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Determination of the molecular mass of the native β-cell sulfonylurea receptor

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Abstract

In the present study we have determined the molecular mass of the β-cell sulfonylurea receptor in its native form by two different experimental approaches; gel filtration chromatography and radiation inactivation analysis. We first confirmed that the denatured photolabelled MIN6 β-cell receptor had a molecular size of 141 ± 2 kDa (mean ± S.E., $n = 8$). Under non-denaturing conditions, using gel filtration chromatography, apparent molecular masses of 166 ± 1 kDa (mean ± S.E., $n = 3$) and 182 ± 5 kDa (mean ± S.E., $n = 4$) were determined for the photoaffinity-labelled and unlabelled sulfonylurea receptor, respectively. We conclude that in the solubilized state the receptor exists as a monomer. Radiation inactivation analysis indicated that the receptor has a target size of 250 ± 30 kDa (mean ± S.E., $n = 7$). This value for the molecular mass is larger than that obtained from SDS-PAGE following photolabelling of the receptor (141 kDa) suggesting that the β-cell sulfonylurea receptor is composed of more than one subunit in the native membrane.

Key words: β-Cell sulfonylurea receptor; [^{3}H]Glibenclamide; Non-denaturing conditions; Gel filtration chromatography; Radiation inactivation analysis

1. Introduction

ATP-regulated K^+ channels, or K-ATP channels, form a heterogeneous family of K^+ channels which are found in a variety of tissues, including pancreatic β-cells, cardiac, smooth and skeletal muscle, and certain neurones [1]. These channels are inhibited by an increase in the intracellular ATP concentration and they thereby link cellular metabolism to electrical activity. This coupling has important functional consequences in many cell types. In the pancreatic β-cell, K-ATP channels control the β-cell resting potential. In response to an increase in the blood glucose concentration, there is increased glucose metabolism within the β-cell which leads to a rise in the intracellular ratio of [ATP]/[ADP] and closure of K-ATP channels. As a consequence of the channels' inhibition, electrical activity, Ca^{2+} influx and ultimately insulin secretion are stimulated (for review, see [1]).

K-ATP channels in pancreatic β-cells are also inhibited by a class of drugs known as sulfonylureas, which are used clinically to treat Type II diabetes. The mechanism involves binding of the sulfonylurea to high-affinity binding sites in the β-cell plasma membrane (for review, see [2]). However, it remains unclear whether the binding site for sulfonylureas lies on the K-ATP channel or on a separate protein with which it interacts.

Several studies have attempted to measure the molecular mass of the sulfonylurea receptor. The approach that the majority of research workers have employed has been to label the receptor covalently with tritiated or iodinated glibenclamide, or glibenclamide analogues, by UV illumination. Following separation of irradiated samples on polyacrylamide gels under denaturing conditions, these studies have reported molecular weights for the sulfonylurea receptor ranging from 60 kDa to 150 kDa [3–7]. However, these studies do not permit conclusions about the molecular mass of the sulfonylurea receptor in its native form, as the experiments were performed under denaturing conditions. In this study we have employed two different experimental approaches to

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Abbreviations: BSA, bovine serum albumin; EDTA ethylenediaminetetraacetic acid; MOPS, 3-[*n*-morpholino]propanesulphonic acid; PMSF, phenylmethyl-sulphonylfluoride; ACHE, acetylcholinesterase.

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determine the molecular mass of the native β-cell sulfonylurea receptor.

2. Materials and methods

2.1. Materials

Tissue culture materials were from Gibco Europe, Paisley, Strathclyde, UK. [^{3}H]Glibenclamide (51.4 Ci/mmol) was purchased from DuPont (Stevenage, Hertfordshire, UK). Glassfibre filters (GF/F) were obtained from Whatman (Maidstone, Kent, UK). Bovine serum albumin (BSA) was from BCL, Lewes, East Sussex, UK. Protease inhibitors were supplied by the Sigma Chemical Co., Poole, Dorset, UK. Optifluor was purchased from Packard Instruments (Downers Grove, IL, USA). Other reagents were from from BDH Ltd, Poole, Dorset, UK. MIN6 β-cells were kindly supplied by Prof J.-I. Miyazaki, University of Tokyo.

2.2. Cell culture

MIN6 β-cells were cultured in DMEM medium containing 15% foetal calf serum and antibiotics (penicillin 100 U/ml), streptomycin 0.1 mg/ml) at 37°C in a humidified atmosphere of air/CO_2 (95:5). HIT-T15 β-cells were cultured in RPMI 1640 medium containing 10% foetal calf serum at 37°C in a humidified atmosphere of air/CO_2 (95:5) as previously described [8]. Cells were passaged weekly and harvested using trypsin-EDTA. After seeding at a density of 5×10^7 cells per flask β-cells were cultured for 6 days before membrane preparation.

2.3. Membrane preparation

β-Cell membranes were prepared essentially as described by Gaines et al. [9]. In brief, after washing twice with 10 ml of phosphate-buffered saline (Dulbecco's), trypsinized cells were resuspended in 10 ml of 5 mM Tris (pH 8.0 with HCl) with protease inhibitors (0.2 mM PMSF, 1 mM EDTA, 1 mM benzamidine, 10 μM leupeptin and 1 mM iodoacetamide), left for 40 min on ice and then homogenized. The homogenate was centrifuged for 10 min at $900 \times g$. The supernatant was then centrifuged for a further 30 min at $96,000 \times g$. The pellets were resuspended at a concentration of 2–4 mg protein/ml in buffer containing 20 mM MOPS (pH 7.4 with NaOH) and the protease inhibitors described above. Protein concentration was determined according to Bradford [10] using BSA as standard. Collected membranes were aliquoted, frozen immediately in liquid nitrogen and stored at −70°C.

2.4. Sulfonylurea binding

MIN6 membranes (200 μg of protein/ml) were incubated with 1–4nM [^{3}H]glibenclamide in 60 mM MOPS pH 7.4 in a total volume of 400 μl in the absence (total binding) or presence of 1 μM glibenclamide (non-specific binding). After 60 min incubation at room temperature, the reaction was terminated by vacuum filtration onto glass fibre (GF/F) filters (Whatman) presoaked in 60 mM MOPS pH7.4. Following filtration, the filters were washed with 4 × 5 ml of ice-cold distilled water and counted for radioactivity in a scintillation counter using aqueous/non-aqueous scintillant (Opti-Fluor).

2.5 Solubilization of the sulfonylurea receptor

MIN6 β-cell membranes were solubilized in 50 mM Tris (pH 8.0 with HCl), 10% (w/v) glycerol, 1.5% (w/v) Triton X-100 (detergent/protein ratio of 8:1) with protease inhibitors (0.2 mM PMSF, 1.0 mM benzamidine). After incubation for 60–90 min at 4°C with gentle stirring, membranes were spun at $100,000 \times g$ for 40 min. The supernatant was retained for gel filtration chromatography. Approximately 65% of [^{3}H]glibenclamide-binding activity was recovered employing this solubilization procedure. Protein was determined using the Pierce Micro BCA Protein Assay Kit with BSA as a standard.

2.6. Binding assay for solubilized receptor

Solubilized membranes (approximately 0.25 mg protein/ml) were incubated in 200 μl of 50 mM Tris (pH 8.0 with HCl) for 60 min at 4°C with 3–4 nM [^{3}H]glibenclamide in the absence (total binding) or presence (non-specific binding) of 1 μM glibenclamide. The final concentration of Triton X-100 in the binding assay was 0.15%. [^{3}H]Glibenclamide binding of solubilized fractions was assayed by rapid gel filtration as described by Niki et al. [6]. Briefly, Sephadex G-50 medium equilibrated overnight with 50 mM Tris (pH 8.0 with HCl) containing 0.1% Triton X-100 was loaded into a 2 ml plastic syringe and spun for 3 min at $111 \times g$. After incubation of solubilized receptor preparations with [^{3}H]glibenclamide for 60 min, aliquots were carefully loaded onto the prespun column. The column was spun again for 3 min at $140 \times g$ and the eluate was collected for counting; 60–70% of bound ligand was collected by this technique.

2.7. Photoaffinity labelling of sulfonylurea receptor

MIN6 β-cell membranes (2 mg/ml) were incubated with 3–4 nM [^{3}H]glibenclamide in 60 mM MOPS (pH 7.4 with NaOH) with protease inhibitors (0.2 mM PMSF, 1.0 mM benzamidine). This concentration of [^{3}H]glibenclamide was chosen because the contribution of low-affinity binding sites is negligible [11]. Non-specific incorporation was measured in a parallel incubation in the presence of 1 μM glibenclamide. After incubation for 60 min at 4°C, each sample was irradiated at 302 nm in an 18 mm diameter Petri dish, in liquid N_2, with an UV lamp (UVM-57 Chromato-Vue Lamp, UVP Inc., San Gabriel, CA, USA) at a distance of 2 cm for 2 min. Membranes were thawed on ice, then pelleted at $100,000 \times g$ for 40 min. Prior to SDS gel electrophoresis, photoaffinity labelled membranes were denatured in SDS sample buffer and heated for 3 min at 100°C.

2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein profiles of membrane fractions photolabelled with [^{3}H]glibenclamide were analysed by discontinuous SDS-PAGE on 7.5% acrylamide/0.2% bisacrylamide gels [12]. Gels were calibrated using the following molecular weight (M_r) markers: myosin (M_r 205,000); β-galactosidase (M_r 116,000); phosphorylase b (M_r 97,400); bovine serum albumin (M_r 66,000); ovalbumin (M_r 45,000).

Following electrophoresis, gels were stained with Coomassie blue. Gel tracks were cut into 2 mm slices and radioactivity extracted with 0.5 ml 30% H_2O_2 at 50°C for 16 h. Aqueous/non-aqueous scintillant (Opti-Fluor) was added to cooled samples and radioactivity determined in a scintillation counter.

2.9. Gel filtration of solubilized MIN6 cell membranes

Gel filtration chromatography was carried out on a Sephacryl S300HR (Pharmacia) column (1.0 × 40 cm). The column was pre-equilibrated in elution buffer (50 mM Tris (pH 8.0 with HCl), 0.5% (w/v) Triton X-100) prior to sample application. Aliquots (600 μl) of solubilized MIN6 β-cell membranes (non-photoaffinity labelled or photoaffinity labelled fractions) were applied to the column and fractions of approximately 500 μl were collected at a flow rate of 3.8–4.4 ml/h at 4°C. Fractions were analysed either for [^{3}H]glibenclamide binding activity as described above or for radioactivity using liquid scintillation spectometry. Columns were calibrated using the following standards: β-amylase, 5 mg (M_r 200,000); alcohol dehydrogenase, 5 mg (M_r 150,000). Detection of these markers was by the Pierce Micro BCA Protein Assay Kit. Blue dextran 2000 (1 mg) was used to determine the void volume (V_o) and vitamin B_{12} (3 mg) to measure the total included volume (V_t) of the column.

2.10. Radiation inactivation

Freshly prepared HIT-T15 β-cell membranes were aliquoted into Eppendorf tubes (approximately 0.8 mg of protein per tube), frozen in liquid N_2 then freeze-dried overnight. Acetylcholinesterase (2 U) (EC 3.1.1.7; Type XII from Bovine Erythrocytes) was included in some samples as an internal standard. Samples were subjected to radiation inactivation in a Gammacell 220 ^{60}Co irradiator (Atomic Energy of Canada, Ottawa) at a dose rate of about 1 Mrads per hour as described by Beauregard et al. [13]. During irradiation, samples were maintained at −78°C with crushed dry ice. Following irradiation, freeze-dried samples were reconstituted in 20 mM MOPS (pH 7.4 with NaOH) with protease inhibitors (0.2 mM PMSF, 1.0 mM benzamidine) and assayed for enzyme activity, protein concentration and [^{3}H]glibenclamide binding activity. The amount of [^{3}H]glibenclamide binding was plotted as a function of the radiation intensity and the molecular mass of the radiolabelled binding component was determined using the following relationship derived by Kepner and Macey [14] which is valid for samples irradiated at −78°C:

$$M_r = 1.29 \times 10^6 / D_{37} \quad \text{(Eqn. 1)}$$

where D_{37} is the dose (in Mrads) at which 37% of the original activity remains.

3. Results

3.1. SDS-PAGE of photoaffinity labelled MIN6 β-cell membranes

We first confirmed the molecular size of the MIN6 β-cell sulfonylurea receptor by photoaffinity labelling under denaturing conditions. [^{3}H]Glibenclamide, at a concentration of 3–4nM, was photoincorporated into MIN6 β-cell membranes by exposure to UV light. Following SDS-PAGE separation of photoaffinity-labelled MIN6 β-cell membranes, the amount of radioactivity in gel slices was determined by liquid scintillation counting (Fig. 1). In all experiments, a single peak of radioactivity was observed corresponding to a polypeptide with an apparent molecular mass of 141 ± 2 kDa (mean ± S.E., n = 8). The incorporation of [^{3}H]glibenclamide into this polypeptide was inhibited by 1 μM glibenclamide (closed circles, Fig. 1). In this study the efficiency of photolabelling of the MIN6 β-cell sulfonylurea receptor was calculated to be approximately 1.4%.

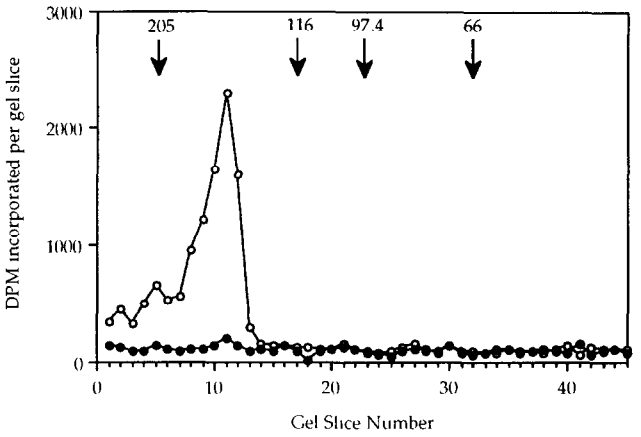

Fig. 1. Photoaffinity labelling of the sulfonylurea receptor with [^{3}H]glibenclamide in MIN6 β-cell membrane preparations. Labelling experiments were performed as described in section 2. Irradiated samples were separated on 7.5% polyacrylamide gels under denaturing conditions. Gels were sliced and the amount of radioactivity in each 2 mm slice was determined as described in section 2. Total (○) (i.e. in the absence of unlabelled drugs) and non-specific (●) (determined in the presence of 1 μM glibenclamide) photo-incorporation are shown. Molecular weight (M_r) markers were: Myosin (M_r 205,000); β-galactosidase (M_r 116,000); phosphorylase b (M_r 97,400); bovine serum albumin (M_r 66,000) and are indicated by arrows.

3.2. Gel filtration chromatography of the sulfonylurea receptor under non-denaturing conditions

To determine the molecular size of the sulfonylurea receptor under non-denaturing conditions, we used gel filtration chromatography of solubilized MIN6 β-cell membranes. Membranes photoaffinity-labelled with [^{3}H]glibenclamide yielded a peak fraction of radioactivity which eluted at a position corresponding to an apparent molecular mass of 166 ± 1 kDa (mean ± S.E., n = 3; Fig. 2A). In parallel experiments with unlabelled membranes a peak of [^{3}H]glibenclamide binding activity was obtained which eluted at a position corresponding to an apparent molecular mass of 182 ± 5 kDa (mean ± S.E., n = 4, Fig. 2B).

3.3. Radiation inactivation analysis of the sulfonylurea receptor

Radiation inactivation provides a means of estimation of the size of a molecule within the native membrane. It is based on the assumption that the biological activity of

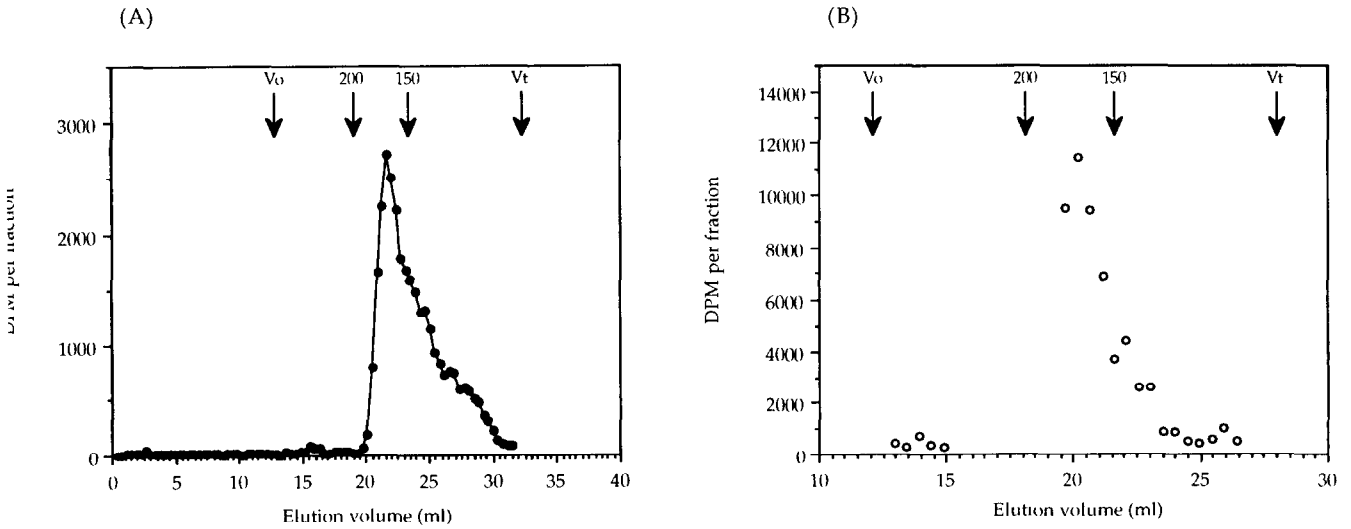

Fig. 2. Gel filtration chromatography of Triton X-100 solubilized photoaffinity-labelled (A) and unlabelled (B) MIN6 β-cell membranes. (A) and (B) show the elution profiles for solubilized photoaffinity-labelled and unlabelled MIN6 β-cell membranes from columns prepared with Sephacryl S300HR media, respectively. Fractions were assayed for either radioactivity (A) or [^{3}H]glibenclamide binding activity (B) as described in section 2. Markers (left to right): blue dextran 2000 (V_o); β-amylase (M_r 200,000); alcohol dehydrogenase (M_r 150,000); vitamin B12 (V_t).

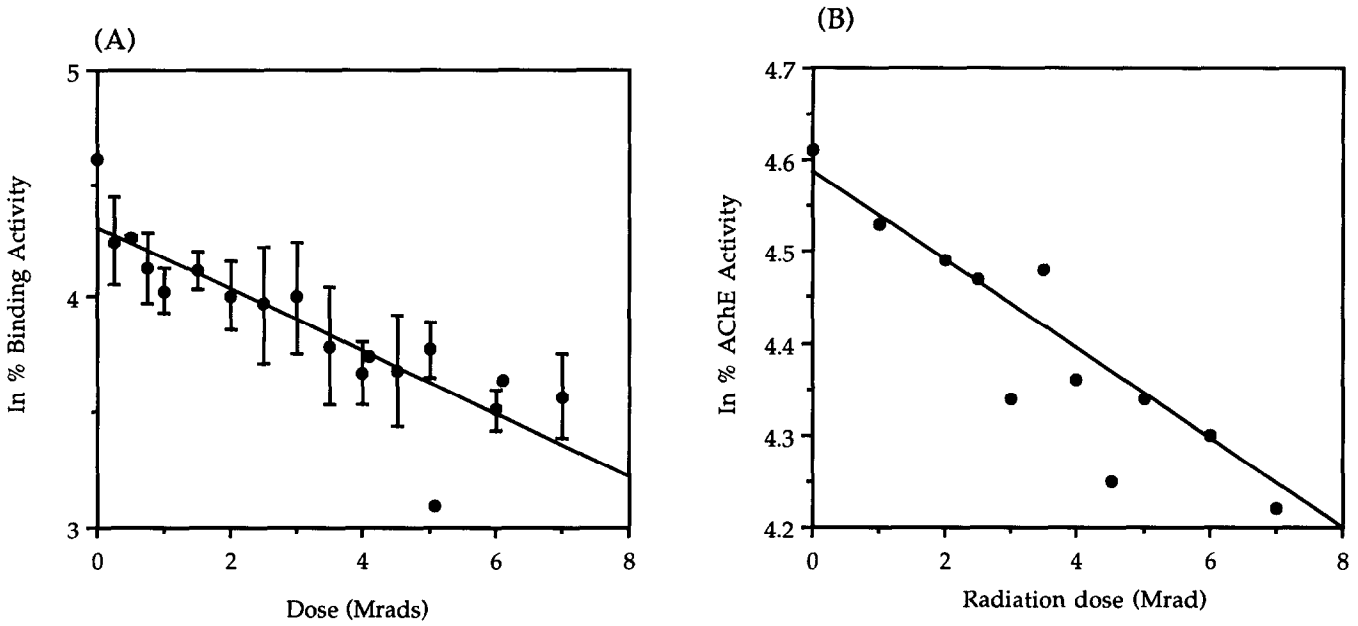

Fig. 3. Effect of radiation on specific [^{3}H]glibenclamide binding to HIT-T15 β-cell membranes (A) and acetylcholinesterase (AChE) activity (B). (A) Freshly prepared HIT-T15 β-cell membranes were freeze-dried and subjected to different irradiation doses. [^{3}H]Glibenclamide binding activity was determined at each irradiation dose and the amount of specifically bound [^{3}H]glibenclamide remaining at different irradiation intensities was calculated and expressed as a percentage of the control (i.e. specific binding in non-irradiated freeze-dried samples, defined as 100% binding). Data is the mean ± S.E.M. of 7 separate experiments. A straight line was fitted to the data using least squares linear regression analysis, the slope of which is proportional to the target size of the molecule. (B) shows the effect of irradiation on AChE from bovine erythrocytes added to the preparation as an internal standard.

the molecule, in this case the sulfonylurea receptor, will be inactivated if the molecule receives a single hit of ionising radiation (for review see [15]). The decrease of binding activity is an exponential function of the radiation dose and is related to the molecular size (by Equation 1).

Fig. 3A plots the natural logarithm of the percent [^{3}H]glibenclamidebinding activity as a function of the radiation intensity. A straight line was fitted to the data using least squares linear regression analysis and the slope of this line was used to calculate the molecular size of the sulfonylurea receptor from Equation 1. The apparent molecular mass for the sulfonylurea receptor using this method was estimated to be 252 kDa when all experiments were pooled (Fig. 3A) and 250 ± 30 kDa (mean ± S.E., $n = 7$) when calculated for each individual experiment. Acetylcholinesterase was included in some samples as an internal standard. The molecular mass of this enzyme estimated from radiation analysis was 68 kDa (Fig. 3B). This value is similar to that reported by previous workers (see [16]) in non-phosphate buffered solutions and corresponds to the molecular mass of a single subunit.

4. Discussion

We have used a range of experimental techniques to estimate the molecular mass of the β-cell sulfonylurea receptor under both denaturing and non-denaturing conditions.

Under denaturing conditions, we identified a molecular species of 141 kDa following gel electrophoresis of MIN6 β-cell membranes photoaffinitylabelled with [^{3}H]glibenclamide. Several other studies, using a variety of different β-cell types, have also concluded that under denaturing conditions a molecular species of approximately 140 kDa represents the high-affinity β-cell sulfonylurea receptor [3,5,7]. In the present study the efficiency of photolabelling of the MIN6 β-cell sulfonylurea receptor was calculated to be 1.4%. This is higher than the previously reported values of approximately 0.4% and 0.75% for photolabelling of the HIT-T15 β-cell sulfonylurea receptor [5,7]. This difference in photolabelling efficiencies may be explained by the 4-fold higher density of glibenclamide-binding sites found in MIN6 β-cells (Skeer, unpublished observations) compared to HIT-T15 β-cells. In previous studies a number of lower molecular weight polypeptide species (the most common being 65 kDa, 50 kDa and 33 kDa) were identified following SDS-PAGE of photolabelled β-cell membranes [3,5–7]. The absence of these polypeptide species in our study may be due to the low concentration of [^{3}H]glibenclamide [11] and the wavelength (302 nm) of the UV light employed for photolabelling [7]. Although the nature of the 65 kDa polypeptide remains unclear, the 50 kDa and

33 kDa polypeptides in HIT-T15 β-cells have recently been identified as calreticulin and malate dehydrogenase, respectively [17].

We used gel filtration chromatography under non-denaturing conditions to obtain an estimate of the molecular size of the active solubilized sulfonylurea receptor. An apparent molecular mass of 166 kDa was determined for the photoaffinity-labelled sulfonylurea receptor, whereas the unlabelled receptor appeared to have a somewhat higher molecular mass of 182 kDa. This difference may be due to modifications in the shape of the photolabelled receptor, as the way proteins migrate through a gel filtration matrix is dependent not only on the size but also on the shape of the protein (see [18]). The estimated molecular weight of the photolabelled receptor is also higher than that determined by SDS-PAGE. In gel filtration studies, unlike SDS-PAGE, the size of an unknown protein can only be estimated accurately from standard proteins run through the same column if the shapes of both the unknown and the calibrating proteins are similar (see [18]). Thus the difference observed in the size of the photoaffinity-labelled sulfonylurea receptor determined by gel filtration chromatography and SDS-PAGE (166 kDa vs. 141 kDa) may be accounted for by the shape of the receptor being dissimilar from that of the proteins employed to calibrate the gel filtration matrix. In addition, the detergent micelle associated with the receptor may also contribute to the higher molecular weight observed with this method (for example see [19]). We therefore conclude that the unlabelled and photoaffinity-labelled receptors have similar molecular weights and thus that photoaffinity labelling does not lead to cross-linking of receptor subunits. Our results thus suggest that in its solubilized state the receptor exists as a monomer with a molecular size of between 140–170 kDa.

We have used radiation inactivation analysis to estimate the molecular size of the sulfonylurea receptor in the native membrane we found that the β-cell sulfonylurea receptor has a target size of ~250 kDa. This value for the molecular mass is larger than that obtained from SDS-PAGE following photolabelling of the receptor (141 kDa) suggesting that the β-cell sulfonylurea receptor may be composed of more than one subunit in the native membrane. However, the data do not allow us to distinguish whether the receptor exists as a dimer or whether it consists of a 141 kDa subunit and a smaller subunit of lower molecular mass. Evidence that the sulfonylurea receptor may consist of two subunits has also been provided by recent binding studies [20].

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