

Differential interaction of glimepiride and glibenclamide with the β -cell sulfonylurea receptor

II. Photoaffinity labeling of a 65 kDa protein by [3 H]glimepiride

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Abstract

Glimepiride is a novel sulfonylurea for the treatment of type II-diabetic patients exhibiting different receptor binding kinetics to β -cell membranes with 8–9-fold higher k_{off} rate and 2.5–3-fold higher k_{on} rate compared to glibenclamide (see accompanying paper (Müller, G. et al. (1994) *Biochim. Biophys. Acta* 1191, 267–277)). To elucidate the molecular basis for this differential behaviour of glimepiride and glibenclamide, direct photoaffinity labeling studies using β -cell tumor membranes were performed. [3 H]Glimepiride was specifically incorporated into a membrane polypeptide of $M_r = 65\,000$ under conditions, which led to predominant labeling of a 140 kDa protein by [3 H]glibenclamide (Kramer, W. et al. (1988) *FEBS Lett.* 229, 355–359). Labeling of the 140 kDa protein by [3 H]glibenclamide was inhibited by unlabeled glimepiride and, vice versa, glibenclamide inhibited labeling of the 65 kDa protein by [3 H]glimepiride. The 65 kDa protein was also specifically photolabeled by the sulfonylurea [125 I]35623, whereas an 4-azidobenzoyl derivative of glibenclamide, N_3 -[3 H]33055, exclusively labeled a 33 kDa protein. Competitive Scatchard analysis of [3 H]glimepiride-binding and [3 H]glibenclamide-binding to RINm5F cell membranes using glibenclamide and glimepiride, respectively, as heterologous displacing compounds yielded non-linear plots. These findings may be explained by cooperative interactions between the 140 and 65 kDa sulfonylurea-binding proteins. The possibility that sulfonylureas of different structure have different access to the 140 and 65 kDa receptor proteins due to the β -cell membrane barrier was investigated by photoaffinity labeling of solubilized β -cell membrane proteins. Interestingly, solubilization of β -cell tumor membranes led to a shift of specific [3 H]glibenclamide binding from the 140 kDa to the 65 kDa binding protein, exclusively, and to an increased labeling of the 65 kDa protein by [3 H]glimepiride. The labeling of a unique protein is in agreement with similar K_d values measured for both sulfonylureas upon solubilization of β -cell tumor and RINm5F cell membranes (see accompanying paper). Furthermore, competitive Scatchard plots of [3 H]glimepiride binding to solubilized RINm5F cell membrane proteins in the presence of glibenclamide and vice versa approximate linearity suggesting loss of cooperativity between the 140 kDa glibenclamide-binding and 65 kDa glimepiride-binding proteins upon solubilization. The physiological significance of the differential interaction of glimepiride and glibenclamide with different binding proteins was also substantiated by photoaffinity labeling of RINm5F cells leading to labeling of a 140 kDa protein by [3 H]glibenclamide and of a 65 kDa protein by [3 H]glimepiride. In conclusion, this report presents the first evidence that different sulfonylurea drugs bind to different components of the sulfonylurea receptor complex which are characterized by different accessibility for the drugs.

Key words: Sulfonylurea receptor; Insulin secretion; Photoaffinity labeling; Glimepiride; Glibenclamide; ATP-regulated K^+ -channel; Potassium ion channel; Beta cell

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Abbreviations: RINm5F cells, rat insulinoma cells; N_3 -[3 H]33055, N -[4-(β -2-(4-azido[3,5- 3 H]benzoyl)aminobenzamidoethyl)benzene-sulfonyl]- N' -cyclohexylurea; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

1. Introduction

Using direct photoaffinity labeling of β -cell tumor membranes (Kramer et al. [1]), HIT T15 cell membranes (Aguilar-Bryan et al. [2,3]) and RINm5F cell

membranes (De Weille et al. [4]) with ^3H - or ^{125}I -labeled glibenclamide analogues, the high-affinity sulfonylurea binding site has been attributed to a 140 kDa membrane protein (for a recent review, see Ashcroft and Ashcroft [5]). The relative ability of various sulfonylureas to displace [^3H]glibenclamide from β -cell membranes parallels their ability to block ATP-regulated K^+ -channels, to stimulate insulin release and to cause reduction of the blood sugar [6–8]. Whether the 140 kDa glibenclamide receptor protein is itself (part of) the ATP-regulated K^+ -channel or whether it is a separate (transiently or permanently) channel-associated protein, has not yet been established.

So far, no reports are available concerning the characterization of binding protein(s) for sulfonylureas of different structure. Since the novel sulfonylurea, glimepiride (Geisen [9]), exhibits a significantly higher exchange rate with the sulfonylurea receptor but a 2.5–3-fold lower binding affinity compared to glibenclamide, we investigated in the present study the molecular interaction of glimepiride with the sulfonylurea receptor by direct photoaffinity labeling with [^3H]glimepiride. In addition to [^3H]glimepiride and [^3H]glibenclamide, two additional photolabile sulfonylureas, N_3 -[^3H]33055 and [^{125}I]35623 were used to identify putative protein components of the sulfonylurea receptor of the pancreatic β -cell.

Parts of the results have been published in abstract form:

Kramer, W., Ökonomopoulos, R., Pünter, J. and Summ, H.-D. (1992) *Diabetologia* 35 (Suppl. 1), A 38;
Müller, G., Hartz, D., Pünter, J., Ökonomopoulos, R., Summ, H.-D. and Kramer, W. (1993) International Conference on ATP-sensitive K^+ -channels and sulfonylurea receptors, Houston, TX, Book of Abstracts, p. 158.

2. Materials and methods

Materials. [^3H]Glimepiride (65 Ci/mmol), [^3H]glibenclamide 33 Ci/mmol), 4- N_3 -[3,5- ^3H]33055 (50.8 Ci/mmol) and [^{125}I]35623 (72.4 Ci/mmol) were synthesized at Hoechst AG, Frankfurt, Germany. 4- N_3 -[3,5- ^3H]33055 was synthesized by reaction of *N*-[4-(β -2-aminobenzamidoethyl)benzenesulfonyl]-*N'*-cyclohexylurea with 4-azido[3,5- ^3H]benzoic acid *N*-hydroxy-succinimide ester (50.8 Ci/mmol) according to the procedure described previously (Kramer [10]). Biolute S was bought from Zinsser Analytik, Frankfurt, Germany. Chemicals for electrophoresis were provided from Serva, Heidelberg, Germany. Molecular mass marker proteins for electrophoresis were obtained from Sigma, Deisenhofen, Germany. All other reagents and materials were purchased as described in the accompanying report [11]. Rats of the strain NEDH (New

England Deaconess Hospital) were kindly donated by S. Warren.

Photoaffinity labeling. For photoaffinity labeling of albumin with [^3H]glimepiride or [^3H]glibenclamide, solutions of human serum albumin (1 mg/ml) in 100 mM sodium phosphate buffer (pH 7.4) were incubated (60 min, 20°C) with 0.25 μM [^3H]glimepiride or 0.25 μM [^3H]glibenclamide in the dark. Irradiation was performed in a Rayonet RPR-100 photochemical reactor (Southern Ultraviolet, Hamden, CT, USA), equipped with 16 RPR-2530 or RPR-3000 Å lamps at a distance of 10 cm from the lamps (according to data of the manufacturer, the RPR-2530 Å lamps emit 84% of their radiation at 2530 Å with an intensity of 12800 $\mu\text{W}/\text{cm}^2$ and $1.65 \cdot 10^{16}$ photons/s per cm^2 ; the RPR-3000 Å lamps have an emission maximum at 3000 Å with a half-bandwidth of 30 nm and a photon intensity of $4 \cdot 10^{17}$ photons/s per cm^2). After definite times aliquots were removed, adjusted to 200 μl with H_2O and protein was precipitated as published [12].

For photoaffinity labeling of β -cell membranes and solubilized β -cell membrane proteins, the 75000 $\times g$ pellet, obtained during preparation of the β -cell tumor membranes, was suspended in 100 mM sodium phosphate buffer (pH 7.4). For labeling of solubilized β -cell membrane proteins, CHAPS or Triton X-100 was added (final concn. 1% w/v). After incubation (60 min, 4°C), the supernatant obtained after centrifugation (48000 $\times g$, 30 min) was used for photoaffinity labeling. For this, 600 μg of β -cell membrane protein (membranes or solubilized membrane proteins) was incubated (60 min, 20°C) in a total volume of 200 μl with 25–60 nM (0.3–0.4 μCi) [^3H]glimepiride, [^3H]glibenclamide, N_3 -[^3H]33055 or [^{125}I]35623 in the dark. After irradiation at 254 or 300 nm for 2 min ([^3H]glimepiride, [^3H]glibenclamide, [^{125}I]35623) or for 30 s (N_3 -[^3H]33055) in the photochemical reactor described above, the samples were diluted with 1 ml of 10 mM Tris-Hepes buffer (pH 7.4), 4 mM EDTA, 4 mM iodoacetamide, 4 mM PMSF, centrifuged (48000 $\times g$, 30 min) and protein was precipitated as above. For photoaffinity labeling with monochromatic light, the samples were irradiated with narrow-bandwidth light for 10 min in a rectangular cuvette (1 $\times$ 0.1 cm) of quartz glass in a sample chamber of a Hitachi F-3000 fluorimeter (Hitachi, Tokyo, Japan) equipped with a 150 W xenon arc lamp. Wavelengths were selected with the excitation monochromator. After irradiation, 100- μl aliquots (40 μg) were removed and protein was precipitated with 500 μl of dioxane.

For photoaffinity labeling of RINm5F cells, (0.5–1) $\cdot 10^6$ cells were incubated (10 min, 20°C) with 25 nM [^3H]glimepiride (0.5 μCi) or [^3H]glibenclamide (0.5 μCi) in the dark and subsequently irradiated at 254 nm for 2 min. The cells were then washed twice with 1 ml of 10 mM sodium phosphate buffer (pH 7.4), 4 mM

PMSF, 4 mM EDTA, 4 mM iodoacetamide. After centrifugation ($48\,000 \times g$, 60 min), the pellets were resuspended in $100\ \mu\text{l}$ H_2O and proteins were precipitated and analyzed by SDS-PAGE.

SDS-PAGE. The dried protein precipitates were dissolved in $70\ \mu\text{l}$ of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 0.005% Bromophenol blue by shaking on a mixer for 60 min. After centrifugation ($15\,000 \times g$, 10 min), the supernatants were submitted to SDS-PAGE on $150 \times 180 \times 1.5$ mm slab gels as described (Kramer et al. [13,14]). After fixing and staining, the gels were scanned with a CD-50 densitometer, Desaga, Heidelberg, Germany and then scanned for radioactivity by liquid scintillation counting of 2-mm gel pieces after digestion of proteins with Biolute S.

Miscellaneous. Culture of RINm5F cells, preparation of β -cell tumor membranes and RINm5F cell membranes and Scatchard plot analysis of specific [^3H]glimepiride and [^3H]glibenclamide binding to RINm5F cell membranes or solubilized RINm5F cell membrane proteins using rapid filtration and precipitation with poly(ethylene glycol) were performed as described in the accompanying report [11].

3. Results

3.1. Competitive Scatchard plot analysis of [^3H]glimepiride and [^3H]glibenclamide binding

Competitive Scatchard plot analysis (Fig. 1) of [^3H]glimepiride (Panel A) and [^3H]glibenclamide (Panel B) equilibrium binding to RINm5F cell membranes in the presence of varying concentrations of unlabeled glibenclamide (Glib.) and glimepiride (Glim.), respectively, resulted in non-linear concavely shaped plots, which could not be attributed to distinct high- and low-affinity binding sites (Panel A, Glib.; Panel B, Glim.). The non-competitive type of binding inhibition observed for this heterologous Scatchard analysis markedly differs from the corresponding ho-

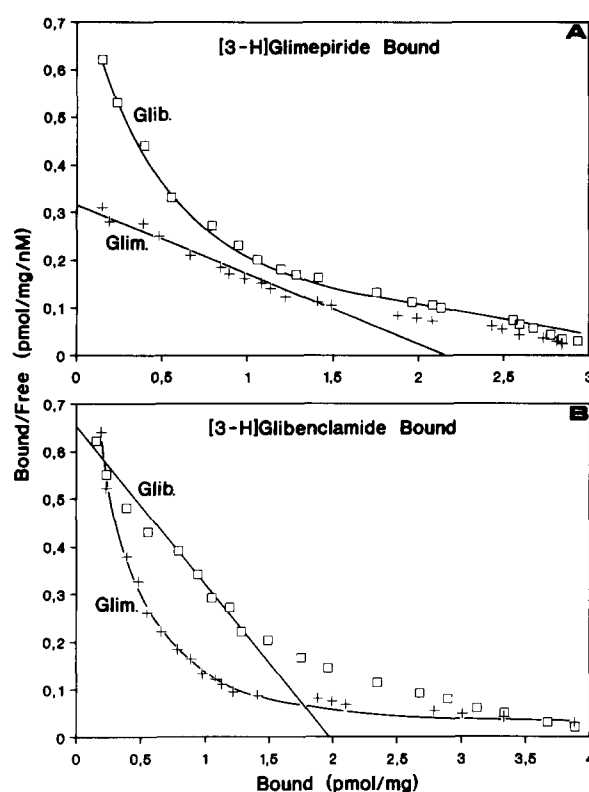


Fig. 1. Competitive Scatchard plot analysis of specific [^3H]glimepiride or [^3H]glibenclamide binding. $200\ \mu\text{g}$ of RINm5F cell membranes were incubated (60 min, 20°) with $0.5\ \text{nM}$ [^3H]glimepiride (Panel A) or [^3H]glibenclamide (Panel B) in the presence of 0.1 – $100\ \text{nM}$ unlabeled glimepiride (+) or glibenclamide ($\square$) and then rapidly filtered. Specific glimepiride-binding and glibenclamide-binding was determined and the means of at least 16 independent determinations obtained with four different membrane preparations were used for the Scatchard plot analysis.

mologous analysis (i.e., [^3H]glibenclamide and [^3H]glimepiride binding in the presence of unlabeled glibenclamide and glimepiride, respectively) (see for a comparison Fig. 1 of the accompanying paper). These findings together with the different binding parameters (K_d , k_{on} , k_{off} values) of the glimepiride and glibenclamide high-affinity binding sites (see Table 1 of the

Table 1
Effect of UV irradiation on viability of RINm5F cells

Time (min)	% Trypan blue-stained RINm5F cells								
	0	0.5	1	2	5	10	15	20	30
Wavelength									
254 nm	2	4	3	5	6	5	7	10	12
300 nm	4	5	4	7	8	7	9	12	15
control	3	3	4	4	5	4	6	8	7

RINm5F cells ($0.75 \cdot 10^6$ cells/ml) were incubated at 25°C in a Rayonet RPR-100 photochemical reactor at a distance of 10 cm from either 16 RPR-100 2530 Å or 16 RPR-3000 Å lamps or were incubated without irradiation (control). After various periods $150\ \mu\text{l}$ aliquots were removed and supplemented with $50\ \mu\text{l}$ Trypan blue solution (0.1%). The number of stained cells was determined under the phase contrast microscope within 2 min. The values calculated as % stained cells for each of the three incubation conditions are derived from one typical experiment repeated three times.

accompanying paper [11]) represent first hints to the existence of distinct binding sites of the two drugs in β -cell membranes (for a discussion of Scatchard plot and heterogeneity in binding affinity of labeled and unlabeled ligand see Refs. [15–18].

3.2. Different proteins of β -cell tumor membranes are photoaffinity labeled by [^3H]glimepiride and [^3H]glibenclamide

To obtain information for the above hypothesis at a molecular level, we performed photoaffinity labeling studies with β -cell tumor membranes using four different photoaffinity probes, [^3H]glimepiride, [^3H]glibenclamide, a 4-azido-[3,5- ^3H]benzoyl derivative of glibenclamide, N_3 -[^3H]33055, and [^{125}I]35623 (Fig. 2). The suitability of [^3H]glibenclamide as a direct photoaffinity probe upon irradiation with UV-light at 254 nm or 300 nm has been demonstrated previously (Kramer et al. [1]) and aromatic azido-compounds are well established as nitrene-generating photoaffinity probes (Bayley [19]). Owing to the α,β -unsaturated carbonyl function in the molecule, a photocatalyzed activation of glimepiride via a $n-\pi^*$ transition state seemed possible (Martyr and Benisek [20]). Therefore, at first the ability of [^3H]glimepiride to act as a direct photoaffinity probe was investigated by irradiation of human

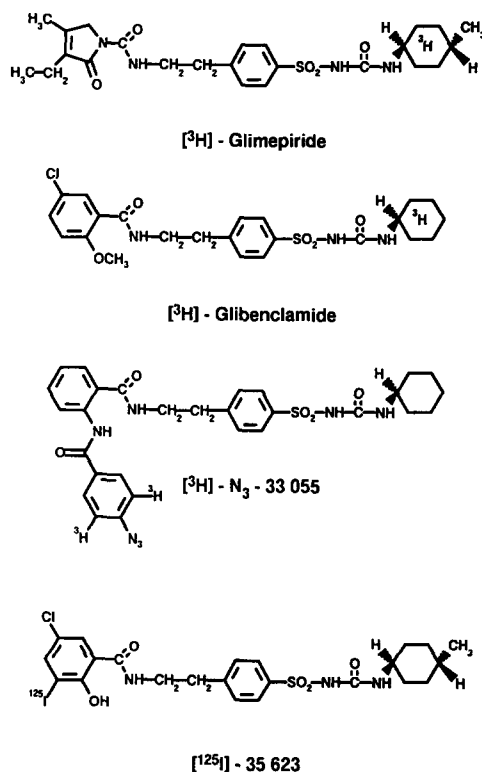


Fig. 2. Chemical structure of [^3H]glimepiride, [^3H]glibenclamide, N_3 -[^3H]33055 and [^{125}I]35623.

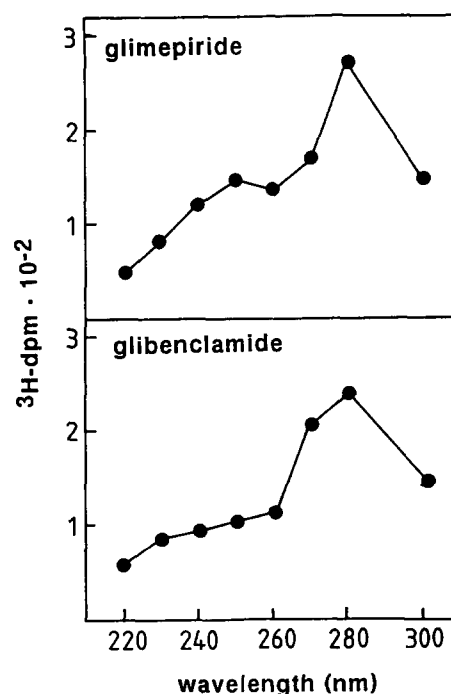


Fig. 3. Photocatalyzed incorporation of [^3H]glimepiride into human serum albumin. Human serum albumin (1 mg/ml) in 100 mM sodium phosphate buffer (pH 7.4) was incubated (10 min, 20°C) with 0.25 μM [^3H]glimepiride (8 $\mu\text{Ci}/\text{mg}$ protein) in the dark. Photoaffinity labeling was carried out at various wavelengths between 220 and 300 nm. After 3 min irradiation, aliquots containing 40 μg protein were removed and protein was precipitated. The covalent attachment of [^3H]sulfonylureas to albumin was determined by scintillation counting after SDS-PAGE and slicing of the gels.

albumin solution in the presence of [^3H]glimepiride with monochromatic light of different wavelength. Fig. 3 shows that photocatalyzed incorporation of [^3H]glimepiride into protein was achieved in the range of 220–300 nm with a maximum at 280 nm (upper panel). [^3H]Glibenclamide showed a nearly identical wavelength dependence for photoincorporation (lower panel). Thus, photoaffinity labeling experiments with β -cell membranes were carried out in a photochemical reactor RPR-100 equipped with 16 RPR-2530 Å lamps having their maximum of light emission at 254 nm. (Identical results were obtained using RPR-3000 Å lamps which have their maximum of emission at 300 nm). Photolabeling with [^3H]glimepiride, [^3H]glibenclamide and [^{125}I]35623 was carried out for 2 min at 254 nm, whereas 30 s at 254 nm was applied for N_3 -[^3H]33055, the half-life time under these conditions being 3.5 s (Kramer et al. [10]). After photoaffinity labeling of β -cell membranes, the membranes were washed and polypeptides were separated by SDS-PAGE followed by determination of radioactively labeled polypeptides after slicing of the gels into 2-mm pieces.

[^3H]Glibenclamide was predominantly incorporated into two membrane polypeptides with apparent M_r of

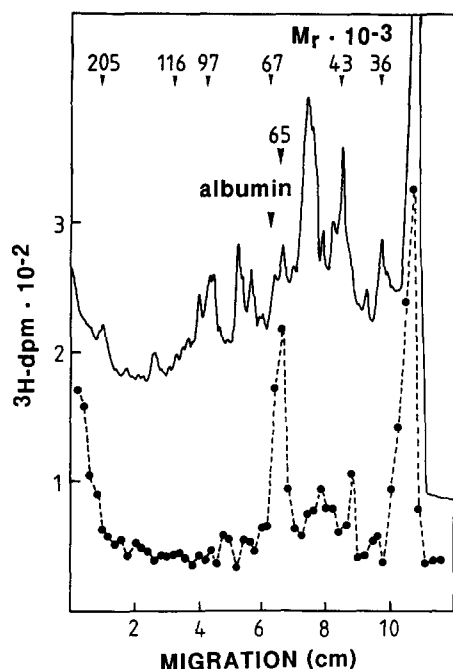


Fig. 4. Photoaffinity labeling of β -cell tumor membranes with [^3H]glimepiride. β -cell tumor membranes (600 μg protein) were incubated (60 min, 20°C) in the dark with 30.76 nM [^3H]glimepiride (0.5 μCi) and subsequently irradiated at 254 nm for 2 min. After washing of the membranes, proteins were separated by SDS-PAGE. The continuous line shows the distribution of Serva-blue R-250 stained polypeptides, the broken line denotes the distribution of ^3H radioactivity as determined by liquid scintillation counting of the sliced gels. Molecular masses (in kDa; at top of the figure) of the marker proteins are indicated by arrows.

140 000 and 33 000 (Kramer et al. [1]). In contrast, photoaffinity labeling with [^3H]glimepiride led to covalent modification predominantly of one polypeptide with an apparent M_r of 65 000, whereas no significant labeling of the 140 kDa polypeptide could be detected (Fig. 4). With the nitrene-generating N_3 -[^3H]33055 a nearly exclusive labeling of only one polypeptide of $M_r = 33$ 000 occurred (Fig. 5, upper panel). Direct photoaffinity labeling with [^{125}I]35623 led to an exclusive incorporation of the photoprobe into one polypeptide of $M_r = 65$ 000, as shown by autoradiography after SDS-PAGE (Fig. 6). The labeling of different binding proteins for [^3H]glimepiride, [^3H]glibenclamide, N_3 -[^3H]33055 and [^{125}I]35623 may reflect different binding affinities of sulfonylureas to one or the other binding site, which determines success or failure of photocrosslinking. The concentrations of the photoprobes in the labeling experiments were in the range of 25–60 nM. Qualitatively, the same labeling pattern was obtained using the probes in the concentration range of 1–10 nM, but the incorporation yields were rather low. In further control experiments, no differences in the labeling patterns of a distinct sulfonylurea photoprobe was found up to concentrations of 100 nM.

In order to elucidate whether the binding proteins for glibenclamide and glimepiride identified in β -cell membranes are also physiologically involved in binding of sulfonylureas, we performed photoaffinity labeling studies with RINm5F cells using [^3H]glibenclamide and [^3H]glimepiride. Since the wavelength necessary for photocrosslinking of these ligands may damage living cells, we measured in a first series of experiments the influence of irradiation on cell viability. Irradiation

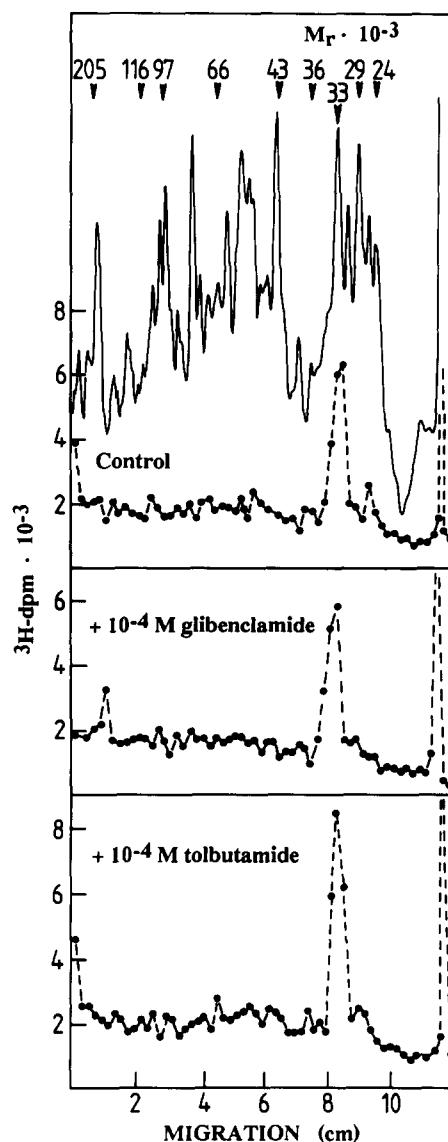


Fig. 5. Photoaffinity labeling of β -cell tumor membranes with N_3 -[^3H]33055. β -cell tumor membranes (500 μg protein) were incubated (15 min, 20°C) and subsequently (5 min, 0°C) with 51 nM N_3 -[^3H]33055 (0.65 μCi) in 100 mM sodium phosphate buffer (pH 7.4) either in the absence (upper panel) or presence of 10^{-4} M glibenclamide (middle panel) or 10^{-4} M tolbutamide (lower panel). After photolabeling at 254 nm for 30 s and washing of the membranes, proteins were separated by SDS-PAGE. The continuous line shows the distribution of Serva-blue R-250 stained polypeptides, the broken line the distribution of radioactivity.

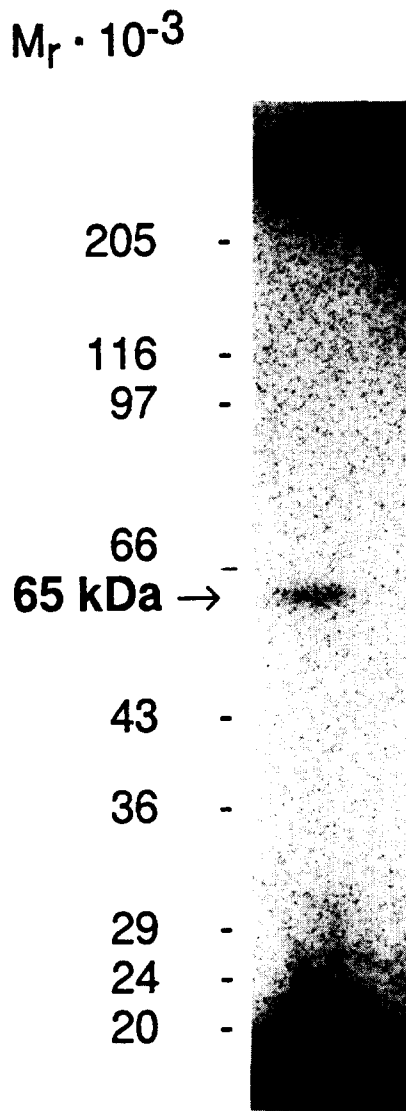


Fig. 6. Photoaffinity labeling of β -cell tumor membranes with [125 I]35623. β -cell tumor membranes (500 μ g protein) were incubated (15 min, 20°C) with 27.6 nM [125 I]35623 (0.4 μ Ci) in the dark and subsequently irradiated at 254 nm for 2 min. After washing of the membranes, proteins were separated by SDS-PAGE. An autoradiography of the dried gel is shown. The molecular masses indicated were derived from marker proteins run in parallel on the same gel.

of RINm5F cells ($10.75 \cdot 10^6$ cells/ml) was performed in a Rayonet RPR-100 photochemical reactor at a distance of 10 cm from the lamps using either 16 RPR-2530 Å or 16 RPR-3000 Å lamps with subsequent determination of cell viability by Trypan blue exclusion. Table 1 shows that within the irradiation times necessary for sufficient photoaffinity labeling with [3 H]glimepiride and [3 H]glibenclamide both at 254 nm and 300 nm, no significant impairment of RINm5F cell viability compared to control cells occurred. Consequently, RINm5F cells ($1 \cdot 10^6$ cells/ml) were incu-

bated for 10 min in the dark either with [3 H]glimepiride or [3 H]glibenclamide and subsequently irradiated at 254 nm for 2 min. After washing, cells were disrupted by osmotic shock and particulate material was collected by centrifugation. Fig. 7 shows that irradiation in the presence of [3 H]glibenclamide led to an incorporation of radioactivity into a 140 kDa protein (lower panel), whereas with [3 H]glimepiride a 65 kDa protein was labeled (upper panel). Therefore, also in intact β -cells, glimepiride and glibenclamide have access to different binding proteins which according to their

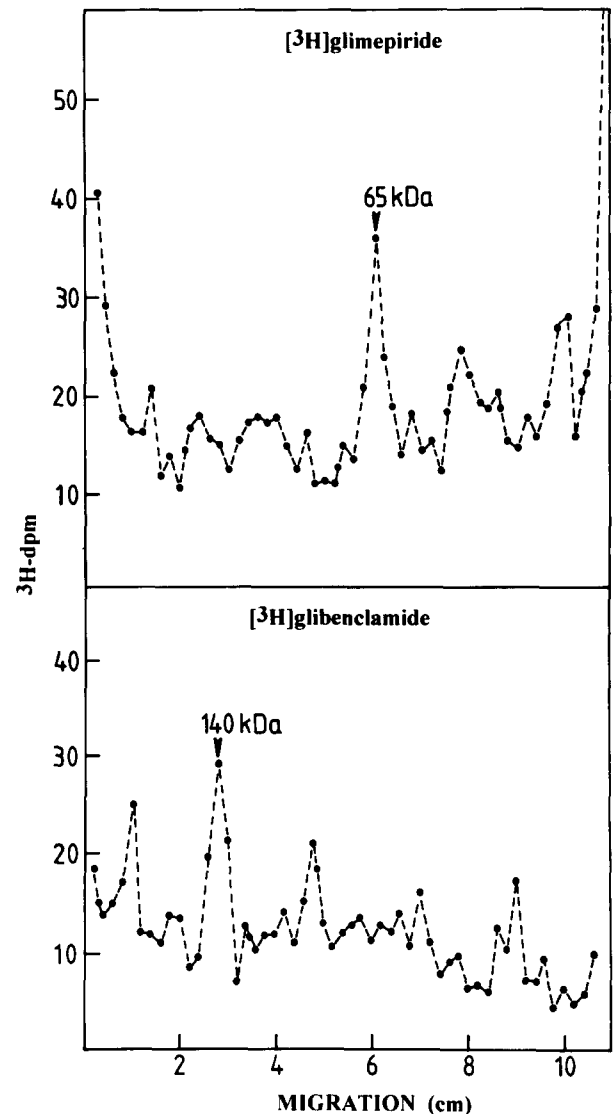


Fig. 7. Photoaffinity labeling of RINm5F cells with [3 H]glimepiride and [3 H]glibenclamide. RINm5F cells ($1 \cdot 10^6$ cells/ml) were incubated (10 min, 20°C) with 25.6 nM [3 H]glimepiride (0.5 μ Ci) or 25.4 nM [3 H]glibenclamide (0.5 μ Ci) in the dark and subsequently irradiated at 254 nm for 2 min. After washing, the particulate material was collected by centrifugation, suspended, precipitated and finally proteins were separated by SDS-PAGE. The distribution of 3 H radioactivity as determined by liquid scintillation counting of the sliced gels.

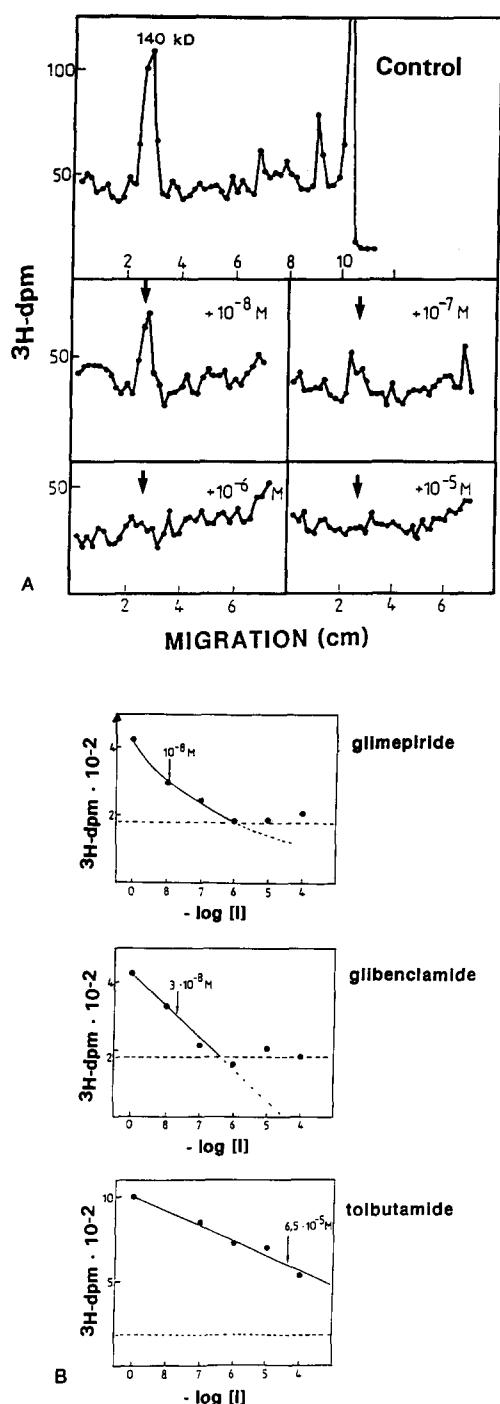


Fig. 8. Competitive photoaffinity labeling of the 140 kDa protein by glimepiride, glibenclamide and tolbutamide. β -cell tumor membranes (600 μ g protein) were incubated (60 min, 20°C) in the dark in the presence of various concentrations (10^{-8} to 10^{-4} M) of glimepiride, glibenclamide or tolbutamide. After incubation (10 min, 20°C) with 60 nM [3 H]glibenclamide (0.37 μ Ci) in the dark, the membranes were irradiated at 254 nm for 2 min. After washing of the membranes, the proteins were separated by SDS-PAGE and counted for radioactivity by liquid scintillation counting of the sliced gels (shown only for glimepiride in Panel A). The logarithmic plots of the drug concentration used for the competition versus the 3 H-radio-label bound to the 140 kDa protein are shown in Panel B. The IC_{50} values for half-maximal inhibition of [3 H]glimepiride binding are indicated by arrows.

apparent M_r may be related to the corresponding binding proteins in the β -cell tumor membrane.

The specificity of the photolabeled sulfonylurea-binding proteins was corroborated by competitive photoaffinity labeling with [3 H]glimepiride, [3 H]glibenclamide or N_3 -[3 H]33055 in the presence of excess of unlabeled sulfonylureas. Photoaffinity labeling of β -cell membranes with [3 H]glibenclamide in the presence of increasing concentrations of unlabeled glimepiride led to a concentration-dependent decrease in the labeling of the 140 kDa protein (Fig. 8, Panel A). Blotting of radioactivity incorporated into the 140 kDa protein

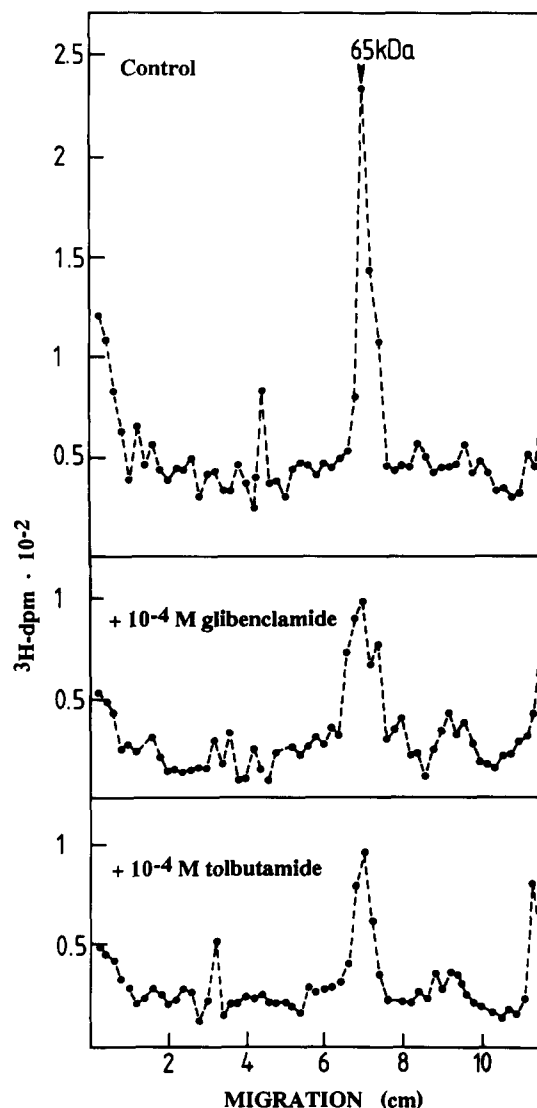


Fig. 9. Competitive photoaffinity labeling of the 65 kDa protein by glibenclamide and tolbutamide. β -cell tumor membranes (500 μ g protein) were incubated (60 min, 20°C) in the dark in the absence (upper panel) or presence of 10^{-4} M glibenclamide (middle panel) or 10^{-4} M tolbutamide (lower panel). After incubation (10 min, 20°C) with 30.76 nM [3 H]glimepiride (0.5 μ Ci) in the dark, the membranes were irradiated at 254 nm for 2 min. After washing of the membranes, proteins were separated by SDS-PAGE and counted for radioactivity by liquid scintillation counting of the sliced gels.

against the concentration of the respective sulfonylureas (Panel B) revealed an IC_{50} value of 10^{-8} M for half-maximal inhibition of photolabeling by glimepiride. Glibenclamide showed an IC_{50} value of $3 \cdot 10^{-8}$ M, whereas for tolbutamide, as expected, much higher concentrations were required ($IC_{50} = 6.5 \cdot 10^{-5}$ M). Analogous experiments for the 65 kDa protein revealed that its labeling by [3H]glimepiride was also concentration-dependently inhibited by other sulfonylureas (Fig. 9). The concentrations of sulfonylurea necessary for inhibition, however, were significantly higher than for the 140 kDa protein. Similar results were obtained using [^{125}I]35623 as photoprobe for labeling the 65 kDa protein (data not shown). The intensive labeling of the 33 kDa polypeptide by N_3 -[3H]33055 was not inhibited by unlabeled sulfonylureas like glibenclamide or tolbutamide to a significant extent (Fig. 5, middle and lower panels). (The labeling of the 33 kDa polypeptide by N_3 -[3H]33055, however, was 'specific' in the sense that photolabeling with 4-azido-[3,5- 3H]benzoic acid *N*-hydroxysuccinimide ester, a precursor in the synthetic procedure, led to unspecific labeling of a variety of membrane proteins; data not shown).

3.3. Effect of solubilization on photoaffinity labeling of the glimepiride-binding and glibenclamide-binding proteins

Since on the one hand glimepiride inhibited labeling of the 140 kDa protein by [3H]glibenclamide without an obvious direct binding to the 140 kDa protein and on the other hand glibenclamide inhibited labeling of the 65 kDa protein by [3H]glimepiride, it seemed possible that both proteins are subunit constituents of the sulfonylurea receptor complex interacting with each other in a cooperative manner. Sulfonylureas of different structure may have different access to these proteins embedded in the β -cell plasma membrane. Consequently, we examined whether photoaffinity labeling of the respective proteins is dependent on the intact structure of the membrane or resists solubilization by detergent.

Fig. 10, Panel A shows that photoaffinity labeling of the 65 kDa protein by [3H]glimepiride was greatly increased by solubilization of the membranes with Triton X-100 (upper diagram) or other non-ionic detergents such as octyl glucoside or CHAPS (data not shown) prior to UV-irradiation compared to the intact

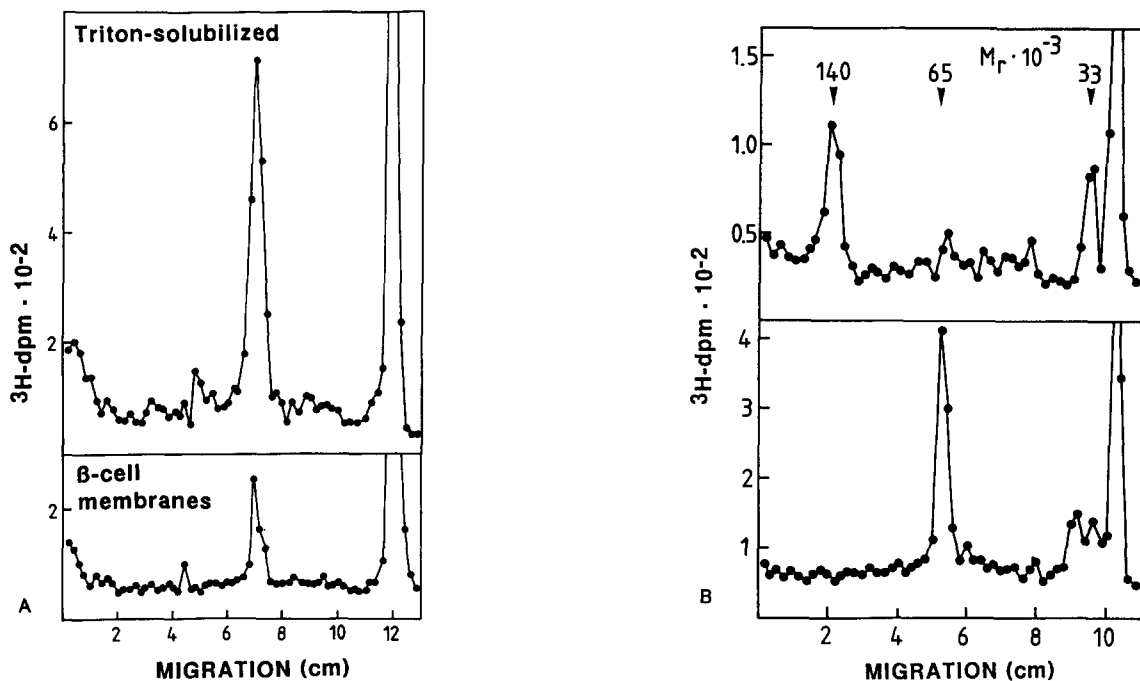


Fig. 10. Photoaffinity labeling of solubilized β -cell membrane proteins with [3H]glimepiride (Panel A) and [3H]glibenclamide (Panel B). β -cell tumor membranes and solubilized β -cell tumor membrane proteins (Panel A: 1% TX-100; Panel B: 1% CHAPS) (600 μ g protein) were incubated (60 min, 20°C) with 30.76 nM [3H]glimepiride (0.5 μ Ci) (Panel A) or 48.3 nM [3H]glibenclamide (0.3 μ Ci) (Panel B) in the dark and subsequently irradiated at 254 nm for 2 min. After washing of the membranes, proteins were separated by SDS-PAGE. The distribution of 3H radioactivity along the gel was determined by liquid scintillation counting of the sliced gels. The molecular weights (at top of the figure) are indicated by arrows.

membrane (lower diagram), and no additional proteins were radiolabeled. This indicates either an increased access of glimepiride to the 65 kDa protein or a tremendous increase of affinity of the 65 kDa protein for glimepiride upon solubilization. Similar experiments with [3 H]glibenclamide as a photoprobe showed (Panel B) that the 140 kDa protein, predominantly labeled in β -cell membranes (upper diagram), was no longer labeled by [3 H]glibenclamide after solubilization of the membrane with nonionic detergents (lower diagram). Instead a nearly exclusive incorporation of [3 H]glibenclamide into the 65 kDa protein occurred indicating a shift of glibenclamide binding from the 140 kDa protein to the 65 kDa binding protein. Competitive photolabeling experiments using structurally different ligands revealed that the solubilized 65 kDa binding protein has similar binding specificity as the 140 kDa binding protein (data not shown).

Upon solubilization of RINm5F cell membranes, the non-linear concavely shaped curve of the competitive Scatchard plots (i.e., [3 H]glimepiride binding in the presence of unlabeled glibenclamide and [3 H]glibenclamide binding in the presence of unlabeled glimepiride; see Fig. 1) were converted to a linear component of the high-affinity binding site (Fig. 11, Panel A, Glib. and Panel B, Glim., respectively). The analogous effect of solubilization was also observed for Scatchard plot analysis of [3 H]glibenclamide binding in the presence of unlabeled iodo-35623 (Fig. 12, Panel B, compare membr. and solub.). This suggests either the disappearance of low/medium affinity binding sites, which could not be resolved from the high-affinity binding sites when embedded within the membrane, or the disruption of the (negative) cooperative interaction between the glimepiride-binding and glibenclamide-binding sites. In contrast to the Scatchard analysis of [3 H]glimepiride binding to RINm5F cell membranes in the presence of glibenclamide and vice versa (Fig. 1), [3 H]glimepiride binding to RINm5F cell membranes in the presence of unlabeled iodo-35623 resulted in linear Scatchard plots (Fig. 12, Panel A, membr.). Thus, solubilization of the membranes is not required to observe competitive binding inhibition between these two drugs and, in fact, did not affect the linearity of the Scatchard plot (Panel A, solub.). Since glimepiride and iodo-35623 presumably bind to the same 65 kDa binding protein, the non-linearity of the Scatchard plots and, thus, the non-competitive nature of binding of glimepiride and glibenclamide to RINm5F cell membranes, seems to rely on binding to different receptor proteins (65 and 140 kDa, respectively) which interact with one another in an allosteric fashion. Solubilization of the β -cell membrane presumably causes dissociation of the putative sulfonylurea receptor complex into monomeric 65 kDa glimepiride-binding and 140 kDa glibenclamide-binding proteins. This might explain the shift from

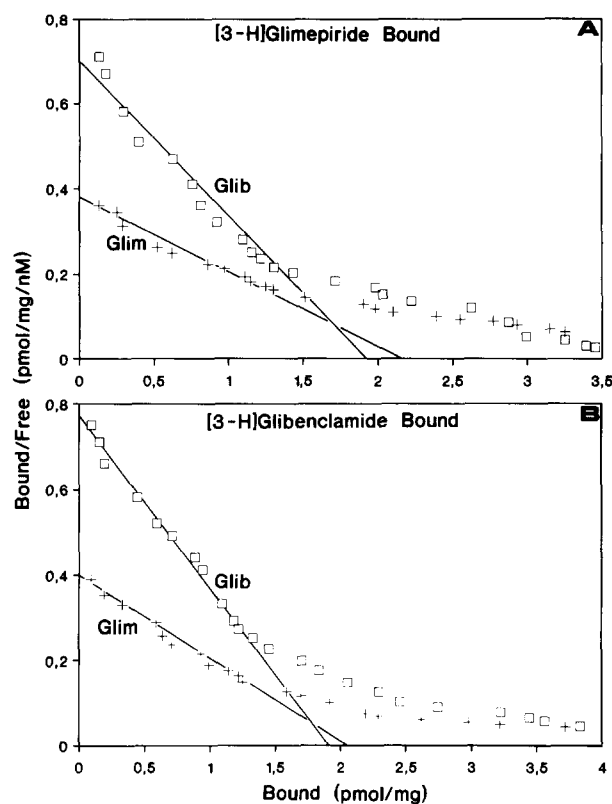


Fig. 11. Competitive Scatchard plot analysis of [3 H]glimepiride (Panel A) and [3 H]glibenclamide (Panel B) binding to solubilized RINm5F cell membrane proteins by glimepiride and glibenclamide. The experiments were carried out as described in the legend to Fig. 1 but using 200 μ g of digitonin-solubilized RINm5F cell membrane proteins and precipitation of the solubilized proteins with poly(ethylene glycol) prior to filtration.

non-competitive to competitive type of inhibition of [3 H]glimepiride-binding by glibenclamide and vice versa in Scatchard plot analysis using heterologous ligands [15–17]. The steady-state and kinetic binding experiments (see accompanying paper [11]) as well as the competitive Scatchard analyses and photoaffinity labeling studies using solubilized membranes hint to an attenuating effect of the membrane environment or of the interaction with the glibenclamide binding protein on the affinity or accessibility of the glimepiride binding protein which can be overcome by solubilization.

4. Discussion

A 140 kDa protein in β -cell tumor membranes has been demonstrated previously to be photoaffinity labeled by [3 H]glibenclamide with high specificity and selectivity (Kramer et al. [1]). With an iodinated analogue of glibenclamide, 5-iodo-2-hydroxyglibenclamide,

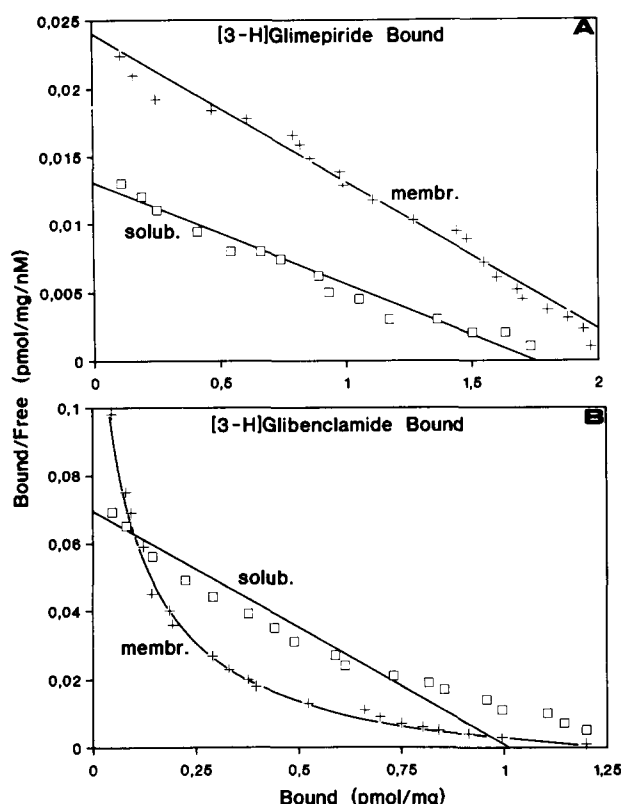


Fig. 12. Competitive Scatchard plot analysis of [^3H]glimepiride (Panel A) and [^3H]glibenclamide (Panel B) binding by iodo-35623. The experiments were carried out as described in the legends to Figs. 1 and 11 by incubation (60 min, 25°C) of 200 μg of RINm5F cell membranes (+, membr.) and digitonin-solubilized RINm5F cell membrane proteins ($\square$, solub.) in the presence of 1 nM–10 μM unlabeled iodo-35623 and then (after precipitation of the solubilized proteins with poly(ethylene glycol)) rapidly filtered. Specific glimepiride-binding and glibenclamide-binding was determined and the means of at least 10 independent determinations were used for Scatchard plot analysis shown in the figure.

four polypeptides with $M_r = 65\,000$, $55\,000$, $43\,000$ and $30\,000$ were additionally labeled in HIT T15 cells [2]. Differential photolabeling by various sulfonylureas revealed half-maximal displacements (and K_i values calculated thereof) for the 30–65 kDa proteins in the low micromolar range. Consequently, these proteins were attributed to low-affinity binding sites (Nelson et al. [21]).

Photoaffinity labeling of β -cell tumor membranes with [^3H]glimepiride led to an exclusive incorporation of the photoprobe into a 65 kDa protein under conditions where [^3H]glibenclamide labeled the 140 kDa protein. Since glimepiride was bound by β -cell tumor and RINm5F cell membranes with K_d values in the low nanomolar range (see accompanying paper [11]), and since labeling of the 65 kDa protein was inhibited dose-dependently by glibenclamide and tolbutamide,

this protein seems to be the high-affinity binding site for glimepiride.

The failure of the 65 kDa protein to become photo-labeled by [^3H]glibenclamide using β -cell membranes may have different reasons.

(i) It may be argued that the photochemistry of the 2-methoxy-5-chlorobenzene ring in glibenclamide and of the 3-ethyl-4-methyl-3-pyrrolin-2-on ring in glimepiride differs completely with respect to addition to double bonds and insertion into single bonds at the binding site of the sulfonylurea binding protein(s). However, since both compounds have a similar structure and space-filling and a nearly identical wavelength dependence for photoincorporation into albumin strongly suggesting a similar photochemical mechanism of covalent crosslinking, this explanation is rather unlikely.

(ii) The 65 kDa protein may be an artefact produced by photochemically induced proteolysis of the 140 kDa protein. This possibility can be excluded based on the following findings. (1) During preparation or electrophoresis of β -cell membranes labeled with [^3H]glimepiride, we never observed the appearance of a radiolabeled 65 kDa band and the disappearance of the 140 kDa band. (2) Addition of proteinase inhibitors during β -cell tumor isolation, membrane preparation, photolabeling or electrophoresis never led to the labeling of the 140 kDa protein by [^3H]glimepiride. (3) Variation of incubation time, photolysis time or irradiation wavelength (254 or 300 nm) affected only the incorporation yield but had no influence on the labeling pattern. (4) Photoaffinity labeling of β -cell membranes in the frozen state at -196°C after incubation of β -cell membranes with ^3H -labeled glimepiride or glibenclamide led to the same labeling patterns as described above excluding the possibility of proteolysis and photodestruction of the 140 kDa protein.

(iii) The respective receptor protein(s) may be inaccessible for the sulfonylureas from the outer face of the membrane. This view is strengthened by the observation that disruption of the membrane barrier by solubilization greatly increased the intensity of photolabeling of the 65 kDa protein by [^3H]glimepiride which was accompanied by a decrease of the K_d value for glimepiride from 1.46 nM with β -cell membranes to 0.55 nM with solubilized β -cell membrane proteins (see accompanying paper [11]). Additionally, the K_d value for glibenclamide (about 0.5 nM) was not changed by solubilization when binding of [^3H]glibenclamide completely shifted from the 140 kDa protein to the 65 kDa protein. As in β -cell membranes, other sulfonylureas were able to displace either [^3H]glibenclamide or [^3H]glimepiride from the solubilized receptor and to inhibit photoaffinity labeling of the 65 kDa protein. Thus, the capability of the two sulfonylureas to gain

access to the 65 kDa and 140 kDa binding polypeptides in the β -cell membrane may rely on the spontaneous insertion of the compounds into the phospholipid bilayer. Interestingly, the partitioning behaviour between an octanol and water phase varies widely within the class of sulfonylurea drugs (Panten et al. [8]). A rapid membrane partitioning of glimepiride may prevent its photocrosslinking to the 140 kDa glibenclamide binding protein if this is amenable to photolabeling only from the outer face of the membrane. Alternatively, the presence of detergent may simply decrease the binding affinity of the 140 kDa and increase the affinity of the 65 kDa sulfonylurea receptor. We think this possibility less likely since, according to competitive photolabeling, the binding selectivity of the 65 kDa protein was not altered after solubilization arguing against gross conformational changes.

The photolabeling studies described above and those performed by others clearly indicate, that the labeling patterns of sulfonylurea-binding proteins greatly depends on the structure of the sulfonylurea photoprobe used. With [^3H]glibenclamide, the radiolabeled form of the therapeutically used sulfonylurea, only two polypeptides of $M_r = 140\,000$ and $33\,000$ were labeled (Kramer et al. [11]). Use of 5-iodo-2-hydroxyglyburide, where the 2-methoxy-5-chlorobenzene ring in glibenclamide was substituted by the more bulky and hydrophobic 5-iodo-2-hydroxybenzene ring, led to labeling of 140 kDa, 65 kDa, 55 kDa and 30 kDa polypeptides with a strong preference for the 140 kDa binding protein (Nelson et al. [21]). The sulfonylurea [^{125}I]35623 containing a 2-hydroxy-5-halobenzene ring of identical photochemical behaviour like glibenclamide or 5-iodo-2-hydroxyglibenclamide (Aquilary-Bryan et al. [2]) led to prominent labeling of a 65 kDa protein in β -cell membranes, exclusively, and also in intact HIT T15 cells (Niki et al. [22]). Thus, a putative different photochemical reactivity of glimepiride and glibenclamide cannot account for the binding of [^3H]glimepiride and [^3H]glibenclamide to different binding proteins. Interestingly, a new nitrene-generating derivative of glibenclamide also predominantly labels a 140 kDa protein in β -cell membranes (Schwanstecher et al. [23]). In contrast, the bulkier N_3 -[^3H]33055 exclusively labeled the 33 kDa protein. The results described here and elsewhere (Nelson et al. [21]) exclude the hypothesis that the 140 kDa protein is a dimer of glibenclamide-binding subunits of $M_r = 65\,000$ as suggested by Niki et al. [22]). With the exception of the 33 kDa protein, the labeling of the sulfonylurea-binding proteins, labeled to different extent by the above-mentioned photolabile glibenclamide and glimepiride probes, was concentration-dependently and specifically inhibited by unlabeled sulfonylureas. These findings with β -cell membranes are reflected by photoaffinity labeling studies in intact cells. Whereas

Table 2

Labeling of sulfonylurea binding proteins in β -cell tumor membranes by different sulfonylurea photoprobes

Compounds	Photolabeled proteins			
	140 kDa	65 kDa	55 kDa	33 kDa
[^3H]Glibenclamide	+	–	–	+
[^3H]Glimepiride	–	+	–	–
[^{125}I]35623	–	+	–	–
N_3 -[^3H]33055	–	–	–	+
[^{125}I]5-iodo-2-hydroxy-glibenclamide	+	(+)	(+)	(+)

glibenclamide labels a 140 kDa protein in RINm5F cells, glimepiride is incorporated into a 65 kDa protein. Furthermore, [^{125}I]35623 exclusively binds to a 65 kDa protein after labeling of HIT T15 cells (Niki et al. [22]).

From the relationship between structure and photolabeling patterns of different sulfonylureas (Table 2), it becomes evident that the photoprobes predominantly labeling the 140 kDa protein contain an cyclohexyl-amino moiety (glibenclamide, 5-iodo-2-hydroxyglibenclamide), whereas the compounds preferably labeling the 65 kDa protein contain a 4-*trans*-methylcyclohexyl amine residue (glimepiride, [^{125}I]35623). This structural difference in the sulfonylurea molecule seems to determine binding to the 65 or 140 kDa sulfonylurea binding proteins, respectively. These data strongly support the hypothesis, that the functional sulfonylurea receptor within the β -cell membrane is composed of subunits, among them the 140 kDa and 65 kDa proteins. The hypothesis that the β -cell sulfonylurea receptor consists of a complex built up by several subunits is a matter of controversy at the moment (see Ozanne et al. [24] vs. Skeer et al. [25] and Nelson et al. [26]). The finding of a functional $M_r = 134\,000$ – $166\,000$ of the sulfonylurea receptor in the native as well as solubilized state as determined by target size analysis and gel filtration, respectively, (Skeer et al. [25]) is not in conflict with our results, since the functional molecular mass was determined using binding and photoaffinity labeling of [^3H]glibenclamide. We also observed a linear Scatchard plot for glibenclamide binding and an almost exclusive incorporation of [^3H]glibenclamide into the 140 kDa protein arguing against allosteric interactions between the glibenclamide-binding and glimepiride-binding proteins if only one binding site is occupied. Further evidence for this interpretation will require radiation inactivation of β -cell membranes followed by competitive Scatchard plot analysis of [^3H]glibenclamide binding in the presence of glimepiride and vice versa or gelfiltration of solubilized [^3H]glibenclamide-labeled β -cell membrane proteins in the presence of glimepiride and vice versa.

Despite the fact that [^3H]glimepiride and [^3H]glibenclamide were crosslinked to two distinct proteins, they competed each other for photolabeling suggesting a direct interaction of both sulfonylurea binding proteins as subunits of a common receptor complex. According to this hypothesis, occupancy of one binding protein decreases the binding affinity of the other in a negative cooperative manner. Thus, the two sulfonylureas seem to inhibit steady-state binding or photoaffinity labeling of each other by allosteric mechanisms rather than by competition for the same binding site. This interpretation is substantiated by Scatchard plot analysis when [^3H]glimepiride was diluted with unlabeled glibenclamide and vice versa. However, this does not explain the discrepancy between the higher potency of glimepiride in inhibiting photolabeling and its lower potency in competing equilibrium binding compared to glibenclamide. Besides other factors, the exchange rate of the ligand with the receptor determines the efficiency of photoaffinity labeling and the inhibitory effect of competing ligands (Pomerantz et al. [27]). In fact, glimepiride binding to β -cell membranes is characterized by drastically higher dissociation and association rates compared to glibenclamide. The resulting ~ 3 -fold higher exchange rate of glimepiride correlates well with the 3-fold lower concentration of this drug required for half-maximal inhibition of photolabeling using [^3H]glibenclamide.

The role for the sulfonylurea receptor of the 33 kDa protein being also photolabelled by [^3H]glibenclamide and exclusively by the radiolabeled azidobenzoyl-derivative of glibenclamide, N_3 -[^3H]33055, remains unclear (see Table 2). Unlabeled N_3 -33055, however, was able to displace [^3H]glibenclamide from β -cell membranes in a competition binding assay. The concentrations necessary were about 100-fold higher than those for unlabeled glimepiride or glibenclamide (data not shown). Attachment of a bulky aromatic residue, such as the azidobenzoyl group, to the aromatic benzamido radical of the glibenclamide molecule leads to a strong decrease in affinity to the sulfonylurea receptor, a finding also observed with a fluorescent fluorescein-derivative of glibenclamide (Müller, G., unpublished results).

We do not know the physiological relevance of the differential photoaffinity labeling of sulfonylureas of different structure. Since the identity of the 140 and 65 kDa components of the putative sulfonylurea receptor complex is unknown so far, it remains unclear whether one or both of them are constituents of the ATP-dependent K^+ -channel in the β -cell plasma membrane or interact as regulatory proteins with the channel in a transient manner. Obviously, occupancy of one component seems to be sufficient for channel inhibition and, as a consequence, stimulation of insulin release. Interestingly, for Ca^{2+} -channels in various tissues charac-

terized with different (photo)-affinity probes, similar molecular masses of 140–170 kDa, 52–60 kDa and 32–35 kDa have been described for the protein components of these channels (Glossmann et al. [28]; Galizzi et al. [29]; Glossmann et al. [30]). Their relationship to the corresponding sulfonylurea-labeled polypeptides has to be elucidated.

References

- [1] Kramer, W., Ökonopulos, R., Pünter, J. and Summ, H.-D. (1988) *FEBS Lett.* 229, 355–359.
- [2] Aguilar-Bryan, L., Nelson, D.A., Vu, Q.A., Humphrey, M.B. and Boyd III, A.E. (1990) *J. Biol. Chem.* 265, 8218–8224.
- [3] Aguilar-Bryan, L., Nichols, C.G., Rajan, A.S., Parker, C. and Bryan, J. (1992) *J. Biol. Chem.* 267, 14934–14940.
- [4] De Weille, J.R., Fosset, M., Mourre, C., Schmid-Antomarchi, H., Bernardi, H. and Lazdunski, M. (1989) *Pflügers Arch.* 414 (Suppl.), S80–S87.
- [5] Ashcroft, S.J.H. and Ashcroft, F.M. (1992) *Biochim. Biophys. Acta* 1175, 45–59.
- [6] Geisen, K., Hitzel, V., Ökonopulos, R., Pünter, J., Weyer, R. and Summ, H.-D. (1985) *Drug. Res.* 35, 707–712.
- [7] Schmid-Antomarchi, H., De Weille, J., Fosset, M. and Lazdunski, M. (1987b) *J. Biol. Chem.* 262, 15840–15844.
- [8] Panten, U., Burgfeld, J., Goerke, F., Rennicke, M., Schwanstecher, M., Wallasch, A., Zünkler, B.J. and Lenzen, S. (1989) *Biochem. Pharmacol.* 38, 1217–1229.
- [9] Geisen, K. (1988) *Drug. Res.* 38, 1120–1130.
- [10] Kramer, W. (1987) *Biochim. Biophys. Acta* 905, 65–74.
- [11] Müller, G., Hartz, D., Pünter, J., Ökonopulos, R. and Kramer, W. (1994) *Biochim. Biophys. Acta* 1191, 267–277.
- [12] Wessel, D. and Flüge, U.I. (1984) *Anal. Biochem.* 138, 141–143.
- [13] Kramer, W., Burckhardt, G., Wilson, F.A. and Kurz, G. (1983) *J. Biol. Chem.* 258, 3623–3628.
- [14] Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal, K., Müller, G., Tripiet, D. and Wess, G. (1993) *J. Biol. Chem.* 268, 18035–18046.
- [15] Ehle, B., Lemoine, H. and Kaumann, A.J. (1985) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 331, 52–61.
- [16] Hogg, P.J. and Winzor, D.J. (1985) *Biochim. Biophys. Acta* 843, 159–164.
- [17] Hollemans, H.J.G. and Berlina, R.M. (1975) *Clin. Chem.* 21, 1769–1780.
- [18] Repke, H. and Liebmann, C. (1987) *Quantitative Analyse der Ligand-Bindungskinetik, in Membranrezeptoren und ihre Effektorsysteme*, Verlag Chemie, Weinheim.
- [19] Bayley, H. (1983) *Photogenerated reagents in biochemistry and molecular biology, in Laboratory Reagents in Biochemistry and Molecular Biology*, Vol. 12 (Work, T.S. and Burdon, R.H., eds.), Elsevier, Amsterdam.
- [20] Martyr, R.J. and Benisek, W.F. (1973) *Biochemistry* 12, 379–395.
- [21] Nelson, D.A., Aguilar-Bryan, L. and Bryan, J. (1992) *J. Biol. Chem.* 267, 14928–14933.
- [22] Niki, I., Welsh, M., Berggren, P.-O., Hubbard, P. and Ashcroft, S.J.H. (1991) *Biochem. J.* 277, 619–624.
- [23] Schwanstecher, M., Löser, S., Chudziak, F. and Panten, U. (1993) *International Conference on ATP-sensitive K^+ channels and sulfonylurea receptors*, Houston, TX, Book of Abstracts, p. 159.
- [24] Ozanne, S.E., Khan, R.N., Adogu, A.A., Hales, C.N. and Ashford, M.L.J. (1993) *International Conference on ATP-sensitive K^+ channels and sulfonylurea receptors*, Houston, TX, Book of Abstracts, p. 158.

- [25] Skeer, J.M., Degano, P., Coles, B., Ashcroft, F.M. and Ashcroft, S.J.H. (1993) International Conference on ATP-sensitive K^+ channels and sulfonylurea receptors, Houston, TX, Book of Abstracts, p. 160.
- [26] Nelson, D.A., Aguilar-Bryan, L., Wechsler, S.W. and Bryan, J. (1993) International Conference on ATP-sensitive K^+ channels and sulfonylurea receptors, Houston, TX, Book of Abstracts, p. 19–26.
- [27] Pomerantz, A.A., Rudolph, S.A., Haley, B.E. and Greengard, P. (1975) *Biochemistry* 14, 3858–3865.
- [28] Glossmann, H.D., Ferry, A., Goll, A., Striessnig, J. and Zernig, G. (1985) *Drug Res.* 35, 1917–1935.
- [29] Galizzi, J.-P., Borsotto, M., Barhanin, J., Fosset, M. and Lazdunski, M. (1986) *J. Biol. Chem.* 261, 1393–1397.
- [30] Glossmann, H., Ferry, D.R., Striessnig, J., Goll, A. and Moosburger, K. (1987) *Trends Pharmacol. Sci.* 8, 95–105.