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Location of the sulphonylurea receptor at the cytoplasmic face of the β-cell membrane

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1 In insulin-secreting cells the location of the sulphonylurea receptor was examined by use of a sulphonylurea derivative representing the glibenclamide molecule devoid of its cyclohexyl moiety (compound III) and a benzenesulphonic acid derivative representing the glibenclamide molecule devoid of its cyclohexylurea moiety (compound IV). At pH 7.4 compound IV is only present in charged form.

2 Lipid solubility declined in the order tolbutamide > compound III > compound IV.

3 The dissociation constant (K_D) for binding of compound IV to the sulphonylurea receptor in HIT-cells (pancreatic β-cell line) was similar to the K_D value for tolbutamide and fourfold higher than the K_D value for compound III.

4 In mouse pancreatic β-cells, drug concentrations inhibiting adenosine 5'-triphosphate-sensitive K^+ channels (K_{ATP}-channels) half-maximally (EC_{50}) were determined by use of the patch-clamp technique. When the drugs were applied to the extracellular side of outside-out or the intracellular side of inside-out membrane patches, the ratio of extracellular to intracellular EC_{50} values was 281 for compound IV, 25.5 for compound III and 1.2 for tolbutamide.

5 In mouse pancreatic β-cells, measurement of K_{ATP}-channel activity in cell-attached patches and recording of insulin release displayed much higher EC_{50} values for compound IV than inside-out patch experiments. A corresponding, but less pronounced difference in EC_{50} values was observed for compound III, whereas the EC_{50} values for tolbutamide did not differ significantly.

6 It is concluded that the sulphonylurea receptor is located at the cytoplasmic face of the β-cell plasma membrane. Receptor activation is induced by the anionic forms of sulphonylureas and their analogues.

Keywords: Sulphonylurea receptor; ATP-sensitive K^+ channel; insulin secretion; pancreatic β-cell; glibenclamide analogues

Introduction

In the plasma membrane of β-cells a K^+ channel has been identified which is inhibited by cytosolic ATP (K_{ATP})-channel) (Cook & Hales, 1984; for reviews see Ashcroft & Rorsman, 1991; Dunne & Petersen, 1991). Activation of β-cell energy metabolism by glucose or other insulin releasing fuels closes K_{ATP}-channels and thereby initiates a chain of events eventually leading to the release of insulin (Ashcroft *et al.*, 1984; Ashcroft & Rorsman, 1991). Sulphonylureas stimulate insulin secretion by inhibiting the K_{ATP}-channel via direct interaction with the β-cell plasma membrane (Sturgess *et al.*, 1985; Trube *et al.*, 1986; for reviews see Ashford, 1990; Edwards & Weston, 1993). In microsomal membranes from insulin secreting cells, a high affinity binding site for sulphonylureas has been found which probably represents the sulphonylurea receptor (Geisen *et al.*, 1985; for reviews see Panten *et al.*, 1992; Ashcroft & Ashcroft, 1992; Edwards & Weston, 1993). However, it is still unclear whether the sulphonylurea receptor is a component of the K_{ATP}-channel or a regulatory protein not permanently associated with the K_{ATP}-channel.

Sulphonylureas and their analogues (e.g. meglitinide) inhibit the K_{ATP}-channel from both sides of the β-cell membrane (Trube *et al.*, 1986; Sturgess *et al.*, 1988). This can be explained by rapid distribution of these drugs within the lipid phase of the cell membrane. Sulphonylureas and their analogues are weak organic acids, and the proportions of their lipophilic undissociated forms decrease with increasing pH. When performing whole-cell clamp experiments and raising the pH value in the extracellular solution at constant total tolbutamide concentration, both the rate of development and the degree of tolbutamide-induced K_{ATP}-channel block is diminished (Zünkler *et al.*, 1989). This finding and additional evidence suggest that the undissociated forms are the effective forms and reach the binding site on the sulphonylurea receptor from the lipid phase of the β-cell membrane. However, the possibility is not ruled out that the binding site is located at the cytoplasmic face of the plasma membrane and that the undissociated forms are only required for transport of externally applied sulphonylureas and related compounds across the membrane.

The aim of the present investigation was to elucidate further the location of the sulphonylurea receptor in the β-cell membrane. We have also sought evidence that the dissociated forms of sulphonylureas and related compounds directly interact with the receptor site. To address these issues we applied glibenclamide analogues (Figure 1) much less lipophilic than the sulphonylureas used so far. The affinities of these compounds for the sulphonylurea receptor were compared with their potencies for K_{ATP}-channel inhibition and stimulation of insulin secretion. Using different configurations of the patch-clamp technique the compounds were applied at the extracellular or intracellular side of the plasma membrane.

Methods

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

Culture of HIT-cells, preparation of microsomes from these cells and measurement of [^{3}H]-glibenclamide binding to microsomes were performed as previously described (Schwanstecher *et al.*, 1992b).

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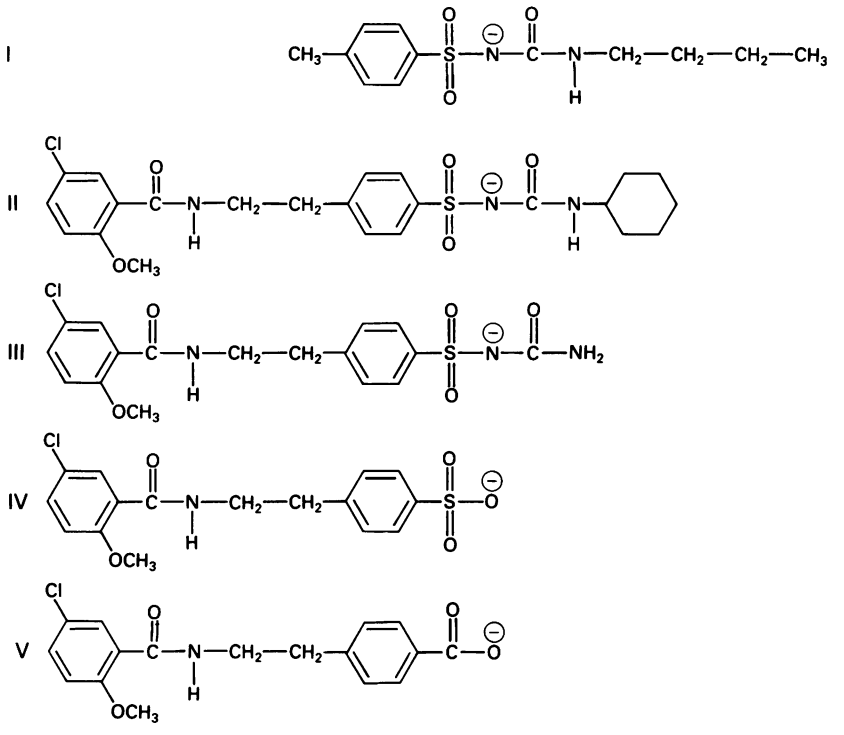

Figure 1 Structures of tolbutamide (I), glibenclamide (II), compound III, compound IV and meglitinide (V). The anionic forms are shown.

Isolation and culture of pancreatic β-cells

Pancreatic islets were isolated from male albino mice (NMRI, 11–15 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 1–4 days on Nunc Petri dishes (35 × 10 mm) in RPMI 1640 tissue culture medium containing 10% foetal calf serum, 100 μg ml^{-1} streptomycin, 100 u ml^{-1} penicillin G and 5 mM D-glucose for cell-attached experiments or 10 mM D-glucose for inside-out and outside-out experiments.

Electrophysiological recording and analysis

The cell-attached, inside-out and outside-out configuration of the patch-clamp technique (Hamill *et al.*, 1981) were used to record currents flowing through K_{ATP}-channels as previously described (Panten *et al.*, 1990; Schwanstecher *et al.*, 1992c). All experiments were performed at room temperature (20–22°C). The bath was perfused continuously at 2 ml min^{-1}. Pipettes were pulled from borosilicate glass and had resistances between 3 and 8 MΩ when filled with pipette solution. At the beginning of an experiment the current output signal of the amplifier was set to zero with the pipette being immersed in extracellular solution (cell-attached and outside-out experiments) or in intracellular solution (inside-out experiments). The zero-current potential served as reference for all measurements and no corrections have been made for the liquid junction potential. In cell-attached patch experiments, the pipette potential was held at 0 mV. The membrane potential was clamped at − 50 mV and 0 mV in inside-out and outside-out patch experiments, respectively. Inward and outward membrane K^+ currents are indicated as downward and upward deflections, respectively, in all current traces.

In cell-attached patch experiments data samples of 1–2 min duration were analyzed with the half-amplitude threshold technique (Colquhoun & Sigworth, 1983) using an interactive graphics-based analysis programme (pCLAMP 5.5.1, FETCH series). Amplitude histograms of sampled cell-attached currents were formed to calculate the mean single-channel current amplitudes (i). Channel activity was defined as the product of N, the number of functional channels and P_o, the open state probability and was determined by dividing the total time the channels spent in the open state by the total sample time. In case of superpositions of channels, the channel activity was calculated by summing the activity for each different current level. In addition, the mean of the channel activity during the control periods (presence of 3 mM D-glucose) before and after application of test substance (0.3–10 μM compound III or 1–100 μM compound IV) was set at 1.0 and the channel activity in the presence of test substance was normalized to this value. Data were sampled during the last minute of a test period (4–13 min duration). Data sampling during the control periods was started 1–2 min before and 5–25 min after the test period.

In inside-out patch experiments the cytoplasmic face of the membrane patch was exposed for 45 s periods to intracellular solution which contained Mg^{2+} and 1 mM ATP and was applied directly to the patch from a U-shaped polythene capillary (Fenwick *et al.*, 1982; Ohno-Shosaku *et al.*, 1987). These 45 s periods alternated with 15 s periods of intracellular solution which was supplemented with or without test substances (ADP and/or drugs) and was applied to the patch by bath perfusion (experimental design shown in Figure 3). Before and after application of test substance-containing bath solution, there were periods during which the same solution was applied except that the test substances were omitted. The mean of the amplitudes of the current responses (current amplitudes) during application of test substances was normalized to the mean current amplitude during ADP- and drug-free control periods in each single experiment. The single-channel current amplitudes of the K_{ATP}-channels were not changed by the applied concentrations of compound III, compound IV and meglitinide.

In outside-out patch experiments K^+ currents were measured during the test period (presence of 1–100 μM tolbutamide, 1–100 μM compound III or 1–1000 μM compound

IV) at the extracellular membrane side and during control periods before and after the test period. In each experiment, test periods with 100 μM tolbutamide were included to detect the base-line (all K_{ATP}-channels closed) for the K^+ current measurements. The mean K^+ current during 1 min intervals was measured during the last minute of the control period before drug application and during the new steady states induced by application and removal of test substance. The K^+ current during a test period was normalized to the mean of the K^+ currents during the control periods before and after application of test substance. Changes of the single-channel current amplitudes of the K_{ATP}-channels were not detected after application of tolbutamide and compounds III and IV.

Recordings were made using an LM-EPC7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Current signals were low-pass filtered at 2 kHz (cell-attached currents) or at 0.5 kHz (inside-out and outside-out currents) with a 4-pole Bessel filter (AF 173, Thomatronik, Rosenheim, Germany), digitized using an A/D converter (Instrutech, New York, U.S.A.) and stored on video tape. For analysis, taped data were replayed, digitized at 10 kHz for cell-attached and at 2 kHz for inside-out and outside-out currents using an Axolab 1100 computer interface (Axon Instruments, Foster City, CA, U.S.A.) and stored in a microcomputer. Analysis of the data was performed with the computer programme pCLAMP 5.5.1 (Axon Instruments). For Figures 3(a), 4(a) and 5(a) taped data were replayed into a chart recorder (220, Gould, Cleveland, OH, U.S.A.).

Measurement of insulin secretion

Measurement of insulin secretion from batches of 50 mouse islets (for animals and isolation see above) and data analysis were performed as described previously (Panten *et al.*, 1989). The islets were perifused at 0.9 ml min^{-1} and 37°C for 60 min in the presence of 10 mM D-glucose and then for 60 min in the presence of 10 mM D-glucose + test substance.

Determination of albumin binding

Binding of drugs to albumin in the perifusion media (37°C) was measured by ultrafiltration as described previously (Panten *et al.*, 1989). The concentration of compound III in the ultrafiltrate was determined by measuring inhibition of specific [^{3}H]-glibenclamide binding to HIT-cell microsomes induced by the ultrafiltrate. Albumin-free media containing known concentrations of compound III served as standards. The concentration of compound IV in the ultrafiltrate was determined by analytical high performance liquid chromatography (h.p.l.c.) on a Kontron 325 system (Kontron Instruments, Neufahrn, Germany) equipped with an ODS-Hypersil 5 μm column (250 × 4 mm, Bischoff, Leonberg, Germany).

Determination of partition coefficients

Determination of apparent octanol-water partition coefficients was performed at room temperature as previously described (Panten *et al.*, 1989). The aqueous phase consisted of 50 mM potassium phosphate and 50 mM boric acid, titrated to pH 7.4 with KOH. Drug concentration in the appropriate phase was measured by u.v. spectrophotometry against the appropriate blank.

Chemicals and solutions

Chemicals for organic syntheses 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). Compound III (known as Hoe 36320; Figure 1) was from Hoechst AG (Frankfurt, Germany). The sodium salt of compound IV (Figure 1) was synthesized as described below and dissolved in the test solutions immediately before use. Stock solutions of compound III, meglitinide and tolbutamide were prepared daily in 50–100 mM NaOH or KOH; if necessary, the pH of the stock solutions was adjusted to 8.5 with HCl. 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 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 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 $MgCl_2$ and the composition of the solutions for pH 7.15 were calculated with a computer programme (Fabiato, 1988) except that some stability constants of the programme were exchanged as detailed previously (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 aforementioned microflow system (Ohno-Shosaku *et al.*, 1987). After addition of 1 mM KADP + 0.1 mM Na_2-ATP, the intracellular solution was used for filling the pipette in outside-out patch experiments. The bath solution in cell-attached and outside-out patch experiments contained (in mM): NaCl 140, KCl 5.6, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10 (titrated to pH 7.40 with NaOH). The pipette solution in cell-attached and inside-out patch experiments contained (in mM): KCl 146, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10 (titrated to pH 7.40 with KOH). In cell-attached patch experiments 3 mM D-glucose and 10 μM nifedipine were added to bath and pipette solutions. Nifedipine was used to prevent action potentials and opening of Ca^{2+}-dependent K^+ channels. Control experiments with nifedipine-free pipette solution showed that switching from a nifedipine-free bath solution containing 3 mM D-glucose to one containing 3 mM D-glucose + 10 μM nifedipine did not significantly alter K_{ATP}-channel activity in cell-attached patches ($n = 5$).

The pH of all solutions was determined after adding D-glucose, nifedipine, tolbutamide, meglitinide, compound III, compound IV or any nucleotides and was readjusted if necessary. Tolbutamide, meglitinide, compound III and compound IV were completely dissolved at all applied concentrations.

Synthesis of compound IV

Synthesis of 2-(5-chloro-2-methoxybenzamido)-ethylbenzene To 18.66 g (0.1 mol) of 5-chloro-2-methoxybenzoic acid was added, while stirring, 72.76 ml (1 mol) of thionyl chloride. After heating for 1 h under reflux, the excess thionyl chloride was removed by vacuum distillation. The resulting 5-chloro-2-methoxybenzoyl chloride was added slowly, while stirring and cooling with ice, to 100 ml dichloromethane containing 12.56 ml (0.1 mol) 2-phenylethylamine and 13.21 ml (0.1 mol) collidine. After stirring for 30 min at room temperature, most of the solvent was removed by vacuum distillation. The remaining reaction mixture was poured into 100 ml icewater (acidified with HCl). The aqueous phase was extracted three times with 50 ml diethyl ether, and the combined ether extracts were washed sequentially with 1 M HCl, 1 M NaOH and water. After drying over anhydrous sodium sulphate and removal of the diethyl ether *in vacuo*, a pale yellow oil (26.6 g) was obtained.

Synthesis of 4-[2-(5-chloro-2-methoxybenzamido)-ethyl]-benzenesulphonic acid (compound IV) To 5.87 g (0.02 mol) of 2-(5-chloro-2-methoxybenzamido)-ethylbenzene, 1.4 ml (0.02 mol) of chlorosulphonic acid was added dropwise at 90°C while stirring. After stirring for 2 h at 90°C and then cooling to room temperature, the reaction mixture was dissolved in a mixture of 80 ml water and 50 ml diethyl ether. The aqueous

phase was washed three times with 50 ml diethyl ether, neutralized with 2 N NaOH and concentrated *in vacuo* to approximately 5 ml. The residue was purified by column chromatography on silica gel (Kieselgel 60, Merck, Darmstadt, Germany). The column was eluted with a mixture of chloroform, methanol and water (13:8:2). After evaporation to dryness, a pale yellow solid (1.90 g) was obtained. The solid was recrystallized from water.

The structure of compound IV shown in Figure 1 was confirmed by ^{1}H-n.m.r. and ^{13}C-n.m.r. (D_6-DMSO, 200 MHz) on a model VXR-200 spectrometer (Varian, Palo Alto, CA, U.S.A.) and IR spectroscopy on a model 297 spectrometer (Perkin-Elmer Corp., Norwalk, CT, U.S.A.), supported by positive-ion fast atom bombardment mass spectrometry on a Finnigan MAT 95 spectrometer.

Elementary analysis: calculated for $C_{16}H_{15}ClNNaO_5S$: C,49.05; H,3.86; Na,5.87. Found: C,49.18; H,3.95; Na,5.93.

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 and Whitney, $P < 0.05$ was considered significant. Relations between drug concentration and effects, specific binding or free drug concentration were analysed by fitting the function

$$(1)\quad E = \frac{a-b}{1 + (EC_{50}/[A])^n} + b$$

to the experimental data by a non-linear least-squares routine where E = effect, specific binding or free drug concentration, EC_{50} = half-maximally effective drug concentration, [A] = drug concentration, n = slope parameter (Hill coefficient), a = maximum value for effect, specific binding or free drug concentration and b = additive constant.

Results

Membrane binding studies

Competitive inhibition assays revealed that compound III and compound IV inhibited specific [^{3}H]-glibenclamide binding to HIT-cell microsomes half-maximally at 4.4 μM (Hill coefficient = − 0.93) and 18.6 μM (Hill coefficient = − 0.92), respectively (Figure 2). These half-maximally inhibitory concentrations, 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 compounds III and IV (1.9 and 7.9 μM, respectively) (Cheng & Prusoff, 1973).

Patch-clamp studies

Figure 3a shows the typical design of our inside-out patch experiments. In order to slow the run-down of channel activity (Ohno-Shosaku *et al.*, 1987), the cytoplasmic face of the membrane patch was exposed for 45 s periods to an intracellular solution containing 0.7 mM free Mg^{2+} and 1 mM ATP, alternating with 15 s periods serving as test or control periods. The example in Figure 3a demonstrates that 1 mM ADP inhibited the K_{ATP}-channel activity in the presence of Mg^{2+} by 32%. Intracellular solution supplemented with Mg^{2+} and ADP contains both the Mg complex of ADP (MgADP) which stimulates the K_{ATP}-channels and free ADP which inhibits the K_{ATP}-channels, but less effectively than free ATP (Ashcroft & Rorsman, 1991). Further addition of 1 μM of compound IV decreased the channel activity to 45% of the activity in control solution (nucleotide- and drug-free). In the presence of Mg^{2+} and 1 mM ADP, compound III (10 μM) and compound IV (100 μM) induced complete block of the K_{ATP}-channels; compound III and compound IV were half-maximally effective at 0.2 μM (Hill coefficient = − 1.20) and 0.8 μM (Hill coefficient = − 1.14), respectively (Figure 3b). However, in the presence of Mg^{2+}, but in the absence of nucleotides, 100 μM concentrations of compound III or compound IV caused only reduction of channel activity to 41 or 33% of the control value (absence of nucleotides and drugs), respectively (Figure 3b). Even 1–3 mM concentrations of compound IV did not lead to closure of all K_{ATP}-channels. As the concentration-response curve showed a turning point at 100 μM compound IV, the additional channel inhibition induced by 1–3 mM compound IV was probably not due to actions at the specific sulphonylurea receptor sites. When curve fitting was restricted to the range of drug concentrations which presumably induced specific effects (up to 100 μM), compound III and compound IV were half-maximally effective in the absence of nucleotides at 0.1 μM (Hill coefficient = − 0.75) and 0.4 μM (Hill coefficient = − 0.85), respectively (Figure 3b).

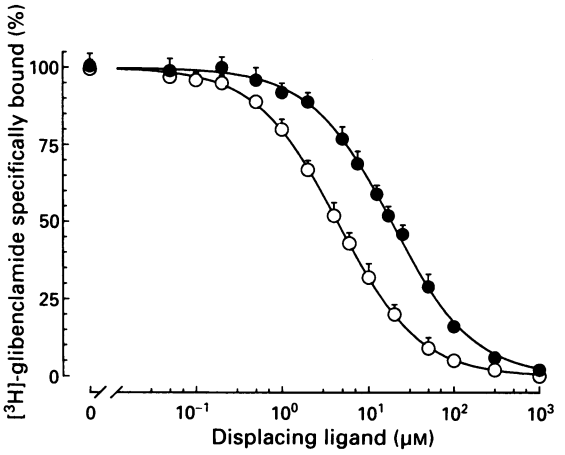

Figure 2 Effects of compounds III and IV on binding of [^{3}H]-glibenclamide to microsomes from HIT-cells. Inhibition of [^{3}H]-glibenclamide binding by compound III (○) or compound IV (●) was measured at room temperature in 1 ml of buffer containing (final concentrations) 0.3 nM [^{3}H]-glibenclamide, 1 mM free Mg^{2+} and the indicated concentrations of inhibiting drug (logarithmic scale). Incubations were started by addition of microsomal protein (200 μg ml^{-1} final concentration) and were terminated after 60 min by filtration. Nonspecific binding was defined by incubations in the presence of 100 nM glibenclamide. Results are presented as percentages of specific binding of [^{3}H]-glibenclamide in the absence of inhibiting drugs. Data analysis was performed as described in the Methods section. Values are given as means (with s.e.mean shown when larger than symbols) for results from four separate experiments using separate membrane preparations.

Using the experimental design shown in Figure 3, the K_{ATP}-channel-inhibiting effect of meglitinide (0.01–10 μM) applied to the cytoplasmic membrane side was examined in the presence of 1 mM ADP at the cytoplasmic membrane side. Meglitinide caused complete channel inhibition at 10 μM and was half-maximally effective at 0.5 μM (Hill coefficient = − 0.97; n = 3–4; data not shown in a figure).

Figure 4a shows the typical design of our outside-out patch experiments with drug application at the extracellular membrane side. Tolbutamide (100 μM) strongly inhibited the K_{ATP}-channel activity. Within 1.5 min after starting the exchange of the bath solution a new steady state was reached. The mean K^+ current measured 4 min after switching to tolbutamide (100 μM) was 3.4% of the mean K^+ current during the control periods (measurements started 1 min before switching to tolbutamide and 4 min after switching back to drug-free solution). Compound IV (100 μM) weakly inhibited the K_{ATP}-channel activity. Within 3 min after switching to compound IV (100 μM) a new steady state was reached. The mean K^+ current measured 5 min after switching to compound IV (100 μM) was 70% of the mean K^+ current during the control periods (measurements started 1 min before switching to compound IV and 4 min after

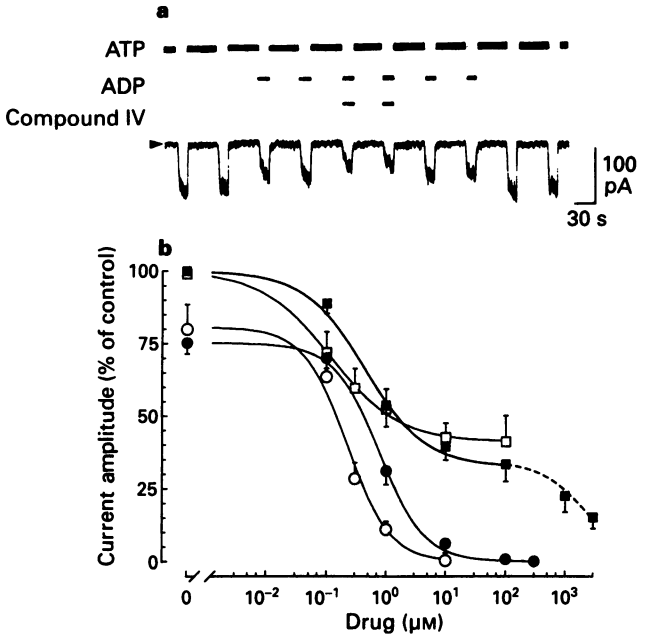

Figure 3 Inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells by compounds III and IV applied to the cytoplasmic membrane side. (a) Current trace obtained from an inside-out patch. The uppermost horizontal bars indicate application of intracellular solution containing 0.7 mM free Mg^{2+} and 1 mM ATP for 45 s by the capillary of the microflow system. The other horizontal bars above the current trace indicate application of intracellular solution containing 0.7 mM free Mg^{2+} and 1 mM ADP (with or without 1 μM compound IV) for 15 s by the bath. The 15 s periods of application of nucleotide- and drug-free intracellular solution (with 0.7 mM free Mg^{2+}) by the bath represent the control periods. (b) Relationship between normalized current amplitude and concentration (logarithmic scale) of compound III (open symbols) or compound IV (solid symbols) during 15 s periods of exposure to intracellular solution (with 0.7 mM free Mg^{2+}) containing no nucleotide (squares) or 1 mM ADP (circles). Using the experimental design shown in (a), the mean current amplitude during drug application was normalized to the mean current amplitude during 15 s periods of exposure to control solution (intracellular solution with 0.7 mM free Mg^{2+}) before and after application of drug or ADP alone in each single experiment. Data analysis was performed as described in the Methods section, except that the dashed line was fitted by eye. Symbols indicate the mean of 4–10 experiments (with s.e.mean shown when larger than symbols).

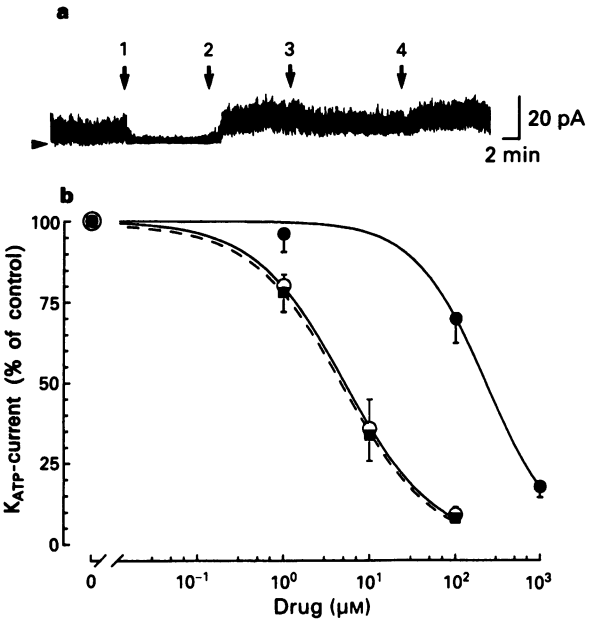

Figure 4 Inhibition of K_{ATP}-channels in outside-out patches of mouse pancreatic β-cells by tolbutamide and compounds III and IV applied to the extracellular membrane side. (a) Continuous chart recorder trace demonstrating the responses of outward K_{ATP}-currents in an outside-out patch when switching from control solution (drug-free bath solution) to a bath solution with 100 μM tolbutamide (arrow 1) and then back to control solution (arrow 2), followed by switching to a bath solution with 100 μM compound IV (arrow 3) and then back to control solution (arrow 4). (b) Relationship between normalized K_{ATP}-current and external concentration (logarithmic scale) of tolbutamide (■—■) compound III (○) or compound IV (●). Using the experimental design shown in (a), the K_{ATP}-current during drug application was normalized to the mean of the K_{ATP}-currents during the control periods before and after drug application in each single experiment. Data analysis was performed as described in the Methods section. Symbols indicate the mean of 3–12 experiments (with s.e.mean shown when larger than symbols).

switching back to drug-free solution). Single-channel current amplitudes ranged between 1.75 and 1.85 pA in the absence of drugs, in the presence of tolbutamide (100 μM) and in the presence of compound IV (100 μM). Tolbutamide, compound III and compound IV were half-maximally effective at 4.8 μM (Hill coefficient = − 0.82), 5.1 μM (Hill coefficient = − 0.83) and 225 μM (Hill coefficient = − 1.02), respectively (Figure 4b).

Figure 5a shows the typical design of our cell-attached patch experiments carried out in the presence of 3 mM D-glucose. On changing to a bath solution containing 30 μM of compound IV (test solution), the K_{ATP}-channel activity slowly decreased from 0.14 (last 1 min interval of the control period before start of bath exchange) to 0.05 during the new steady state (last 1 min interval of the test period). After switching back to drug-free bath solution, it took 12 min until a steady state was reached again (channel activity = 0.15). Single channel current amplitudes were − 4.5 pA during the test period and − 4.7 pA during the control periods before and after the test period, respectively. Compound III and compound IV were half-maximally effective at 0.6 μM (Hill coefficient = − 1.44) and 34 μM (Hill coefficient = − 1.46), respectively (Figure 5b).

Insulin secretion studies

In perifused mouse islets compounds III and IV stimulated a steep increase in insulin release reaching a peak rate at 2.5–3.5 min (compound III) or 2.5–5.5 min (compound IV), then declining rapidly within the next few min and slowly or not at all from 18 to 58 min (Figure 6). This secretory pattern was seen in each single experiment with the indicated concentrations of compounds III and IV. At 58 min, 0.1 mM of compound III and 3 mM of compound IV produced secretory rates which did not differ significantly.

The relationships between total and free drug concentrations in the perifusion media (Figure 7) and the secretory rates shown in Figure 6 were used to establish concentration-response curves for free compound III and IV (Figure 8). Half-maximally effective concentrations of free compound III and IV were 3.5 μM (Hill coefficient = 0.90) and 141 μM (Hill coefficient = 0.76) at min 2.5, respectively, (analysis not shown) and 1.3 μM (Hill coefficient = 0.90) and 68.2 μM (Hill coefficient = 0.76) at min 58, respectively (Figure 8).

Lipophilicity of compounds III and IV

To characterize the lipophilicity of compounds III and IV, the partition of the compounds between octanol and an aqueous phase was measured at pH 7.4. Apparent partition coefficients of 0.6 (compound III) and 0.4 (compound IV) were observed (means of results from two separate experiments).

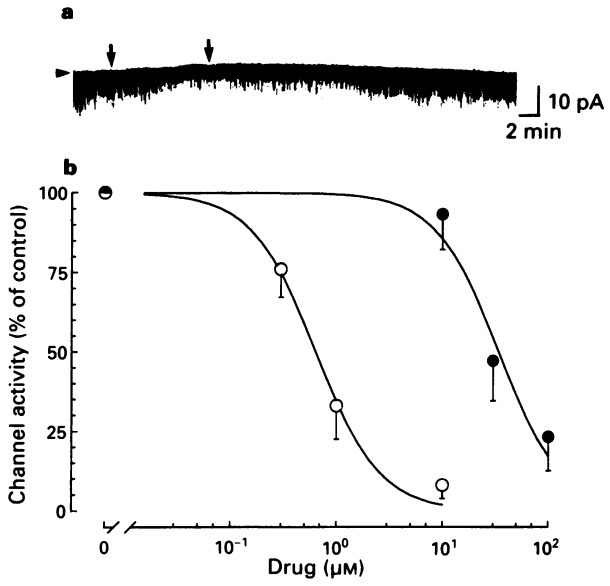

Figure 5 Inhibition of K_{ATP}-channels in cell-attached patches of mouse pancreatic β-cells by compounds III and IV. (a) Continuous chart recorder trace demonstrating the responses of single K_{ATP}-channel currents in a cell-attached patch when switching from control solution (drug-free bath solution) to a solution with 30 μM compound IV (first arrow) and then back to control solution (second arrow). The pipette solution and all bath solutions were supplemented with 3 mM D-glucose and 10 μM nifedipine. The arrowhead indicates the zero current level (all channels closed). (b) Relationship between normalized channel activity and concentration (logarithmic scale) of compound III (○) or compound IV (●). Using the experimental design shown in (a), channel activity during drug application was normalized to the mean channel activity during the control periods before and after drug application in each single experiment. Data analysis was performed as described in the Methods section. Symbols indicate the mean of 4–7 experiments (with s.e.mean shown when larger than symbols).

Discussion

Compound IV can be regarded as a derivative of *p*-toluenesulphonic acid. The pK_a value of compound IV has not been measured, but is probably close to that of *p*-toluenesulphonic acid (1.55; Schawartz & Ladik, 1953). Compound IV is therefore completely dissociated at pH 7.4 and barely dissolves in the lipid phase of cell membranes. The present study shows that this anionic sulphonylurea analogue binds to the sulphonylurea receptor, inhibits the K_{ATP}-channel and stimulates insulin secretion. These findings reflect interaction of compound IV with membrane-bound sulphonylurea receptors and thus argue against direct accessibility of the receptor binding sites from the lipid phase of the β-cell plasma membrane. Furthermore, the receptor binding of compound IV suggests that the anionic forms of hypoglycaemic sulphonylureas bind to the sulphonylurea receptor.

The benzoic acid derivative, meglitinide (Figure 1), represents another sulphonylurea analogue which is effective at the sulphonylurea receptor and initiates insulin release (Ashcroft & Rorsman, 1991). Brown & Foubister (1984) proposed that the COOH group of meglitinide and the SO_2NHCO group of sulphonylureas are isosteres and that these acidic groups are required for hypoglycaemic activity. The effects of compound IV also demonstrate that the urea group of sulphonylureas is not a prerequisite for interaction of these drugs with their receptor. The urea group is mainly a means of attaching a hydrophobic residue to benzenesulphonic acid with conservation of an acidic group. Unlike the urea group, the hydrophobic cyclohexyl group strongly enhances receptor binding. This is reflected in the finding that the binding affinity of compound III is not much higher than that of compound IV (Table 1), whereas the binding affinity of glibenclamide is higher by several orders of magnitude (K_D = 0.2–0.8 nM in HIT-cell microsomes; Gaines *et al.*, 1988; Schwanstecher *et al.*, 1992a).

When compound III or IV is applied to the cytoplasmic face of inside-out patches in the absence of nucleotides, the

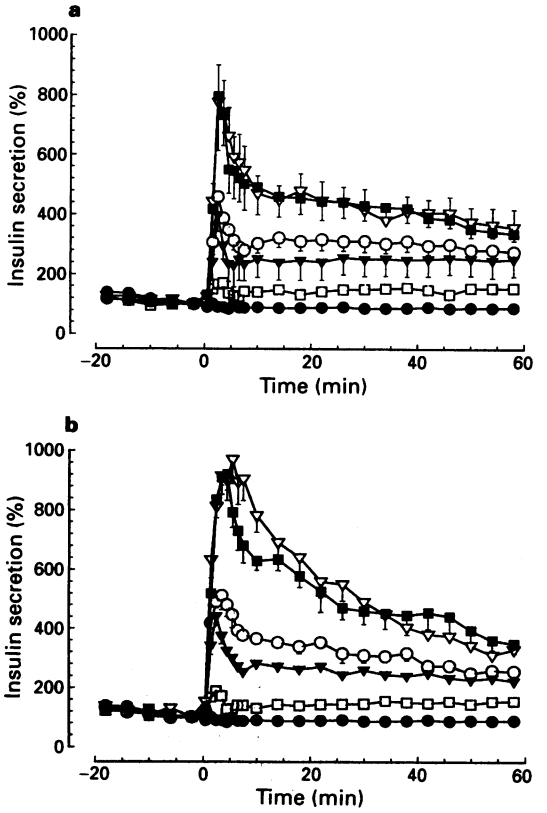

Figure 6 Concentration-dependent effects of compounds III (a) and IV (b) on the kinetic of insulin secretion from mouse pancreatic islets. All media contained 10 mM D-glucose. In addition, from zero time to min 60 the test media contained 0 (●), 1 (□), 3 (▼), 10 (○), 30 (■) or 100 (▽) μM of compound III or 0 (●), 30 (□), 100 (▼), 300 (○), 1000 (■) or 3000 (▽) μM of compound IV. Symbols indicate the mean of results from 4–8 separate experiments (with s.e.mean shown when larger than symbols).

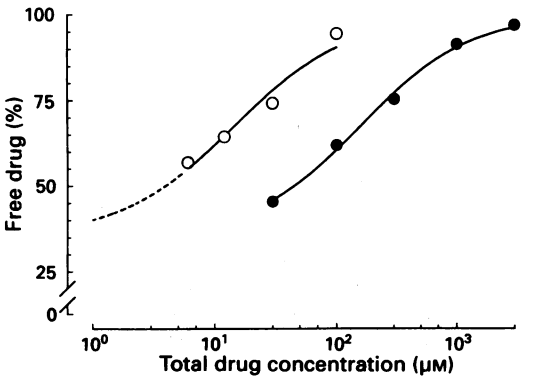

Figure 7 Binding of compounds III (○) and IV (●) to bovine serum albumin in basal perifusion medium (modified Krebs bicarbonate buffer containing 20 mM bicarbonate, 10 mM HEPES and 2 mg ml^{-1} albumin, pH adjusted to 7.4; Panten *et al.*, 1989). The percentages of free drug at the indicated total drug concentrations (logarithmic scale) were measured at 37°C. Symbols indicate the mean of 2–3 separate experiments. Data analysis was performed as described in the Methods section and the calculated constants were used to extrapolate the curve for compound III (------) into the range of concentrations which could not be measured.

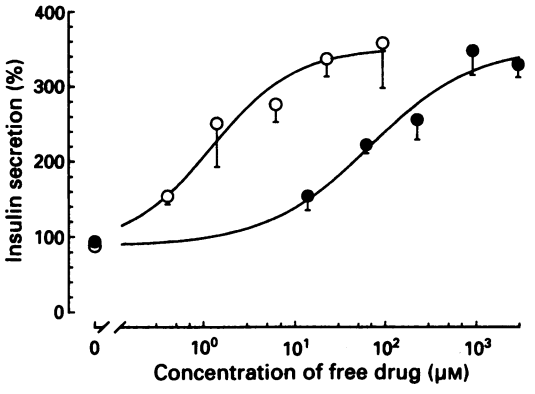

Figure 8 Dependence of insulin secretion from mouse pancreatic islets on the free concentrations (logarithmic scale) of compounds III (○) and IV (●). The secretory rates at 58 min were taken from the experiments shown in Figure 6. The free drug concentrations were calculated using the relations shown in Figure 7. Data analysis was performed as described in the Methods section. Symbols indicate the mean of results from 4–8 separate experiments (with s.e.mean shown when larger than symbols).

effects of millimolar drug concentrations probably result from occupation of both the specific sulphonylurea receptor sites and low affinity binding sites (Figure 3). Occupation of all specific sites by compound III or IV appears to cause only partial inhibition of the K_{ATP}-channels in the absence of nucleotides, but complete channel-block in the presence of Mg^{2+} and 1 mM total ADP (0.5 mM MgADP + 0.5 mM free ADP) (Figure 3). A similar ADP effect has previously been observed for tolbutamide and meglitinide and is due to the simultaneous presence of MgADP which stimulates the channel and free ADP which inhibits the channel (Zünkler *et al.*, 1988b; Panten *et al.*, 1990; Schwanstecher *et al.*, 1992d). Binding of sulphonylureas to their specific receptor increases the potency of inhibitory nucleotides and shifts the competitive interaction between stimulatory and inhibitory nucleotides towards the inhibitory nucleotides (Schwanstecher *et al.*, 1994). The K_D values for binding of tolbutamide, meglitinide, compound III and compound IV to microsomal membranes are 3 to 10 fold higher than the corresponding EC_{50} values for channel inhibition in inside-out patches exposed to ADP (Table 1). This finding is consistent with the previous conclusion that spare receptors for sulphonylureas may exist in the β-cell membrane (Panten *et al.*, 1989). All of these findings indicate that compounds III and IV control the K_{ATP}-channel via the specific sulphonylurea receptor in the same way as more lipophilic sulphonylureas and analogues.

In the outside-out patch experiments of the present study channel run-down was reduced by including 0.09 mM Mg-ATP (0.1 mM total ATP) in the cytosol-like pipette solution. In the inside-out patch experiments, channel run-down was reduced by 0.9 mM MgATP (1 mM total ATP) present in the cytosol-like bath solution except for 15 s test periods. In addition, the cytoplasmic membrane side was exposed to MgADP (0.5 mM) and free ADP (0.5 mM) when comparing the K_{ATP}-channel-inhibiting potencies of drugs applied to the extracellular side of outside-out membrane patches with the potencies of drugs applied to the intracellular side of inside-out membrane patches. Under these conditions the ratio of extracellular to intracellular EC_{50} value was 281 for compound IV, 25.5 for compound III and 1.2 for tolbutamide (Table 1). This finding can be explained by location of the sulphonylurea receptor at the cytoplasmic face of the β-cell plasma membrane and the additional assumption that the externally applied drugs are transported across the plasma membrane at rates increasing in the order compound IV < compound III < tolbutamide. In our outside-out patch experiments the rate of membrane transport controls the drug concentration effective at the internal membrane side, since the drug molecules diffuse rapidly into the recording pipette which represents an effectively infinite volume (Penner *et al.*, 1987; Pusch & Neher, 1988). Compound IV which is only present in charged form does not cross the β-cell membrane by rapid diffusion through the lipid phase of the membrane. There is some partition of compound IV into the octanol phase which is saturated with buffer (Table 1). This partition does not indicate similar affinity of compound IV for the lipid phase of plasma membranes but probably reflects that the partitioning process into octanol is an imperfect model for drug distribution between buffer and natural membranes. Carriers which remain to be characterized for β-cells might mediate the membrane transport of compound IV and the anionic forms of sulphonylureas. These carriers might resemble the transporters for the anionic forms of sulphonylureas detected in the plasma membrane of hepatocytes (Petzinger & Fückel, 1992). Whatever is the system transporting compound IV, its capacity seems to be too low to compete efficiently with the diffusion of compound IV into the pipette. However, a high octanol-water partition coefficient for undissociated tolbutamide (350; Panten *et al.*, 1989) is calculated from the pK_a value and the apparent partition coefficient (P) for tolbutamide suggesting that nonionic diffusion of the undissociated species mediates rapid membrane transport of tolbutamide. Therefore, tolbutamide applied to the external side of outside-out membrane patches appears to cross the β-cell membrane so rapidly that its rate of diffusion into the pipette does not cause a significant decrease in the tolbutamide concentration at the internal membrane side. Hence, similar tolbutamide concent-

Table 1 pK_a values, partition coefficients, binding affinities and β-cytotropic potencies of tolbutamide and glibenclamide analogues

	Tolbutamide	*Meglitinide*	*Compound III*	*Compound IV*
pK_a	5.3[a]	4.4[a]	5.3[a]	1.6[b]
P (octanol-water)	2.9[a]	2.7[a]	0.6	0.4
K_D (binding)	11.9[c]	4.0[a]	1.9	7.9
EC_{50} (inside-out)	4.0[d]	0.5	0.2	0.8
EC_{50} (outside-out)	4.8	ND	5.1	225.0
EC_{50} (whole-cell)	4.1[e]	0.5[e]	ND	ND
EC_{50} (cell-attached)	2.2[f]	ND	0.6	34.0
EC_{50} (secretion)	5.2[a]	1.6[a]	1.3	68.2

The pK_a values for compound III and IV are assumed to be similar to the pK_a values for tolbutamide and *p*-toluenesulphonic acid, respectively. Apparent octanol-water partition coefficients (P) were measured at pH 7.4. The pK_a values, the K_D values for binding to microsomes from insulin-secreting cells, the EC_{50} values for inhibition of K_{ATP}-channels in mouse β-cells (patch-clamp configuration in parentheses) and the EC_{50} values for insulin secretion in mouse pancreatic islets were taken from the Results section or were previously published ([a]Panten *et al.*, 1989; [b]Schawartz & Ladik, 1953; [c]Schwanstecher *et al.*, 1992e; [d]Schwanstecher *et al.*, 1992c; [e]Zünkler *et al.*, 1988a; [f]Panten *et al.*, 1990). The EC_{50} values for drug application to the cytoplasmic membrane side (inside-out configuration) or to the extracellular membrane side (outside-out configuration) were determined in the presence of 1 mM ADP at the cytoplasmic membrane side. K_D and EC_{50} values are given as μM of free drug. ND, not determined.

rations are probably present at the external and internal membrane side. Unlike the apparent partition coefficient (P) for compound IV, the coefficient for compound III reflects not only distribution of the charged form but also distribution of the undissociated form. Since undissociated compound III crosses the cell membrane via non-ionic diffusion, the rate of membrane transport of compound III is probably higher than that of compound IV. In comparison with undissociated tolbutamide, the undissociated form of compound III is much less lipophilic (see P values in Table 1) and its rate of non-ionic diffusion across the cell membrane is therefore much slower. Hence, in excised membrane patches no major deviation of extracellular from intracellular EC_{50} value is expected for tolbutamide whereas pronounced deviations are expected for compound III and in particular for compound IV.

Findings with meglitinide are also in accord with a location of the sulphonylurea receptor at the cytoplasmic membrane side (Table 1). Rapid membrane transport of meglitinide is suggested by the very high partition coefficient of its undissociated form (2500; Panten *et al.*, 1989) and can explain that the EC_{50} value for meglitinide externally applied in whole-cell clamp experiments is identical with the EC_{50} value for meglitinide applied to the cytoplasmic membrane side in inside-out experiments (Table 1). In the whole-cell configuration of the patch-clamp technique small molecules (M_r < 1000) diffuse rapidly from the intracellular space into the recording pipette (Penner *et al.*, 1987; Pusch & Neher, 1988).

Owing to the inside negative membrane potential, passive anion distribution across the plasma membrane leads to free anion concentrations lower at the internal than at the external membrane side of intact cells (Murer & Burckhardt, 1983). In accordance with such a transmembrane concentration gradient and location of the sulphonylurea receptor at the cytoplasmic face of the β-cell plasma membrane, cell-attached patch experiments and recordings of insulin release displayed much higher EC_{50} values for the anionic compound IV than inside-out patch experiments (Table 1). However, much less pronounced differences in EC_{50} values were observed for compound III and particularly for meglitinide and tolbutamide. When an organic acid crosses the plasma membrane of intact cells solely by diffusion of its nonionic undissociated form and not by transport of its anionic form, no transmembrane concentration gradient results for the organic acid. Thus the findings are consistent with the view that nonionic diffusion plays a major role in the transport of compound III and nearly completely controls the flux of meglitinide and tolbutamide.

In conclusion, there is strong evidence for location of the sulphonylurea receptor at the cytoplasmic face of the β-cell plasma membrane and for receptor activation by the anionic forms of sulphonylureas and their analogues.

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