Intracellular localization and molecular heterogeneity of the sulphonylurea receptor in insulin-secreting cells

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Summary Sulphonylureas stimulate insulin secretion by binding to a receptor in the pancreatic beta-cell plasma membrane resulting in inhibition of ATP-sensitive K^+ channels, membrane depolarization and thus influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. Sulphonylureas can also induce hormone release at fixed membrane potentials without Ca^{2+} entry suggesting that these drugs may have other modes of action. We have determined whether different forms of sulphonylurea-binding proteins are present in insulin-secreting cells and their subcellular localization by density gradient centrifugation. Binding studies using [3H]-glibenclamide showed that islet and insulinoma membranes contained a single high affinity sulphonylurea binding site ($K_d = 1$ nmol/l). Photo-crosslinking of the drug to the membranes resulted in labelling of two proteins with ap-

parent molecular weights of 170 and 140 kDa. The same analyses of insulinoma subcellular fractions showed that the majority (>90%) of binding proteins were localized to intracellular membranes with only minor levels (<10%) on plasma membranes. The 170 kDa sulphonylurea binding protein was present in both plasma and granule membrane fractions whereas the 140 kDa form was not present in the plasma membrane fraction. The differences in the molecular forms and subcellular distribution of the receptor are consistent with sulphonylureas having multiple sites of action in the pancreatic beta cell. [Diabetologia (1995) 38: 277–282]

Key words Non-insulin-dependent diabetes mellitus, insulin, sulphonylurea receptors, islets, glibenclamide, secretory granule.

Sulphonylureas have been used to treat non-insulin-dependent diabetic patients for over 30 years. These drugs stimulate insulin secretion by inhibiting ATP-sensitive potassium (K_{ATP}) channels which leads to depolarization of the plasma membrane, activation of voltage-dependent Ca^{2+} channels and an increase in cytoplasmic Ca^{2+} levels [1]. High-affinity sulphonylurea binding sites have been reported in a number of insulin-secreting cells including HIT cells and murine islets [2–4]. Photoaffinity labelling studies us-

ing [3H]-glibenclamide [5] and [^{125}I]-glibenclamide [6, 7] have shown that the binding protein has an apparent molecular weight of 140 kDa in HIT cells, as determined by SDS-PAGE under reducing conditions. Recent radiation inactivation studies have shown that the native sulphonylurea receptor in MIN6 beta-cell membranes is a multimer with a molecular mass of 250 kDa [8]. The mechanism by which sulphonylurea binding elicits K_{ATP} -channel inhibition has not been elucidated, although patch clamp studies have shown that the process is Mg^{2+} dependent [9]. Studies combining patch clamp, drug binding and secretion analyses of the CRI-G1 and CRI-D11 cell lines, which differ in their sensitivity to sulphonylureas, have shown that the receptor and channel moieties are closely-associated in the plasma membrane although they appear to reside on separate polypeptide chains [10].

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Abbreviations: NEDH rats, New England Deaconess Hospital rats; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulphonyl fluoride; ER, endoplasmic reticulum.

Apart from the inhibitory effect of sulphonylureas on plasma membrane K_{ATP} channels, sulphonylureas may stimulate insulin secretion by other mechanisms. Studies using electrically-permeabilized RIN-m5f cells have shown that tolbutamide can stimulate insulin secretion by a mechanism which is not dependent on changes in membrane potential [11]. Similarly, Ammala et al. [12] have shown that tolbutamide enhanced the rate of exocytosis from depolarized mouse beta cells without any change in the existing Ca^{2+} current. Since ultrastructural studies show that [3H]-glibenclamide associates with secretory granules in rat islets of Langerhans [13], it is proposed that the drug may interact with the secretory machinery. However, the nature of such intracellular sulphonylurea binding sites and their relationship to the receptor involved in K_{ATP} channel regulation have not been characterized.

The aim of the present study was to investigate the molecular complexity of sulphonylurea binding proteins in insulin-secreting cells by glibenclamide binding studies and photoaffinity-labelling analyses. The subcellular distribution of different molecular forms was determined by density gradient centrifugation analyses of transplantable insulinoma tissue.

Materials and methods

Materials. Analytical grade biochemicals were obtained from Sigma Chemical Co. or BDH Chemicals (both of Poole, Dorset, UK), unless stated otherwise. [3H]-glibenclamide (50.7 Ci/mmol) was obtained from NEN-Dupont (Stevenage, Herts., UK).

Tissues. Insulinomas were propagated in New England Deaconess Hospital (NEDH) rats as described previously [14]. Tumours were removed and tissue was scraped from the fibrous capsules into Hanks saline [(in mmol/l) 137 NaCl, 5.4 KCl, 1.67 $MgSO_4$, 4 $CaCl_2$, 0.34 Na_2HPO_4 and 4.2 $NaHCO_3$ (pH 7.4)]. All subsequent procedures were performed at 4°C. Cells were isolated from insulinoma tissue by Percoll density gradient centrifugation as described previously [15] and used immediately. The CRI-G1 and CRI-D11 insulin-secreting cell lines, derived initially from transplantable rat insulinoma cells, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (both from Flow Laboratories, Irvine, Scotland, UK) and passaged at 4–7 day intervals as described by Carrington et al. [16]. Islets of Langerhans were isolated from the pancreata of 8–12-week-old NEDH rats by collagenase digestion followed by purification on Histopaque density gradients as described by Guest et al. [17]. Islets were cultured overnight in DMEM containing 11 mmol/l glucose and 10% fetal calf serum.

Subcellular fractionation of insulinomas. Insulinoma tissue was homogenized in isotonic media and fractionated by density gradient centrifugation on Nycodenz step gradients as described by Hutton et al. [18]. This yielded four membrane fractions designated as A, B, C and D in order of increasing density. Fractions were stored in the presence of 100 μ mol/l phenylmethylsulphonyl fluoride (PMSF), 20 μ mol/l trans-Epoxy-

succinyl-L-leucylamido(4-guanidino)-butane (E-64) and 10 μ mol/l pepstatin A under liquid nitrogen prior to analysis.

Membrane preparation. Islets, CRI-G1 and CRI-D11 cells were lysed and crude membrane fractions prepared by ultracentrifugation as described previously [10]. Samples were resuspended in 50 mmol/l Tris/HCl (pH 7.4) containing 100 mmol/l PMSF, 10 mmol/l Pepstatin A and 20 mmol/l E64 and stored under liquid nitrogen prior to use.

Salt washing of insulinoma membranes. Insulinoma membrane fractions were pelleted by centrifugation for 30 min at $100,000 \times g$ (Sorvall T-1270; Du Pont (U.K) Ltd., Stevenage, Merts, UK), then resuspended in 2 ml of 10 mmol/l NH_4HCO_3 and sonicated for 10 s at 3 μ m (MSE Sonifier, Crawley, Sussex, UK). Centrifugation was repeated and the pellets were resuspended in 2 ml of 10 mmol/l NH_4HCO_3 containing 1 mol/l NaCl and 1 mmol/l EDTA, sonicated and centrifuged as above. The pellet was resuspended in 2 ml of 10 mmol/l NH_4HCO_3 and the sonication and centrifugation steps were repeated. The membranes were stored under liquid nitrogen in 1 ml of 50 mmol/l Tris/HCl pH 7.4 containing the protease inhibitors described above. Control (non-salt washed) membranes were treated in an identical manner except all procedures were performed in 50 mmol/l Tris/HCl (pH 7.4).

[3H]-glibenclamide binding studies. Membranes prepared from islets, insulinoma, CRI-G1 and CRI-D11 cells (25 μ g protein) were incubated for 2 h at room temperature in 0.5 ml assay buffer (50 mmol/l Tris/HCl pH 7.4) containing the indicated concentrations of [3H]-glibenclamide (50.9 Ci/mmol, Du Pont [UK] Ltd). Incubations were terminated by addition of 2 ml of ice-cold buffer and bound ligand was collected by rapid vacuum filtration onto 2.5-cm diameter GF/B filter disks (Whatman International Ltd, Maidstone, Kent, UK). Filters were washed with 4×2 ml of ice-cold buffer and the bound radioactivity determined by liquid scintillation counting using OptiPhase HiSafe II (LKB Scintillation Products, Loughborough, Leicestershire, UK) and a Packard liquid-scintillation spectrometer (Canberra Packard, Slough, Berkshire, UK).

Assay protocols. Protein was determined by a dye binding method using bovine serum albumin as standard [19]. Insulin was measured by radioimmunoassay as described previously [20]. Spectrophotometric assays were performed for cytochrome oxidase [21], NADPH-cytochrome c reductase [22] and alkaline phosphatase [23]. β -N-acetylglucosaminidase was determined by a fluorometric method [24].

Photoaffinity labelling. Membrane fractions from each cell type (100 μ g protein) were incubated for 2 h at room temperature in 60 μ l of sodium phosphate buffer (pH 7.5) containing 10 nmol/l [3H]-glibenclamide. The samples were irradiated for 15 min at 312 nm in a Stratalinker (Stratagene, La Jolla, Calif., USA) and then combined with an equal volume of $2 \times$ concentrated sample loading buffer (0.25 mol/l Tris/HCl pH 9.2, 2% SDS, 12% sucrose, 20 mmol/l EDTA, 130 mmol/l dithiothreitol). Samples were subjected to SDS-polyacrylamide gel electrophoresis on slab gels (15 $\times$ 15 $\times$ 0.15 cm) polymerized from 10% (weight/volume) acrylamide and 0.13% NN'-methylenebisacrylamide using the discontinuous buffer system of Laemmli [25]. Gels were impregnated for fluorography with 20% (weight/volume) 2, 5-diphenyloxazole in acetic acid, vacuum dried and exposed to Kodak Scientific Imaging Film (Eastman Kodak Company, New York, N. Y., USA) at -70°C.

Fig. 1. Effect of NaCl and EDTA washes on [³H]-glibenclamide binding to insulinoma membranes. Insulinoma membranes were washed with 10 mmol/l NH₄HCO₃ containing 1 mol/l NaCl and 2 mmol/l EDTA and incubated with the indicated concentrations of [³H]-glibenclamide as described in the methods section. Bound radioactivity was collected by vacuum filtration onto GF/B disks and determined by liquid scintillation counting. Non-specific binding was determined in the presence of 1 μmol/l glibenclamide and was always less than 10 % of the total bound counts. Results are expressed as fmol [³H]-glibenclamide bound/mg initial membrane protein after subtraction of non-specific binding and are the mean ± SEM of three experiments, each performed in triplicate —■— washed; —□— control

Results

Binding studies on total membranes. High affinity glibenclamide binding sites were detected in total membranes prepared from transplantable rat insuli-

noma cells (Fig. 1). The affinity of the insulinoma receptors (1.17 ± 0.18 nmol/l) was similar to those expressed in the CRI-G1 cell line ($K_d = 1.31 \pm 0.06$ nmol/l), although the B_{max} was approximately three-fold higher (4.3 ± 0.2 vs 1.4 ± 0.1 pmol/mg). Washing of the insulinoma membranes with NaCl and EDTA-containing solutions had no effect on the affinity of the receptor for glibenclamide (K_d of 1.23 ± 0.3 nmol/l) and only slightly reduced the number of receptors detected (B_{max} of 3.8 ± 0.1 pmol/mg initial protein). However, the specific activity of receptor binding increased from 4.3 ± 0.2 to 16.5 ± 0.5 pmol/mg following membrane washing.

Subcellular fractionation of insulinoma membranes. Nycodenz density gradient centrifugation of insulinoma tissue produced four membrane fractions designated A, B, C and D, in order of increasing density. Analysis of selected marker proteins showed that fraction A was enriched in plasma membranes, fraction B was a mixture of plasma membranes and secretory granules, fraction C was enriched in secretory granules and fraction D was a mixture of granules, mitochondria, lysosomes and endoplasmic reticulum (ER) (Table 1). Glibenclamide binding studies showed that the sulphonylurea receptor was distributed across the gradient with slightly higher specific activities detected in fractions B and C (Table 1). Thus, from total recoveries of each fraction it was estimated that fractions A, B, C and D contained 1 %, 3 %, 30 % and 66 % of the total recovered cellular binding sites, respectively. This was similar to the distribution of insulin secretory granules as fractions A, B, C and D contained 0.5 %, 2 %, 35 % and 62.5 % of the total recovered immunoreactive insulin, respectively, whereas ER, mitochondria

Table 1. Distribution of sulphonylurea receptors and marker proteins in subcellular fractions. Subcellular fractions prepared from rat insulinoma tissue from 15 rats were assayed for glibenclamide binding and the following marker proteins:

Marker	Specific activity units	Fraction				Total activity units	Fraction			
		A	B	C	D		A	B	C	D
Insulin	(nmol/mg)	5	12	21	15	(nmol)	2	9.6	168	300
β-N-Acetylglucosaminidase	(arbitrary units)	24	25	30	100	(arbitrary units)	9.6	20	240	2000
Alkaline phosphatase	nmol · min ⁻¹ · mg	930	467	127	52	(nmol/min)	372	373.6	1016	1040
NADPH-cytochrome c reductase	nmol · min ⁻¹ · mg	4	7	38	102	(nmol/min)	1.6	5.6	304	2040
Cytochrome c oxidase	nmol · min ⁻¹ · mg	26	27	28	94	(nmol/min)	10.4	21.6	224	1880
Glibenclamide binding	(fmol/mg)	664	1025	925	825	(fmol)	267	820	7400	16500
Protein		—	—	—	—	(mg)	0.4	0.8	8	20

(1) secretory granules (insulin), (2) lysosomes (β-N-acetylglucosaminidase), (3) mitochondria (cytochrome c oxidase), (4) Endoplasmic reticulum (NADPH-cytochrome c reductase) and (5) plasma membranes (alkaline phosphatase)

Fig. 2. Comparison of insulin content and sulphonylurea binding in insulin-secreting cells. The indicated membranes were incubated in 0.2 nmol/l [³H]-glibenclamide for 2 h and then bound radioactivity was collected by vacuum filtration onto GF/B disks and determined by liquid scintillation counting as described in the methods section. Non-specific binding (NSB) was determined in the presence of 1 μ mol/l glibenclamide. Results are expressed as fmol bound/mg protein after subtraction of NSB and are the mean $\pm$ SEM of three experiments. Insulin was determined by radioimmunoassay and results are the mean $\pm$ SEM of three experiments each performed in triplicate. Sulphonylurea receptors; Insulin

and lysosomes showed a different distribution (Table 1).

Insulin and sulphonylurea receptor levels in different beta-cell sources. Insulin radioimmunoassay and glibenclamide binding studies showed that there was a strong correlation ($R = 0.92$) between cellular insulin content and number of sulphonylurea receptors present in the corresponding total cellular membranes with the highest levels of each found in pancreatic islets and the lowest found in CRI-D11 cells (Fig. 2).

Photoaffinity labelling studies. Ultraviolet-cross-linking of [³H]-glibenclamide to membranes prepared from pancreatic islets, insulinoma and CRI-G1 cells labelled two proteins of apparent molecular weights 140 kDa and 170 kDa (Fig. 3). There was a difference in the relative levels of these two proteins in the different cell types. In CRI-G1 cells the 140 kDa species was the predominant component, whereas the 170 kDa form was more abundant in islets and insulinoma tissue. The two forms of the receptor were also distributed differently in different insulinoma subcellular membrane fractions (Fig. 4). The plasma membrane- and secretory granule-enriched fractions A, B and C contained only the 170 kDa form of the receptor whereas fraction D, which contained lysosomes, mitochondria and ER in addition to secretory granules, had both the 170 kDa and 140 kDa forms of the receptor. Neither of the

Fig. 3. Photoaffinity labelling of sulphonylurea receptors present in islets, insulinoma and CRI-G1 membranes. Membranes (50 μ g) were incubated with 10 nmol/l [³H] glibenclamide and irradiated at 302 nm for 15 min as described in the methods section. Labelled proteins in islets (track 1), insulinoma (track 2) and CRI-G1 (track 3) membranes were detected by SDS-PAGE and fluorography

proteins were labelled in the presence of 1 μ mol/l unlabelled glibenclamide (Fig. 4).

Discussion

Sulphonylurea receptors have been identified in a variety of tissues including pancreatic beta cells, ventricular myocytes and cerebral cortex [1]. The molecular structure of the receptor is poorly understood, although it has been suggested that it is glycosylated based on its ability to bind wheatgerm agglutinin and its apparent increase in mobility on SDS-PAGE gels following treatment with endoglycosidase F [26]. The present study showed that tumours propagated in NEDH rats express sulphonylurea receptors with a single type of high affinity binding site. These receptors remain associated with the membrane following stringent washing procedures suggesting that they might be either integral membrane proteins or covalently attached to the membrane. This observation is consistent with recent structural studies which proposed that the receptor contains multiple transmembrane spanning domains [27].

Sulphonylurea binding studies of insulinoma subcellular fractions showed that there was a high speci-

Fig. 4. Photoaffinity labelling of sulphonylurea receptors in insulinoma membrane fractions. Membranes (50 μ g) were incubated with 10 nmol/l [3 H] glibenclamide and irradiated at 302 nm for 15 min as described in the methods section. Labelled proteins in insulinoma membrane fractions A (track 1), B (track 2), C (track 3) and D (track 4) were detected by SDS-PAGE and fluorography. Labelling of fraction D was also carried out in the presence of 1 μ mol/l glibenclamide (track 5)

fic activity of receptors on secretory granule membranes. This is consistent with the observation that the levels of sulphonylurea receptors and insulin were correlated in a number of different sources of beta cells which differ markedly in their extent of granulation. The finding that most of these binding sites were intracellular is consistent with previous studies which have shown that the receptors are not sensitive to trypsinolysis of intact cells [28]. These findings support the hypothesis that sulphonylurea receptors might regulate insulin secretion by more than one mechanism and raises the possibility that this could occur through direct interaction of the receptor with granule membrane proteins such as those involved in granule movement or membrane fusion.

Little is known about the structural organization of the sulphonylurea receptor in membranes. Numerous studies employing photoaffinity labelling have shown that the high affinity sulphonylurea binding site is present on a polypeptide chain with an apparent molecular weight of 140 kDa to 150 kDa [5–7, 29]. The present photoaffinity labelling approach demonstrated that there are actually two species of sulphonylurea receptor with molecular weights of approximately 140 and 170 kDa in crude membrane preparations of rat islet. CRI-G1 and insulinoma cells. The subcellular distribution of these components in the insulinoma tissue was consistent with that of sulphonylurea binding sites, with a predominant localization to intracellular membranes. A small proportion of the 170 kDa form of the receptor was found in association with subcellular fractions enriched in plasma membrane and the 140 kDa form was present only in the subcellular fraction associated with dense organelles.

The relationship between the two receptors is not clear. It is possible that they are two forms of the same protein which arise from either differential mRNA splicing or post-translational modifications. It is unlikely that the 140 kDa species arises from non-specific proteolysis of the 170 kDa protein because protease inhibitors were present throughout membrane preparation and the relative proportion of the proteins was similar in separate experiments. Electrophysiological studies have shown that application of sulphonylureas to excised plasma membrane patches results in inhibition of K_{ATP} channel activity [30]. The presence of the 170 kDa form of the receptor in a subcellular fraction enriched in plasma membranes suggests that it might be the form responsible for regulation of K_{ATP} channels. It is less clear why most of the 170 kDa form and all of the 140 kDa receptor are localized to fractions containing a mixture of granules, mitochondria, ER and lysosomes. One possibility is that one or both of the receptors directly mediate exocytotic release of insulin. Another explanation is that these receptors could be associated with granule membrane ion channels which are involved in the regulation of the intragranular environment. Sulphonylurea-sensitive K^+ identified previously on mitochondrial membranes [31] and zymogen granules [32]. It has been suggested that these channels may play an important role in the fusion of zymogen granule membranes with the plasma membrane. It is possible that sulphonylureas also regulate the fusion of insulin secretory granule membranes with the plasma membrane. The presence of a large intracellular pool of sulphonylurea receptors may also provide a versatile mechanism by which interaction of sulphonylurea receptors with plasma membrane K_{ATP} channels could be regulated. For example, stimulation of the cell might result in exocytotic deposition of the granule membrane proteins in the vicinity of K_{ATP} channels, and removal of the stimulus might lead to their endocytotic retrieval for intracellular storage. Further studies are warranted to test these possibilities and to determine whether the two forms of the receptor have different activities.

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