Characterization of the Sulfonylurea Receptor on Beta Cell Membranes*

(Received for publication, September 11, 1987)

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Specific, high affinity sulfonylurea receptors were characterized on membranes of an insulin-secreting hamster beta cell line (HIT cells). Saturable binding of the sulfonylurea, [3H]glyburide, was linear up to 0.8 mg/ml membrane protein. Scatchard analysis of equilibrium binding data at room temperature indicated the presence of a single class of saturable, high affinity binding sites with a $K_D$ of 0.76 ± 0.04 nM and a $B_{max}$ of 1.09 ± 0.13 pmol/mg protein, $n = 9$. The insulin secretory potency of glyburide, glipizide, tolbutamide, tolazamide, and carboxytolbutamide was compared to the ability of these ligands to displace [3H]glyburide from the sulfonylurea receptor. Tolbutamide, tolazamide, and glipizide demonstrated reasonable agreement with $ED_{50}$ values of 15 µM, 3 µM, and 30 nM and $K_I$ values of 25.3 µM, 7.2 µM, and 45 nM, respectively. The inactive tolbutamide metabolite, carboxytolbutamide, at the highest concentration tested, only partially displaced [3H]glyburide from the receptor and was a very poor secretagogue. At 37 °C the affinity of [3H]glyburide binding, $K_D = 2.0$ nM, was similar to the $ED_{50}$ of 5.5 nM when the free glyburide concentrations were corrected for binding of the drug to albumin. These studies suggest that sulfonylureas initiate their biologic effect through a high affinity, specific interaction with sulfonylurea receptors on the beta cell membrane.

A variety of biologically active molecules, including certain drugs, elicit their cellular effects by first binding to specific receptors on the plasma membrane (reviewed in Ref. 1). The receptor ligand interaction then results in the generation of intracellular signals which activate the physiologic response characteristic of each cell type. Sulfonylureas have been used for almost three decades to treat non-insulin-dependent diabetestes. We have recently shown that they signal insulin release from the pancreatic beta cells by allowing $Ca^{2+}$ to enter through voltage-dependent $Ca^{2+}$ channels, increasing the free cytosolic calcium level ([Ca$^{2+}$]) (2). However, the mechanism by which these drugs first interact with the beta cell remain controversial. In isolated pancreatic islets the volume of distribution of the sulfonylurea, tolbutamide, only slightly exceeds that of extracellular space markers (3), suggesting an interaction with the beta cell membrane. Nevertheless, it has proven difficult to identify and characterize a sulfonylurea receptor. Although putative sulfonylurea receptors have been identified on crude rat brain membranes (4), this tissue is not known to be activated by these drugs. In the same study, binding of the sulfonylurea, glikinone, to a rat beta cell tumor was of high affinity and saturable, suggesting an interaction with specific membrane receptors. However, Hellman has questioned the existence of specific sulfonylurea receptors. He points out that saturable and presumed specific binding of these compounds to nonbiologic systems can be demonstrated (5). Moreover, Hellman's studies have not shown a correlation between the secretory potency of individual sulfonylureas and their ability to bind to isolated pancreatic islets (6). He has suggested that these drugs are coupled to the insulin secretory process by a lipophilic interaction with the phospholipid domain in the membrane (7) that then activates voltage-dependent Ca$^{2+}$ channels.

To determine if the sulfonylureas signal insulin release by a specific interaction with a putative sulfonylurea receptor, we have used membranes isolated from the HIT cells to identify and characterize the receptor. These experiments provide clear evidence for the existence of specific, high affinity sulfonylurea receptors on beta cell membranes which initiate the biologic effects of these important drugs.

**EXPERIMENTAL PROCEDURES**

Materials—[3H]Glyburide (2 Ci/mmol), tolbutamide, glyburide, tolazamide, and carboxytolbutamide were generous gifts from the Upjohn Company. Glipizide was kindly provided by Roerig. The radioactive purity of [3H]glyburide, which was tritiated in the benzamide ring, was 98% as judged by thin layer chromatography. Tris base, bovine serum albumin (fraction V, radiiodimunassay grade), and disopropyl fluorophosphate were obtained from Sigma, and MOPS* was from Gibco. Whatman GF/F glass microfiber filters were obtained from Fisher.

**Cell Culture and Insulin Secretory Studies**—HIT cells were cultured and insulin secretion was monitored in static incubation as previously described (2, 8, 9). In brief, HIT cells were plated into 12-well Castar plates (3 x 10$^5$ cells/25-mm well) and grown for 3 days. On the day of the experiment, growth medium was removed, and the cells were preincubated for 45 min in Krebs-Ringer bicarbonate basal buffer. This was followed by a second incubation in basal buffer for 15 min, and a third incubation, containing the sulfonylurea, for 10 min. The buffer was saved and insulin content was measured by radioimmunoassay (2). All secretory experiments were performed at nonstimulatory concentrations of glucose (1.7 mM). During the period of these experiments, insulin secretion by these cells was monitored weekly by radioimmunoassay in response to the sulfonylureas, glyburide, and tolbutamide, glucose, and high K$^+$ concentration to verify the continued differentiated function of the HIT cells. All of these studies were performed between passages 61 to 77 and insulin release to the sulfonylurea was similar between these passages.

**Membrane Preparation**—Membranes were prepared from HIT cells collected from confluent flasks and washed twice with phosphate-buffered saline, pH 7.4. Cells were frozen at −70 °C until sufficient cell numbers were collected. The pellets were resuspended in ice-cold 5 mM Tris base, pH 8.0, containing 0.1 mM disopropyl fluorophosphate and transferred to a Kontes glass homogenizer. The cell suspension was incubated on ice in this hypotonic buffer for 40

*This work was supported by National Institutes of Health Grants DK34447 and DK72665 (to A. E. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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* The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.
min and then homogenized. The resulting homogenate was centrifuged for 10 min at 900 × g. The supernatant was collected and centrifuged at 96,000 × g for 30 min in a Beckman L8-55M ultracentrifuge. The pellets from the second centrifugation were resuspended in 20 mM MOPS, pH 7.4, containing 1 mM diisopropyl fluorophosphate and rehomogenized. The membranes were immediately frozen in liquid nitrogen until binding studies were performed. Membrane protein concentrations were determined according to the method of Lowry (10) using bovine serum albumin as a standard.

\[ ^\text{[H]}\text{Glyburide} \] Binding—The binding assay was performed in 50 mM MOPS, pH 7.4, 0.1 mM CaCl\(_2\), in a total volume of 1 ml/tube. Membrane protein (200 µg/ml) was incubated with \[ ^\text{[H]}\text{Glyburide} \] at concentrations between 0.15 and 10 nM for 2-3 h at room temperature (26 °C). Each concentration was performed in duplicate and experiments were replicated at least three times. Stock solutions of unlabeled drugs were prepared in dimethyl sulfoxide which had no effect on binding. Nonspecific binding was determined in the presence of 1 µM unlabeled glyburide and accounted for 5-15% of total binding. Binding was terminated by rapid filtration through Whatman GF/F glass fiber filters followed by five washes with ice-cold distilled water. Filters were counted wet in 10 ml of Beckman HP/B scintillant.

Competitive inhibition assays were performed with the concentrations of \[ ^\text{[H]}\text{Glyburide} \], inhibitors, and protein indicated in the figure legends. Buffer concentrations and incubation conditions were the same as those of the direct binding assays.

Data Analysis—Direct binding data were fit to a one-site model based on a Marquardt nonlinear least squares method to estimate the free parameters \( K_d \) and \( B_{\text{max}} \). (11). Competitive inhibition data was also fit by this method to estimate the free parameter \( K_i \) using \( K_d \) and \( B_{\text{max}} \), values obtained from direct binding studies. Parameter uncertainties were provided by \( \chi^2 \) values. Scatchard analysis of direct binding data was performed using a least squares fit linear regression program (12). Correlation coefficients were greater than 0.95. The insulin secretory dose response curves were calculated as previously described (2).

RESULTS

Time Course and Effect of Protein on \[ ^\text{[H]}\text{Glyburide} \] Binding to HIT Cell Membranes—In preliminary experiments, we found the saturable or specific binding of 1 nM \[ ^\text{[H]}\text{Glyburide} \] to HIT cell membranes at room temperature reached equilibrium by 2 h. The specific binding of \[ ^\text{[H]}\text{Glyburide} \] to HIT cell membranes was linear with protein concentration up to 0.8 mg/ml HIT cell membrane protein (data not shown).

Concentration Dependence of Equilibrium Binding to HIT Cell Membranes—Fig. 1 shows the binding of increasing concentrations of \[ ^\text{[H]}\text{Glyburide} \] to HIT cell membranes at room temperature. As the concentration of \[ ^\text{[H]}\text{Glyburide} \] was increased from 0.15 to 10 nM, specific binding to 200 µg of membrane protein increased up to 5 nM of the radioligand. The nonspecific binding was minimal and rose from 5 to 15% of total binding with increasing \[ ^\text{[H]}\text{Glyburide} \] concentrations.

Scatchard Analysis of \[ ^\text{[H]}\text{Glyburide} \] Binding—Scatchard analysis of the equilibrium binding data revealed a single population of high affinity binding sites with a dissociation constant \( K_d \) of 0.76 ± 0.04 nM and a maximum number of binding sites \( B_{\text{max}} \) of 1.09 ± 0.13 pmol/mg protein, \( n = 9 \) (Fig. 1, inset). A Hill plot yielded a slope of 0.98, suggesting a lack of cooperativity in the binding. Binding studies in the presence of tolbutamide indicated that this ligand is competitive with \[ ^\text{[H]}\text{Glyburide} \] (data not shown).

To test the structural requirements for receptor binding, we examined the ability of the sulfonylureas, tolanazamide, tolbutamide, carboxytolbutamide, glyburide, and glipizide to inhibit the binding of \[ ^\text{[H]}\text{Glyburide} \] binding to HIT cell membranes. Fig. 2 shows the inhibition curves of these sulfonylureas. The \( K_i \) for tolbutamide was calculated to be 25.3 µM. Tolanazamide, which is reported in clinical studies or in isolated islets to be slightly more potent than tolbutamide as an insulin secretagogue (13), gave a \( K_i \) of 7.2 µM. Carboxytolbutamide, a relatively inactive tolbutamide metabolite, pos-

![Fig. 1. \[ ^\text{[H]}\text{Glyburide} \] binding to HIT cell membranes, HIT cell membranes (200 µg of protein) were incubated in 1 ml of 50 mM MOPS (pH 7.4) with \[ ^\text{[H]}\text{Glyburide} \] at concentrations from 0.15 to 10 nM for 2 h at room temperature. Bound radioligand was separated from free by filtration through Whatman GF/F filters. Specific pico- moles of \[ ^\text{[H]}\text{Glyburide} \] bound per milligrams of protein (O) was determined by subtracting nonspecific binding (●) (those counts remaining in the presence of 1 µM unlabeled glyburide from the total amount bound). Each point represents the mean ± S.E. of nine different experiments performed in duplicate. Inset, Scatchard analysis of specific \[ ^\text{[H]}\text{Glyburide} \] binding to HIT cell membranes with and without unlabeled glyburide. Each point represents the mean of nine experiments with \[ ^\text{[H]}\text{Glyburide} \].](image)

![Fig. 2. Effect of other sulfonylureas on \[ ^\text{[H]}\text{Glyburide} \] binding. \[ ^\text{[H]}\text{Glyburide} \] (1.25 nM) was added to HIT cell membranes (200 µg of protein) in the presence of 1 pmol to 1 µM cold glyburide (A), 1 nM to 1 mM concentrations of glipizide (Δ), tolanazamide (□), tolbutamide (○), and carboxytolbutamide (●) and incubated for 3 h at room temperature. \( K_i \) values were 7.2 µM, 25.3 µM, and >1 mM for tol- amide, tolbutamide, and carboxytolbutamide, respectively, and 45 nM for glipizide and 0.24 nM for glyburide.](image)

| \( K_i \) | \( ED_{50} \) |
|---|---|
| Tolanazamide | 7.2 µM | 3.0 ± 0.3 µM |
| Tolbutamide | 25.3 µM | 15.0 ± 3 µM |
| Carboxytolbutamide | >1 mM | >1 mM |
| Glyburide | 0.7 nM | 112.0 ± 18 nM |
| Glipizide | 45 nM | 30 nM |

TABLE I

Comparison of \( K_i \) values for binding displacement to \( ED_{50} \) values for insulin secretion by HIT cells

Varying concentrations of sulfonylureas were added to HIT cell membranes (200 µg of protein) and incubated with \[ ^\text{[H]}\text{Glyburide} \]. \( K_i \) values were calculated from computer-generated curves as described under “Experimental Procedures.” \( ED_{50} \) values were mean ± S.E. values determined from two to five static secretion experiments on HIT cell monolayers as described previously (2).
The receptor at all concentrations of the drug (Fig. 3) and the binding curve to the right, which more closely approximates the glyburide insulin secretory dose response curve. The $K_i$ of binding at room temperature is $0.76 \pm 0.04 \text{nM}$ versus a $K_i$ of 2.0 nM at $37^\circ C$ with no change in the number of binding sites, suggestive of an enthalpy-driven interaction. Sulfonylurea Receptor on Beta Cell Membranes

**Figure 3.** $[^{3}H]$Glyburide binding to HIT cell membranes at room temperature and at $37^\circ C$ compared to glyburide stimulation of insulin secretion. HIT cell membranes were incubated at either room temperature (○) or $37^\circ C$ (•) for 2 h with varying concentrations of $[^{3}H]$glyburide. Bound and free counts were separated as described under "Experimental Procedures." HIT cell monolayers were grown in 12-well plates as described in Ref. 2 and incubated with varying concentrations of glyburide, and the insulin concentration was measured in the incubation buffer for a 10-min period. Insulin secretion (□) was determined as described in Ref. 9. Each point is the mean of five to eight determinations. Determination of $[^{3}H]$glyburide binding to albumin and are used at much higher concentrations than glyburide in insulin secretory studies (micromolar versus nanomolar). There was also excellent agreement in glipizide and biologic activity without correction for albumin.

Upon increasing the temperature to $37^\circ C$, the affinity of glyburide binding was decreased by approximately 35%. This shifts the binding curve to the right (see Fig. 3), which more closely approximates its biologic effect. Decreased affinity of a ligand for its receptor with increases in temperature is sometimes characteristic of agonist-receptor interactions which are enthalpy-driven. Temperature is known to alter both the number of binding sites and the dissociation constant of certain receptors. For example, the number of β-receptors identified by $[^{3}H]$-hydroxybenzyl-pindolol binding decreases with increasing temperature. This effect appears to be due to, at least in part, changes in the fluidity at the lipid bilayer with some of the receptors becoming increasingly cryptic and undetectable (15).

A great deal of data using a variety of techniques now indicate that sulfonylurea signal insulin release through an effect on an ATP-sensitive K+ channel. Early studies of ion flux using $[^{86}Rb]$ to measure K+ efflux from isolated islets demonstrated that the first effect of these drugs was to decrease K+ permeability leading to depolarization (16). Recent studies utilizing the cell-attached patch clamp technique have identified a potassium channel in both dispersed beta cells and insulin-secreting cell lines which is active at the

**DISCUSSION**

These studies characterize for the first time sulfonylurea receptors on the membranes of an SV40-transformed hamster beta cell line (HIT cells). A great deal of evidence implicates this receptor as necessary for the biologic action of these drugs. In both clinical and in _vitro_ studies, glyburide and glipizide are much more potent insulin secretagogues than tolbutamide, which is slightly more potent than tolbutamide. Carboxytolbutamide is a relatively inactive metabolite of tolbutamide (13). These five ligands demonstrated that same rank order of binding to the sulfonylurea receptor on HIT cell membranes. Furthermore, the biologic potency of glipizide, tolbutamide, and tolazamide, as reflected by the $ED_{50}$ values on insulin secretion of 30 nM and 15 and 3 μM, was closely related to the inhibitor constants for concentration displacement of $[^{3}H]$glyburide from the receptor of 45 nM and 25.3 and 7.2 μM, respectively. Carboxytolbutamide had very little effect on insulin secretion and only weakly displaced $[^{3}H]$glyburide from its binding site.

The binding constant of glipizide $K_i = 0.76 \text{nM}$ did not as closely approximate its $ED_{50}$ (112 nM) as did the other sulfonylureas. However, all of the sulfonylureas bind to albumin, and glyburide is the most strongly bound. The interaction of glyburide appears nonionic, whereas the other drugs may interact by ionic mechanisms (14). Insulin secretory studies must be performed with albumin present in the buffers to decrease the nonspecific sticking of insulin to the surfaces of the Petri dishes or perfusion chambers. At 0.1% albumin used in the secretory studies, the binding of glyburide to albumin (14) would greatly reduce the free concentration available to stimulate insulin release. Correcting the added concentration of glyburide for the amount bound to albumin, using the binding constants reported by Crooks and Brown (14), gives rise to free concentrations of glyburide which shifts the dose response curve for insulin secretion to the left. The corrected concentration response curve (Fig. 3) with an $ED_{50} = 5.5 \text{nM}$ more closely correlates with the $[^{3}H]$glyburide binding curve of 2 nM at $37^\circ C$. This is a much less significant factor using tolbutamide or tolazamide, which bind with less avidity to albumin and are used at much higher concentrations than glyburide in insulin secretory studies (micromolar versus nanomolar). There was also excellent agreement in glipizide binding and biologic activity without correction for albumin.

Upon increasing the temperature to $37^\circ C$, the affinity of glyburide binding was decreased by approximately 35%. This shifts the binding curve to the right (see Fig. 3), which more closely approximates its biologic effect. Decreased affinity of a ligand for its receptor with increases in temperature is sometimes characteristic of agonist-receptor interactions which are enthalpy-driven. Temperature is known to alter both the number of binding sites and the dissociation constant of certain receptors. For example, the number of β-receptors identified by $[^{3}H]$-hydroxybenzyl-pindolol binding decreases with increasing temperature. This effect appears to be due to, at least in part, changes in the fluidity at the lipid bilayer with some of the receptors becoming increasingly cryptic and undetectable (15).
resting membrane potential (17, 18) and is closed by the metabolism of glucose or other metabolizable insulin secretagogues, mannose, leucine or glyceroldehyde (19), ATP (19, 20) or the sulfonylureas (21–24). The channel reopens on addition of the appropriate metabolic inhibitors which block utilization of that particular substrate (19). The effect of sulfonylureas can be demonstrated in outside-out patches of membrane, suggesting that the effect of sulfonylureas on K+ channels is not due to a second messenger. We have confirmed the patch-clamp study using the HIT cells and have shown that the inactivation of the ATP-sensitive K+ channel by either glyburide or tolbutamide occurs at drug concentrations over the range which saturates the sulfonylurea receptor (25).

Based on patch-clamp studies, Sturgess and co-workers (21) have hypothesized that the sulfonylurea receptor or a closely associated protein is the ATP-sensitive K+ channel. The binding data in our study indicate this hypothesis is reasonable and the hypothesis is now subject to direct experimentation. There is precedent for a receptor being an ion channel since the nicotinic acetylcholine receptor and the dihydropyridine/benzodiazepine receptor isolated from bovine brain shows homology with other ligand-gated receptor subunits, suggesting there is a superfamily of ion channel receptors (28).

The vectorial process by which the biochemical expression of sulfonylurea receptor occupation is transmitted into an intracellular signal appears clear. Upon binding of the drug to the receptor, the ATP-sensitive K+ channel is inactivated, decreasing K+ efflux. This is followed by cell depolarization which opens voltage-dependent Ca2+ channels, allowing the entry of extracellular Ca2+. The rise in [Ca2+]i is the signal which triggers insulin release (2).

In summary, these studies demonstrate that sulfonylureas bind with high affinity to specific receptors on the beta cell membrane. The affinity of binding of the sulfonylureas to the receptor reflects the ability of these compounds to stimulate insulin secretion from the beta cell.

Acknowledgments—We would like to thank Kevin Brush, Cheryl Neal, and Farideh Bagheri for technical assistance, and Joni Kellen and Tammy Cummings for preparation of the manuscript.

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