acylphenylalanines with the sulphonylurea receptor does not induce complete inhibition of K_{ATP}-channel activity in the absence of inhibitory cytosolic nucleotides.

Keywords: N-benzoyl-D-phenylalanine; sulphonylurea receptor; K_{ATP}-channel; pancreatic β-cell; cytosolic nucleotides

Introduction

Sulphonylureas inhibit adenosine 5′-triphosphate-sensitive K^+ channels (K_{ATP}-channels) in the pancreatic β-cell plasma membrane and thereby initiate a chain of events leading to the release of insulin (Sturgess *et al*., 1985; for a review see Ashcroft & Rorsman, 1991). These channels are also inhibited when glucose metabolism of the β-cell increases (Ashcroft *et al*., 1984). The response of K_{ATP}-channels to changes in glucose metabolism is mediated by cytosolic nucleotides. Adenosine 5′-triphosphate (ATP) and some related nucleotides (e.g. free ADP) inhibit, whereas the Mg complexes of nucleoside diphosphates (e.g MgADP and MgGDP) activate the K_{ATP}-channels (Ashcroft & Rorsman, 1991). The β-cell K_{ATP}-channel is composed of two proteins, an inwardly rectifying K_{ATP}-channel subunit (K_{IR}6.2) and the sulphonylurea receptor (SUR1) (Aguilar-Bryan *et al*., 1995; Inagaki *et al*., 1995). Four K_{IR}6.2 subunits form the K^+-conducting pore and are associated with four SUR1 subunits (Clement *et al*., 1997). SUR1 endows the β-cell K_{ATP}-channel with sensitivity to sulphonylureas, diazoxide (K_{ATP}-channel opener which inhibits insulin release) and stimulant nucleoside diphosphates (Aguilar-Bryan *et al*., 1995; Inagaki *et al*., 1995; Nichols *et al*., 1996). It has been proposed that the recognition site for ATP and other inhibitory nucleotides is located on K_{IR} 6.2 (Tucker *et al*., 1997).

In β-cells, complete block of K_{ATP}-channels induced by inhibitory nucleotides can be relieved by channel-activating nucleoside diphosphates (e.g. MgADP and MgGDP) (Ashcroft & Rorsman, 1991). The resulting K_{ATP}-channel openings are completely suppressed when tolbutamide or related compounds are applied at concentrations saturating binding to the sulphonylurea receptor (Schmid-Antomarchi *et al*., 1987; Zünkler *et al*., 1988; Panten *et al*., 1989; Schwanstecher *et al*., 1992d; 1994a,b). This finding appears to be due to elimination of the channel-activating effects of nucleoside diphosphates by sulphonylureas (Schwanstecher *et al*., 1994a; Schwanstecher & Panten, 1994). In the absence of inhibitory cytosolic nucleotides, sulphonylurea concentrations saturating receptor binding do not completely suppress K_{ATP}-channel activity (Zünkler *et al*., 1988; Schwanstecher *et al*., 1992c,d; 1994a,b).

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N-acylphenylalanines (e.g. A-4166, Figure 1) and several related derivatives of 3-phenyl-propionic acid or benzoic acid (e.g. meglitinide, Figure 1) have been shown to stimulate insulin release and to induce hypoglycaemia (Garrino *et al*., 1985; Shinkai *et al*., 1989; Sato *et al*., 1991; Bakkali-Nadi *et al*., 1994; Ohnota *et al*., 1994; 1995; Hirose *et al*., 1995; Seto *et al*., 1995). Studies investigating the structural requirements for hypoglycaemic activity of N-acylphenylalanines revealed that the D-isomers of these compounds were much more potent than the corresponding L-isomers (Shinkai *et al*., 1988; 1989). It is not known whether these differences in potency reflect differences in pharmacokinetics or stereospecific interaction of the D- and L-isomers with the sulphonylurea receptor. The D-isomer A-4166 (Figure 1) has been shown to bind to the sulphonylurea receptor and to inhibit the K_{ATP}-channels of β-cells (Akiyoshi *et al*., 1995; Fujita *et al*., 1996) whereas L-isomers have not been tested. Hypoglycaemic effectiveness of N-acyl-D-phenylalanines requires the carboxyl group of the phenylalanine moiety and increases with increasing lipophilicity of the acyl moiety (Shinkai *et al*., 1988; 1989).

Which structural features of N-acylphenylalanines determine their interaction with the sulphonylurea receptor of the β-cell has not been investigated *in vitro*. Elucidation of this information should elaborate the biochemical properties of the receptor site and might facilitate the development of ligands specific for extrapancreatic sulphonylurea receptors. Apart from SUR1 two other sulphonylurea receptor isoforms (SUR2A and SUR2B) have been detected (Inagaki *et al*., 1996; Isomoto *et al*., 1996). For these reasons, we examined the effects of N-acylphenylalanines on receptor binding of [^{3}H]-glibenclamide and K_{ATP}-channel activity in β-cells.

A-4166 has been shown to increase $[Ca^{2+}]_i$ in β-cells more effectively than sulphonylureas under conditions of reduced intracellular ATP level (Fujitani *et al*., 1997). This finding and the structural differences between sulphonylureas and N-acylphenylalanines led us to examine whether the latter drugs are more effective than sulphonylureas in suppressing K_{ATP}-channel activity in the absence of inhibitory cytosolic nucleotides.

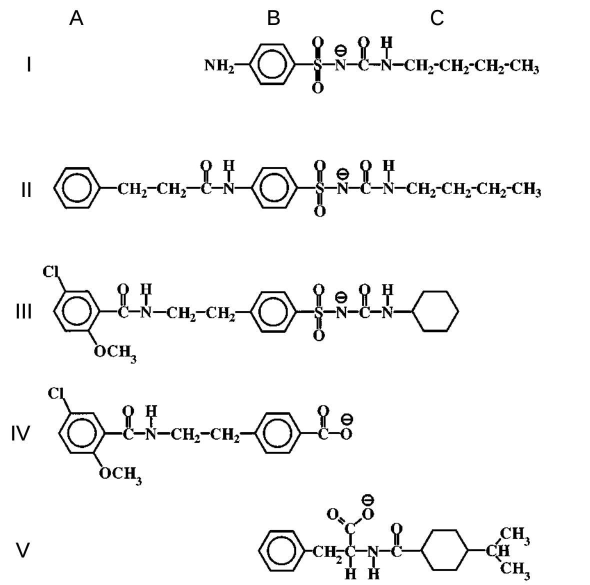


Figure 1 Structures of carbutamide (I), N-[p-(3-phenyl-propionylamido)benzene-sulphonyl]-N′-butylurea (II), glibenclamide (III), meglitinide (IV) and A-4166 (V). The anionic forms are shown. A, B and C label the lipophilic areas in the molecules.

Methods

Measurement of [^{3}H]-glibenclamide binding to microsomes

Culture of HIT-T15 cells (SV-40 transformed hamster pancreatic β-cells; Santerre *et al*., 1981), preparation of microsomes from these cells and measurement of [^{3}H]-glibenclamide binding to microsomes were performed as previously described (Schwanstecher *et al*., 1992b), except that the media for incubation of the microsomes were based on 200 mM Tris (adjusted with HCl to pH 7.4). Inhibition of [^{3}H]-glibenclamide binding by test compounds (final concentrations in the incubations indicated in the legends for the figures) was measured at room temperature in 1 ml of buffer containing (final concentration) 0.3 nM [^{3}H]-glibenclamide. Incubations were started by addition of microsomal protein (200 μg ml^{-1}) and were terminated after 60 min by filtration. Non-specific binding was defined by incubations in the presence of 100 nM unlabelled glibenclamide.

Isolation and culture of pancreatic β-cells

Pancreatic islets were isolated from male albino mice (NRMI, 8–12 weeks old, fed *ad libitum*) as previously described (Panten *et al*., 1989). The islets were dissociated into single cells by shaking in a solution without Ca^{2+} (Lernmark, 1974). The cells were cultured for 1–2 days on Nunc Petri dishes (35 × 10 mm) in RPMI 1640 tissue culture medium containing 15% foetal calf serum, 100 μg ml^{-1} streptomycin, 100 units ml^{-1} penicillin G and 10 mM D-glucose.

Electrophysiological recording and analysis

The inside-out configuration of the patch-clamp technique was used to record currents flowing through K_{ATP}-channels as previously described (Schwanstecher *et al*., 1994b). The membrane potential was clamped at −50 mV and inward membrane currents were measured. All experiments were performed at room temperature (20–22°C). The single-channel current amplitudes of the K_{ATP}-channels were not changed by the applied concentrations of nucleotides and drugs.

Chemicals and solutions

Chemicals for organic syntheses, N-benzoyl-D-phenylalanine, N-benzoyl-L-phenylalanine and carbutamide were purchased from Aldrich Chemical Co. (Steinheim, Germany) and Fluka Chemie AG (Buchs, Switzerland). [^{3}H]-glibenclamide (51 Ci mmol^{-1}, 96% radiochemical purity) was purchased from NEN (Dreieich, Germany). All other chemicals were obtained from sources described elsewhere (Panten *et al*., 1989; 1990; Schwanstecher *et al*., 1992a,b).

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′-tetra-acetic acid (EGTA) 10 and 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (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*. (1992d). 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 (Ohno-Shosaku *et al*., 1987). 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).

Stock solutions (20 – 50 mM) of the tested compounds were prepared daily in DMSO for patch-clamp experiments or in NaOH (100 mM) for binding experiments. The pH of all test solutions was determined after addition of test substances and was readjusted if necessary. All tested compounds were completely dissolved at the applied concentrations.

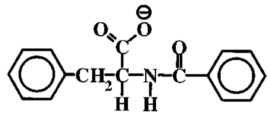
Syntheses

General The structural formulae of the synthesized N-acyl-amino acids are depicted in Figure 2. The α-amino group of an amino acid was allowed to react with benzoyl chloride or cyclohexane-carboxylic acid chloride in the presence of NaOH. The product was collected after acidification with HCl to pH 1 – 3.

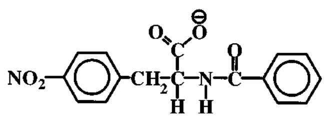
Unless stated otherwise, chemicals (pure grade) were used as received. Solvents were dried and distilled before use. All melting or decomposition points were determined on a Linström melting point apparatus (Bühler, Tübingen, Germany) and are uncorrected. Optical rotations were measured on a Perkin Elmer 241 polarimeter. Elementary analyses were performed by the analytical laboratory of the Institute of Pharmaceutical Chemistry, Technical University of Braunschweig. The structures of the synthesized compounds were confirmed by ^{1}H-n.m.r. (DMSO, 400 MHz) and ^{13}C-n.m.r. (DMSO, 100 MHz) spectroscopy on a model AM400 spectrometer (Bruker, Karlsruhe, Germany) and IR spectroscopy on a model FTIR spectrometer (ATI Unicam, Kassel, Germany),

A B C

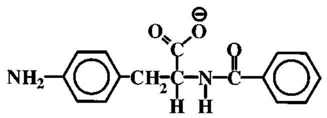
VI



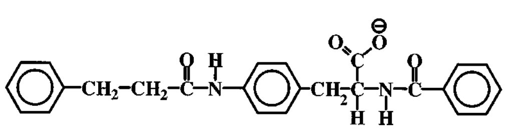
VII



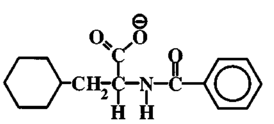
VIII



IX



X



XI

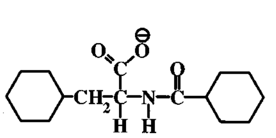


Figure 2 Structures of N-benzoyl-phenylalanine (VI), N-benzoyl-p-nitro-phenylalanine (VII), N-benzoyl-p-amino-phenylalanine (VIII), N-benzoyl-3[p-(3-phenyl-propionylamido) benzene]-alanine (IX), N-benzoyl-3-cyclohexyl-alanine (X) and N-cyclohexylcarbonyl-3-cyclohexyl-alanine (XI). The anionic forms are shown. A, B and C label the rings in the molecules.

supported by electron-impact mass spectrometry on a Finnigan MAT 8430 spectrometer.

N-[p(3-phenyl-propionylamido)benzenesulphonyl]-N'-butylurea (compound II) Compound II was prepared by the addition of 3-phenylpropionyl chloride to carbutamide: mp 196°C. Elementary analysis: calculated for $C_{20}H_{25}N_3O_4S$: C, 59.53; H, 6.25; N, 10.41. Found: C, 59.50; H, 6.32; N, 10.10.

N-Benzoyl-p-nitro-D-phenylalanine (compound VII) Compound VII was prepared by the addition of benzoyl chloride to p-nitro-D-phenylalanine: mp 165°C (160 – 162°C, Kameda *et al.*, 1958); $[\alpha]^{20}_D + 58°$ (c 1.0, 70% ethanol). Elementary analysis: calculated for $C_{16}H_{14}N_2O_5$: C, 61.14; H, 4.49; N, 8.91. Found: C, 61.15; H, 4.44; N, 8.80.

N-Benzoyl-p-amino-D-phenylalanine (compound VIII) Compound VIII was prepared via catalytic hydrogenation of the nitro group of compound VII: mp 205°C (dec) (212°C, Kameda *et al.*, 1958); $[\alpha]^{20}_D - 14.7°C$ (c 1.0, 1 N NaOH). Elementary analysis: calculated for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.57; H, 5.70; N, 9.84.

N-Benzoyl-3-[p-(3-phenyl-propionylamido)benzene]-D-alanine (compound IX) Compound IX was prepared by the addition of 3-phenylpropionyl chloride to compound VIII: mp 220°C; $[\alpha]^{20}_D + 18.9°$ (c 0.1, 1 N NaOH). Elementary analysis: calculated for $C_{25}H_{24}N_2O_4$: C, 72.10; H, 5.81; N, 6.73. Found: C, 71.98; H, 5.74; N, 6.75.

N-Benzoyl-3-cyclohexyl-D-alanine (compound X) Compound X was prepared by the addition of benzoyl chloride to 3-cyclohexyl-D-alanine: mp 124°C; $[\alpha]^{20}_D + 3.28°$ (c.1.0, 1 N NaOH). Elementary analysis: calculated for $C_{16}H_{21}NO_3$: C, 69.80; H, 7.69; N, 5.09. Found: C, 69.55; H, 7.71; N, 4.89.

N-Cyclohexylcarbonyl-3-cyclohexyl-D- alanine (compound XI) Compound XI was prepared by the addition of cyclohexanecarboxylic acid chloride to 3-cyclo-hexyl-D-alanine: mp 82°C; $[\alpha]^{20}_D + 11.18°$ (c 1.0, 1 N NaOH). Elementary analysis: calculated for $C_{16}H_{27}NO_3H_2O$: C, 64.17; H, 9.76; N, 4.68. Found: C, 64.35; H, 9.82; N, 4.53.

Treatment of results

Values are presented as mean ± s.e.mean. Relations between drug concentration and specific binding or channel activity were analysed by fitting the function

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

to the experimental data by a non-linear least-squares method where y = specific binding or K_{ATP}-channel activity, EC_{50} = half-maximally effective drug concentration, x = drug concentration, n_H = slope parameter (Hill coefficient), a = maximum value for specific binding or K_{ATP}-channel activity and b = additive constant.

Results

Effects of stereoisomers of N-benzoyl-phenylalanine on [^{3}H]-glibenclamide binding and on K_{ATP}-channel activity

Competitive inhibition assays revealed that N-benzoyl-D-phenylalanine and N-benzoyl-L-phenylalanine (compound VI,

Figure 2) inhibited specific [^{3}H]-glibenclamide binding to HIT-cell microsomes half-maximally at 25 μM (Hill coefficient = -1.0) and 485 μM (Hill coefficient = -1.0), respectively (Figure 3a). These half-maximal inhibitory concentrations (IC_{50}), the dissociation constant (K_D) for glibenclamide (0.22 nM, Schwanstecher *et al.*, 1992a) and the free [^{3}H]-glibenclamide concentration in the incubations were used to calculate the K_D values for the D-isomer and the L-isomer (10.6 and 206 μM, respectively) (Cheng & Prusoff, 1973).

Figure 3b 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 30 s periods to an intracellular solution containing 1 mM ATP, alternating with ATP-free 15 s periods serving as test or control (absence of nucleotides and drugs) periods. Except for the experiments shown in Figure 4, 1 mM ADP was always present at the cytoplasmic membrane side during the test periods. Intracellular solution supplemented with Mg^{2+} and 1 mM ADP contains both MgADP (0.5 mM) which stimulates and free ADP (0.5 mM) which inhibits the K_{ATP}-channels. The example in Figure 3b demonstrates that 1 mM ADP reduced K_{ATP}-channel activity to 78% of control. Further addition of 3 μM of the D-isomer of N-benzoyl-phenylalanine decreased K_{ATP}-channel activity to 37% of control; 100 μM of the D-isomer induced complete inhibition of all K_{ATP}-channels (Figure 3c). In the presence of ADP (1 mM), the D-isomer and the L-isomer of N-benzoyl-phenylalanine were half-maximally effective at 2.4 μM (Hill coefficient = -1.4) and 211 μM (Hill coefficient = -0.8), respectively.

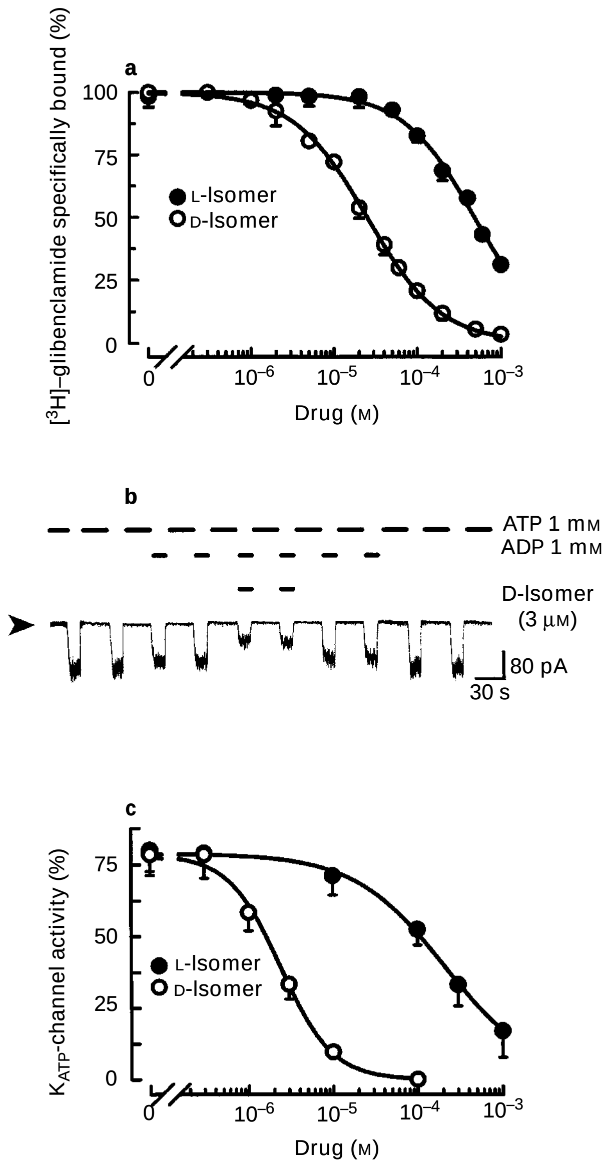


Figure 3 Effects of the D- and L-isomers of N-benzoyl-phenylalanine on [^{3}H]-glibenclamide binding to membranes from HIT-cells and on K_{ATP}-channel activity in inside-out patches of mouse pancreatic β-cells. (a) Inhibition of [^{3}H]-glibenclamide binding by the D-isomer or the L-isomer. Results are presented as percentage of specific binding of [^{3}H]-glibenclamide in the absence of inhibiting drugs. Values are given as means (with s.e.mean (vertical lines) shown when larger than symbols) for results from four separate experiments. (b) 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. The other horizontal bars above the current trace indicate application of intracellular solution containing 1 mM ADP (with or without 3 μM NBDP) for 15 s by the bath. The 15 s periods of application of nucleotide- and drug-free intracellular solution by the bath represent the control periods. The arrowhead denotes the zero-current level (all K_{ATP}-channels closed). (c) Relationship between normalized K_{ATP}-channel activity and concentration of the D-isomer or L-isomer in the presence of 1 mM ADP. By use of the experimental design shown in (b), K_{ATP}-channel activity during drug application was normalized to K_{ATP}-channel activity during 15 s control periods before and after drug application in each single experiment. Symbols indicate the mean of 4–11 experiments (with s.e.mean shown when larger than symbols).

K_{ATP}-channel inhibition by N-benzoyl-D-phenylalanine in the absence of nucleotides or presence of GDP

In the absence of cytosolic nucleotides, N-benzoyl-D-phenylalanine was half-maximally effective at 1.5 μM (Figure 4). However, under these conditions maximally effective concentrations of the D-isomer (100 μM – 1 mM) reduced channel activity to only 47% of control.

Intracellular solution supplemented with Mg^{2+} and 100 μM GDP contains both MgGDP (50 μM) which stimulates the channel and free GDP (50 μM) which is ineffective (Schwanstecher *et al.*, 1994a). GDP (100 μM total concentration) increased K_{ATP}-channel activity to 253 ± 38% of control ($n = 6$) and maximally effective concentration of the D-isomer (1 mM) reduced K_{ATP}-channel activity to 44% of control. In the presence of GDP (100 μM total concentration), the D-isomer

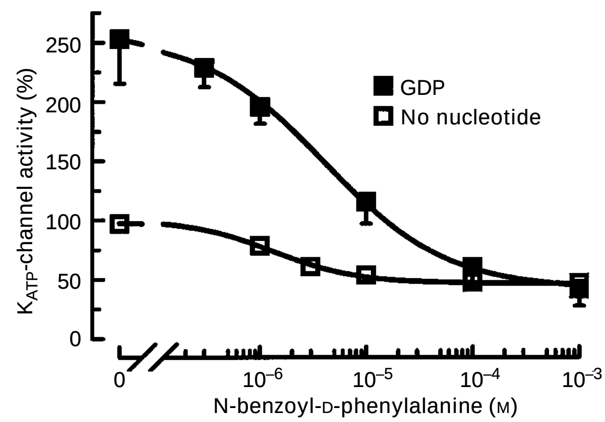


Figure 4 Inhibition of K_{ATP}-channel activity in inside-out patches of mouse pancreatic β-cells by N-benzoyl-D-phenylalanine in the absence of nucleotides or presence of 0.1 mM GDP. Symbols indicate the mean of 4–8 experiments (with s.e.mean (vertical lines) shown when larger than symbols). For further details see legend of Figure 3.

was half-maximally effective at 4 μM (Hill coefficient = −0.75) (Figure 4).

Effects of derivatives of N-benzoyl-D-phenylalanine on [^{3}H]-glibenclamide binding and on K_{ATP}-channel activity

Further structure-activity investigations were performed with derivatives of the D-isomer of N-benzoyl-phenylalanine (compounds VII to XI, Figure 2). Figure 5 shows the influence of substituents in the phenyl ring of the D-phenylalanine moiety of N-benzoyl-D-phenylalanine. Introduction of a p-nitro (compound VII) or a p-amino (compound VIII) substituent strongly increased the concentrations required for half-maximal inhibition of [^{3}H]-glibenclamide binding (801 μM, Hill coefficient = −1.0 and 272 μM, Hill coefficient = −1.0, respectively) and K_{ATP}-channel activity (83 μM, Hill coefficient = −1.1, and 31 μM, Hill coefficient = −1.1, respectively). The loss of binding affinity and potency could be reversed by formation of the phenylpropionic acid derivative of N-benzoyl-p-amino-D-phenylalanine (compound IX; IC_{50} = 28 μM, Hill coefficient = −0.9; EC_{50} = 3.5 μM, Hill coefficient = −1.1).

Figure 6 shows that exchange of the benzene ring in the D-phenylalanine moiety of N-benzoyl-D-phenylalanine by a cyclohexyl ring (compound X) slightly decreased the concentrations required for half-maximal inhibition of [^{3}H]-glibenclamide binding (9.8 μM, Hill coefficient = −1.0) or K_{ATP}-channel activity (1.2 μM, Hill coefficient = −1.1). Similar half-maximal inhibitory concentrations (6.9 μM, Hill coefficient = −1.0, and 1.2 μM, Hill coefficient = −1.7, respectively) were observed for a derivative produced by exchange of both benzene rings by cyclohexyl rings (compound XI; Figure 6).

Effects of sulphonylureas on [^{3}H]-glibenclamide binding

For comparison with compounds VIII and IX, the binding affinities of two structurally related sulphonylureas were determined. Carbutamide (compound I, Figure 1) and its phenylpropionic acid derivative (compound II, Figure 1) inhibited [^{3}H]-glibenclamide binding half-maximally at 173 μM (Hill coefficient = −0.9) and 17 μM (Hill coefficient = −1.0), respectively (mean values from four separate experiments; results not shown in a figure). From these IC_{50} values K_D values of 73 μM and 7.2 μM, respectively, were calculated.

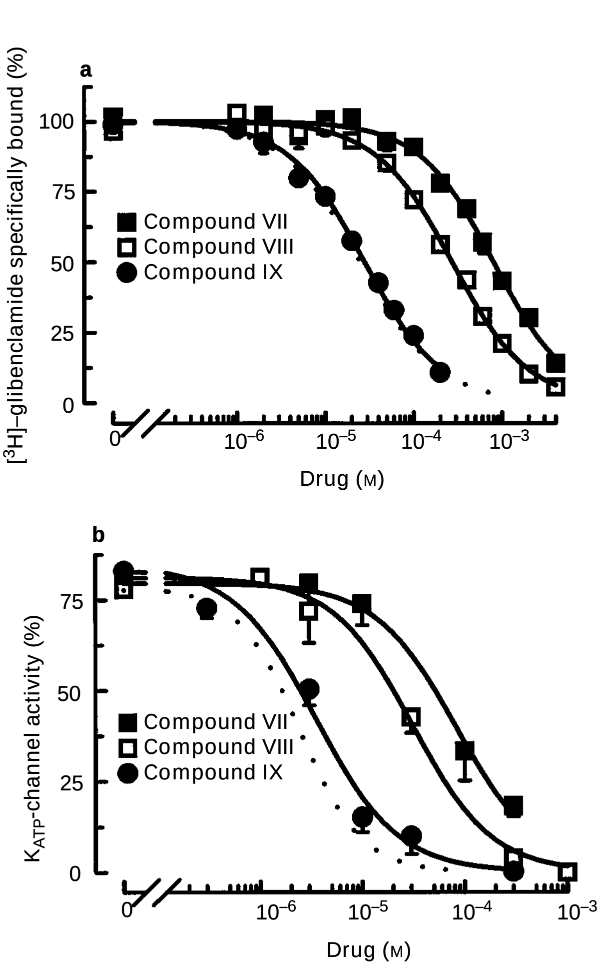


Figure 5 Effects of compound VII, compound VIII and compound IX on [^{3}H]-glibenclamide binding to membranes from HIT-cells (a) and on K_{ATP}-channel activity in inside-out patches of mouse pancreatic β-cells (b). Symbols indicate the mean (with s.e.mean shown by vertical lines when larger than symbols) of 4 separate binding experiments or 4–11 patch-clamp experiments. To facilitate comparison, the concentration-dependent effects of N-benzoyl-D-phenylalanine are shown as dashed lines (taken from Figure 3). For further details see legend of Figure 3.

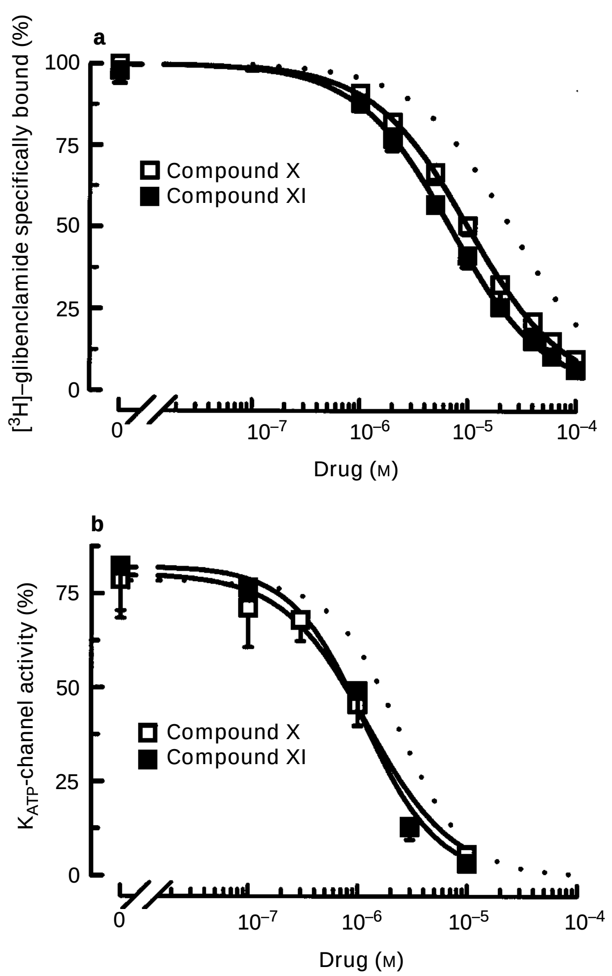


Figure 6 Effects of compound X and compound XI on [^{3}H]-glibenclamide binding to membranes from HIT-cells (a) and on K_{ATP}-channel activity in inside-out patches of mouse pancreatic β-cells (b). Symbols indicate the mean (with s.e.mean shown by vertical lines when larger than symbols) of 4 separate binding experiments or 4–12 patch-clamp experiments. To facilitate comparison, the concentration-dependent effects of N-benzoyl-D-phenylalanine are shown as dashed lines (taken from Figure 3). For further details see legend of Figure 3.

Discussion

The data documented here support the previous conclusion that the N-acylphenylalanine A-4166 and related compounds inhibit the K_{ATP}-channel of β-cells by binding to the same receptor site that mediates the responses to hypoglycaemic sulphonylureas (Fujita et al, 1996; Panten et al., 1996; Ikenoue et al., 1997). The K_D values for receptor binding of NBDP and related N-acyl-D-amino acids were 2–4 fold higher than the corresponding EC_{50} values for channel inhibition (Table 1). Similar differences (3–10 fold higher) have been obtained for the K_D and EC_{50} values of tolbutamide, meglitinide and two glibenclamide derivatives (Schwanstecher et al., 1994b). These differences might indicate that more receptor sites are present in the β-cell membrane than required for maximal inhibition of the K_{ATP}-channels (Panten et al., 1989).

In the absence of cytosolic nucleotides, maximally effective concentrations of N-benzoyl-D-phenylalanine (NBDP) produced incomplete inhibition of K_{ATP}-channel activity (Figure 4). The proportion of channel activity resistant to NBDP was not smaller than the corresponding proportions observed for tolbutamide and related compounds (Zünkler et al., 1988; Schwanstecher et al., 1994b). Hence, greater effectiveness at the sulphonylurea receptor was probably not the reason why A-4166 increased the $[Ca^{2+}]_i$ of metabolically compromised β-cells more effectively than sulphonylureas (Fujitani et al., 1997).

Tolbutamide has been shown to inhibit channel activation induced by MgGDP (Schwanstecher et al., 1994b). The same holds true for NBDP (Figure 4). In the absence of inhibitory nucleotides, MgGDP did not significantly affect the EC_{50} value of NBDP and the proportion of channel activity resistant to maximally effective concentrations of NBDP. This suggests that NBDP inhibits channel openings in a uniform manner, regardless of whether they occur spontaneously or are evoked by nucleoside diphosphates.

In the presence of 1 mM cytosolic ADP (0.5 mM MgADP + 0.5 mM free ADP), maximally effective concentrations of NBDP completely suppressed K_{ATP}-channel activity (Figure 3), as previously observed for sulphonylureas and related compounds (Zünkler et al., 1988; Schwanstecher et al., 1992d; 1994a,b). This finding is probably due to elimination of the channel-activating effect of 0.5 mM MgADP by NBDP, as suggested for sulphonylureas (Schwanstecher et al., 1994a; Schwanstecher & Panten, 1994). The remaining inhibitory effect of ADP (Panten et al., 1990) is expected to cause complete channel closure.

Table 1 Interaction of N-benzoyl-D-phenylalanine and related compounds with the sulphonylurea receptor of insulin-secreting cells

Compound	K_D	EC_{50}
A-4166	0.25; 0.43	0.23; 0.005[1]
N-Benzoyl-D-phenylalanine	10.6	2.4
Compound VII	339	83
Compound VIII	115	31
Compound IX	11.8	3.5
Compound X	4.2	1.2
Compound XI	2.9	1.2

The EC_{50} values for inhibition of K_{ATP}-channels from β-cells were taken from the Results section or are those previously published for A-4166 (Akiyoshi et al. 1995. [1] The values have been obtained in the whole-cell and inside-out configurations, respectively). The K_D values for binding to microsomes from HIT-cells were calculated as described in the Results section or are those previously published for A-4166 (Fujita et al., 1996; Ikenoue et al., 1997). EC_{50} and K_D values are given in μM.

There is evidence that the phenylpropionic acid moiety of N-acyl-D-phenylalanines corresponds to the benzenesulphonamide moiety of hypoglycaemic sulphonylurea derivatives with regard to interaction with the sulphonylurea receptor. Firstly, introduction of a p-nitro-substituent in the D-phenylalanine moiety of N-benzoyl-D-phenylalanine (NBDP) led to a derivative (compound VII, Figure 2) with much lower binding affinity and K_{ATP}-channel-inhibiting potency than NBDP (Figure 5), but not with lower lipophilicity. Hansch hydrophobic constants π for the p-nitrobenzyl fragment and the benzyl fragment are around 1.9 and 1.7, respectively (Pliška & Escher, 1996). Probably the high electronegativity of the p-nitro group hinders contact with the receptor site. It has been shown previously that a p-nitro-substituent causes loss of the hypoglycaemic effect of the first-generation sulphonylurea N-(benzenesulphonyl)-N′-butylurea (Ruschig et al., 1958; Dorche et al., 1959). Secondly, introduction of a p-amino substituent in the D-phenylalanine moiety of NBDP (compound VIII, Figure 2), was also associated with a decrease in affinity and potency (Figure 5). This can be explained by the lower lipophilicity of the p-amino derivative (π value for the p-aminobenzyl fragment is around 1.0, Pliška & Escher, 1996). Formation of the more lipophilic phenylpropionic acid derivative of N-benzoyl-p-amino-D-phenylalanine (compound IX, Figure 2) compensated for the decrease in affinity and potency (Figure 5). A corresponding dependence on substitution of the p-amino group was demonstrated for the binding affinity of sulphonylureas (compounds I and II, Figure 1; see Results).

The D-isomer of N-benzoyl-phenylalanine displayed K_D and EC_{50} values which were 20 and 80 fold lower, respectively, than those of the L-isomer (Figure 3). This finding suggests that the position of the anionic COO^- group relative to the neighbouring lipophilic areas of the molecule (labelled B and C in Figure 2) is critical for interaction with the sulphonylurea receptor site. Receptor binding of sulphonylurea derivatives could induce a position of their anionic group relative to the neighbouring lipophilic areas which resembles the position of the COO^- group of N-acyl-D-phenylalanines.

Replacement of the benzene ring of the phenylalanine moiety of NBDP by a cyclohexyl ring produced a more lipophilic compound (π value for the cyclohexylmethyl fragment is around 2.7; Pliška & Escher, 1996) which displayed K_D and EC_{50} values only slightly lower than those for NBDP (Figure 6). It may be that a stronger increase in receptor binding affinity corresponding to the gain in lipophilicity was prevented by steric hindrance due to the more bulky structure of the cyclohexyl ring. Exchange of both benzene rings in NBDP by cyclohexyl rings further increased the lipophilicity (π values for benzene and cyclohexane are 2.1 and 3.5, respectively, Recker & Mannhold, 1992), but not the binding affinity and the K_{ATP}-channel-inhibitory potency of the derivative (Figure 6). The latter observations, too, suggest steric hindrances, but also indicate that interaction with the sulphonylurea receptor does not require an aromatic ring in the ligand.

In conclusion, the present study shows that N-acylphenylalanines interact with the sulphonylurea receptor of β-cells in a stereospecific manner. Lipophilic but not aromatic properties of their benzene rings are essential for activity. The phenylpropionic acid moiety of N-acyl-D-phenylalanines corresponds to the benzenesulphonamide moiety of sulphonylurea derivatives with regard to interaction with the sulphonylurea receptor. As observed for sulphonylureas and their analogues, maximally effective concentrations of N-acyl-D-phenylalanines cause incomplete inhibition of K_{ATP}-channel activity in the absence of inhibitory cytosolic nucleotides.

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