Characterization and Significance of Sulfonylurea Receptors

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This study describes and characterizes a putative sulfonylurea receptor. The radioligand used was [^{3}H]glipizide (9 Ci/mmol). The β-cell plasma membranes were derived from a transplantable rat insulinoma generated by subcutaneous injection of RINm5F cells and purified by ultracentrifugation on a 15–55% sucrose gradient. Specific binding of [^{3}H]glipizide to purified β-cell plasma membranes was determined to be maximal at temperatures of 4–23°C, pH 7.3, and an incubation of 2 h. Scatchard analysis indicated a single binding site with K_d = 7 nM and sulfonylurea binding of 0.93 pmol/mg membrane protein. Displacement of [^{3}H]glipizide from the purified β-cell plasma membranes by various sulfonylureas and their analogues correlated well with their known hypoglycemic and insulin-releasing activities. Various agents, including nutrients, agents affecting Ca^{2+} flux, gastrointestinal hormones, and pancreatic hormones, had no effect on [^{3}H]glipizide binding to the β-cell plasma membranes. Putative sulfonylurea receptors on β-cell and brain cell plasma membranes have been reported by several groups of investigators. Sulfonylurea binding to the β-cell is hypothesized to close an ATP-sensitive K^+ channel, which leads to depolarization of the membrane and activation of a voltage-dependent Ca^{2+} channel.
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The mechanisms by which sulfonylureas exert their various pancreatic and extrapancreatic actions have eluded delineation for >40 yr. Several early observations suggested that sulfonylurea stimulation of insulin secretion might involve an action at the plasma membrane. Initial studies on the in vivo distribution of tolbutamide led to the conclusion that tolbutamide was restricted to the extracellular compartment, except perhaps in the liver (1). Fifteen years later, when an appropriate in vitro system was available, the uptake of [^{35}S]tolbutamide into microdissected pancreatic islets from *ob/ob* mice was compared with that of [^{3}H]sucrose, [^{3}H]mannitol, and [^{3}H]-3-*O*-methylglucose (2). The sulfonylurea was found to equilibrate with the extracellular space markers (2,3). Subsequently, dextran-linked tolbutamide was perfused through isolated rat pancreas at concentrations equivalent to those of the free sulfonylurea and caused comparable increases in first-phase insulin secretion (4).

Therefore, we initiated studies in the early 1980s to see whether we could identify a specific plasma membrane sulfonylurea binding site on the β-cell and, if so, what its significance might be. Herein, we describe our results and review the results of several other investigations.

RESEARCH DESIGN AND METHODS

[^{3}H]glipizide was synthesized utilizing intermediates provided by Pfizer (New York). Its specific activity was 9 Ci/mmol, and purity was proved by chromatography. Subsequent high-performance liquid chromatography analyses revealed three major peaks that had approximately equal binding activity. Glipizide and chlorpropamide were provided by Pfizer. Tolbutamide and carboxytolbutamide were gifts of Upjohn (Kalamazoo, MI). Glyburide and HB 699 were obtained from Hoechst (Frankfurt, FRG).

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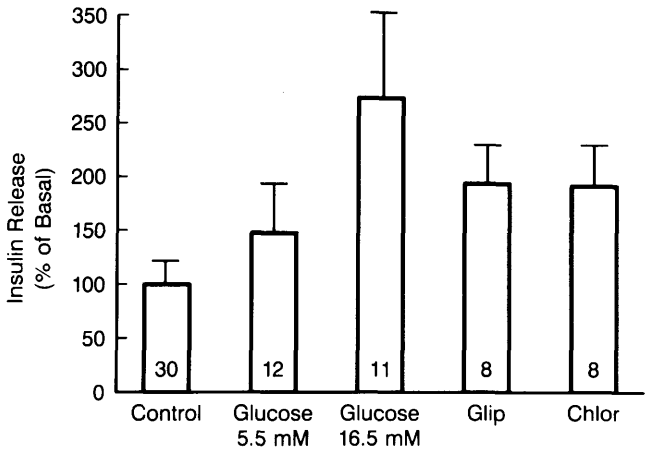

FIG. 1. Stimulation of insulin secretion by sulfonylureas in RINm5F cells in culture. Experimental design is described in RESEARCH DESIGN AND METHODS. Insulin release was measured after incubation in RPMI-1066 medium with 3 mM glucose. Glip, 10 μM glipizide; chlor, 350 μM chlopropamide. Numbers of observations are given within bars. Data are means ± SE.

For preparation of β-cell plasma membranes, cloned RINm5F cells were injected subcutaneously into an inbred strain of rats initially obtained from the New England Deaconess Hospital. After several weeks, a palpable mass developed, and it was surgically removed when it was sufficiently large to cause the rat to have a random plasma glucose ≤2.78 mM. After the tumor tissue was carefully dissected away from connective tissue and blood vessels, 1 g of it was homogenized at 4°C per 10 ml of a buffer, pH 7.5, containing 10 mM imidazole, 250 mM sucrose, and 5 mM EDTA. The homogenate was centrifuged for 10 min at 4°C and 1000 × *g* to remove cellular debris. The supernatant was then centrifuged at 4°C and 12,000 × *g* for 40 min to pellet the membrane and organelle fractions. The pellets were then resuspended in 1 mM imidazole, pH 7.5, and layered on top of a continuous 15–55% sucrose gradient. Plasma membrane fractions were collected after a 16-h 100,000 × *g* centrifugation at 4°C. The plasma membrane fraction was identified by the presence of a peak of 5′-nucleotidase activity and the absence of significant NADH oxidase activity. Protein content was determined by the method of Lowry (4a).

For insulin secretory studies, RINm5F cells were grown in RPMI-1066 medium plus 10% newborn calf serum in 250-ml flasks at 37°C in an atmosphere of 10% CO_2/90% O_2 to confluence. They were then transferred to plates with 25 wells (1 cm diam) and again grown to confluence. The cells were then preincubated in RPMI-1066 medium with 3 mM glucose for 2 h at 37°C in 10% CO_2/90% O_2, after which the medium was changed, and they were incubated in RPMI-1066 medium that contained either 3 (control), 5.5, or 16.5 mM glucose or 3 mM glucose with glipizide or chlorpropamide for 1 h. The medium was removed and assayed for insulin by radioimmunoassay. The cells were digested in 0.1 M NaOH and assayed for protein.

For binding studies, plasma membranes were used at a final concentration of 250 μg protein/ml. [^{3}H]glipizide radioligand was used at a concentration of 50 nM. Binding was carried out in 10 × 75-mm glass tubes precoated with 5% bovine serum albumin in a buffer of 100 mM HEPES and 1% bovine serum albumin. Binding parameters were chosen after appropriate studies. We used the following conditions for incubation: pH 7.4, temperature 23°C, and 2-h incubation. At the end of the reaction period, plasma membranes were sedimented by centrifugation at 16,000 × *g* for 10 min at 4°C. The supernatants were aspirated, and the tubes were rinsed rapidly with iced buffer (100 mM HEPES and 0.3% bovine serum albumin at pH 7.5). Membranes were solubilized with Protosol (Du Pont-NEN, Boston, MA), transferred to a toluene-based scintillar, and counted by liquid-scintillation spectrometry. All experiments included controls for background binding in which no membranes were added to the incubation and controls in which nonspecific binding was measured by adding 1000-fold excess of 50 μM unlabeled glipizide.

RESULTS

Plasma membranes from transplantable rat islet cell tumor tissue generated from the m5 clone of RIN cells were chosen for the binding studies because this clone shows a classic insulin secretory response to sulfonylureas. Figure 1 shows the in vitro effect of glipizide and chlorpropamide in stimulating insulin secretion from these cells in vitro. Both increase insulin secretion to twice that of the basal state.

A series of experiments was carried out to determine the appropriate conditions for studying the binding of

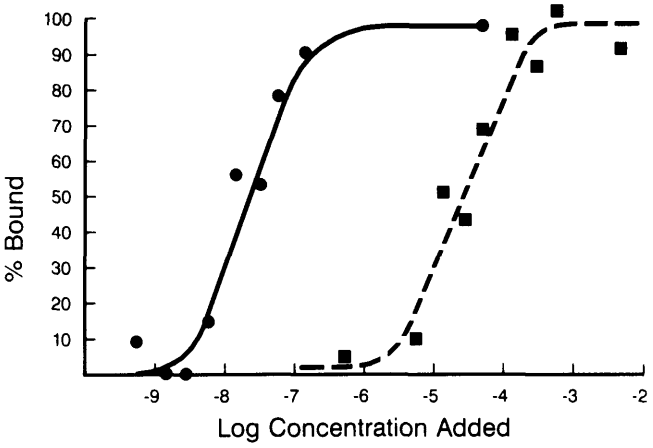

FIG. 2. Specific binding of glipizide (*solid line*) and tolbutamide (*dashed line*) to purified plasma membranes from transplantable RINm5F-generated insulinomas. Curves are derived from displacement of radiolabeled glipizide by glipizide or tolbutamide. Concentration is molar.

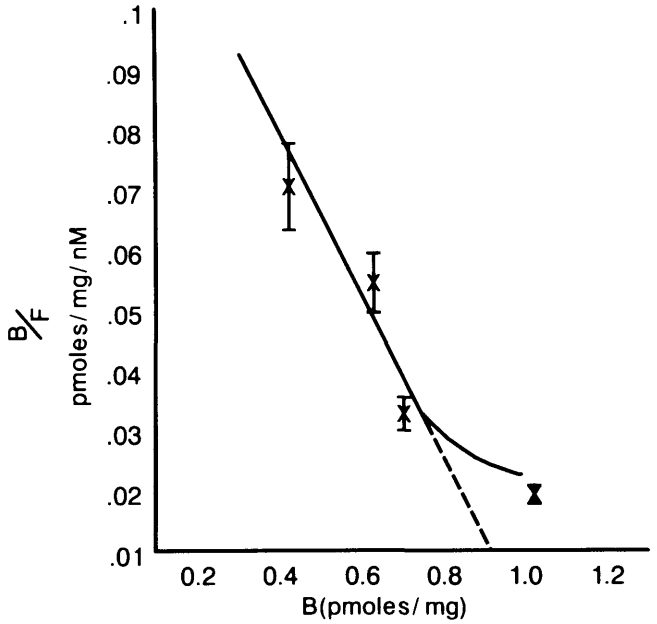

FIG. 3. Scatchard plot of [³H]glipizide binding to purified plasma membranes from RINm5F-generated insulinomas. X, mean ± SE for each group; B/F, ratio of bound to free sulfonylurea; B, bound sulfonylurea. Data show single class of binding sites. K_d = 7 nM; R_o (no. of binding sites), 0.93 pmol/mg.

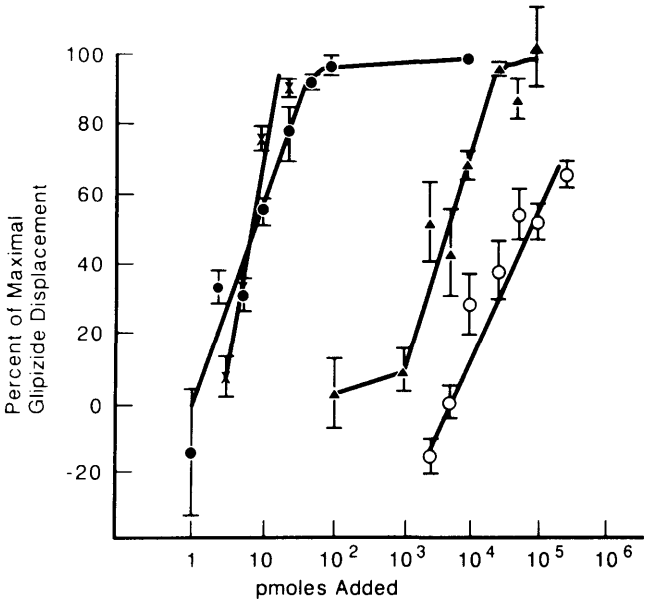

FIG. 4. Displacement of [³H]glipizide from purified plasma membranes from RINm5F-generated insulinomas by glipizide (●), glyburide (X), tolbutamide (▲), and chlorpropamide (○). Data are means ± SE of 3 determinations.

[³H]glipizide to the purified insulinoma plasma membranes. The maximum specific binding of [³H]glipizide was noted at pH 7.3. Binding falls sharply on either side of pH 7.3 (pH 7, 80% of maximal binding; pH 7.8, 77% of maximal binding). A time course of [³H]glipizide binding to the plasma membranes showed that specific binding is 90% complete in 1 h and peaks at 2 h. Longer incubations (≥4 h) result in a marked decrease of specific [³H]glipizide binding. At 10 h, 58% of the specifically bound glipizide remains, but the nonspecific binding component rises to 75% of the total picomoles bound. Using a 2-h incubation, we then characterized the temperature dependence of [³H]glipizide binding to the plasma membranes at 4, 23, and 37°C. Maximal specific binding was noted at 4°C with a 15% decrease at 23°C and a 40% decrease at 37°C. Nonspecific bind-

TABLE 1
Correlation between in vivo hypoglycemic activity and in vitro [³H]glipizide displacement from purified tumor plasma membranes derived from RINm5F insulinomas

Agent	Hypoglycemic activity (relative to glipizide)	Concentration giving 50% [³H]glipizide displacement (M)
Glipizide	1.0	3.2×10^{-8}
Glyburide	1.0	3.2×10^{-8}
HB 699	0.005–0.001	2.2×10^{-5}
Chlorpropamide	0.02	3.6×10^{-4}
Tolbutamide	0.005	2.2×10^{-5}
Carboxytolbutamide	Inactive (<0.00001)	1.0×10^{-2}

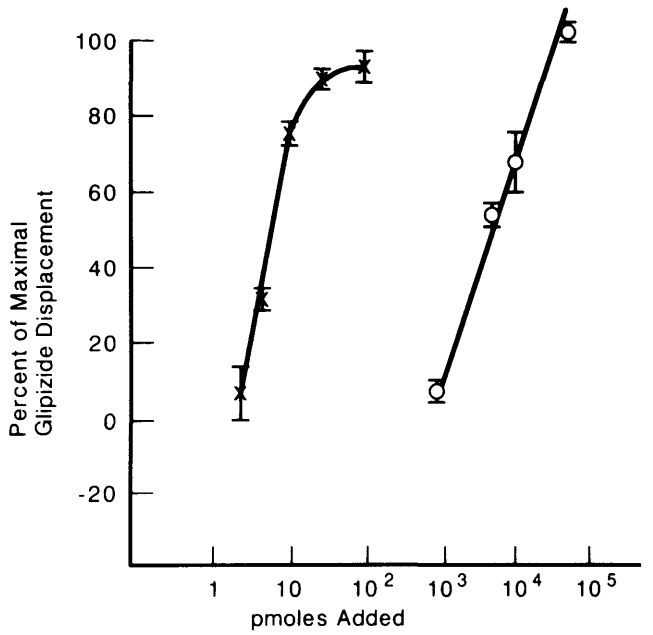

FIG. 5. Displacement of [³H]glipizide from purified plasma membranes from RINm5F-generated insulinomas by glyburide (X) and its nonsulfonylurea analogue, HB 699 (○). Data are means ± SE of 3 determinations.

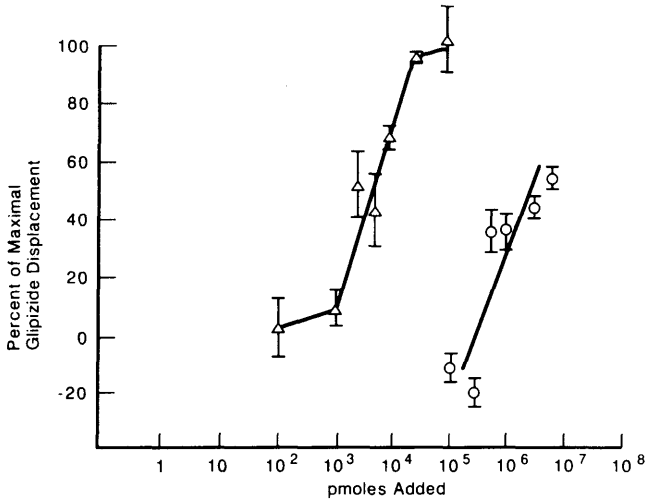

FIG. 6. Displacement of [^{3}H]glipizide from purified plasma membranes from RINm5F-generated insulinomas by tolbutamide (△) and its metabolite carboxytolbutamide (○). Data are means ± SE of 3 determinations.

ing was ~50% at 4°C and increased to 73% at 37°C. Thus, it appeared that 4 and 23°C were more appropriate temperatures for binding studies than was 37°C. All subsequent binding studies were carried out at room temperature for 2 h in a buffer of pH 7.4.

Specific glipizide binding to the plasma membranes of RINm5F insulinoma cells was demonstrated. Figure 2 plots the effect of increasing concentrations of unlabeled glipizide or tolbutamide on sulfonylurea binding utilizing [^{3}H]glipizide as the radioligand. Saturation of the specific sites occurs at a concentration 2 orders of magnitude greater than that at which the binding can first be demonstrated. These sigmoidal curves have the characteristics of a hormone–hormone receptor interaction.

If the binding of glipizide is carried out under conditions in which the amount of sulfonylurea added to the plasma membranes is varied, a B/F versus B plot can be defined, where B/F is the ratio of the bound to free sulfonylurea, and B is bound sulfonylurea. The results of one such experiment are shown in Fig. 3. It appears that the binding site is a single site, has a K_d = 7 nM, and can bind up to 0.93 pmol/mg membrane protein. Because of the biological variation among individual transplanted tumors, the high nonspecific binding component (30–50%) and the relatively low specific activity of the radiolabeled ligand, these binding constants must be considered tentative.

We next addressed the issue of whether this binding site is relevant to sulfonylurea antidiabetic action in vivo. We reasoned that, if the binding site mediates sulfonylurea action, there should be a high degree of correlation between the binding affinities of the various sulfonylureas and their known insulin-releasing and/or hypoglycemic activities. We subsequently studied the ability of other sulfonylureas and related drugs to compete with [^{3}H]glipizide for the presumed sulfonylurea binding site. Table 1 lists the approximate hypoglycemic potency of several sulfonylureas and related compounds. Figure 4 shows the displacement of [^{3}H]glipizide from the purified plasma membranes isolated from the RINm5F insulinoma by glyburide, tolbutamide, and chlorpropamide. The degree of displacement correlates well with the hypoglycemic activity of the specific sulfonylurea (Table 1).

Figure 5 shows that the nonsulfonylurea analogue of glyburide, HB 699, which has weak hypoglycemic activity, also weakly displaces [^{3}H]glipizide binding. This indicates that it is the hypoglycemic activity site and not the sulfonylurea grouping that is responsible for interacting with the binding site. Figure 6 shows that the inactive metabolite of tolbutamide, carboxytolbutamide, has very little activity in displacing [^{3}H]glipizide binding.

If these data are accepted as evidence that a specific sulfonylurea binding site exists on β-cell plasma membranes and that the initial event in sulfonylurea action on the β-cell is binding to this receptor, then we must ask, what is the naturally occurring ligand for this site? We examined several agents, e.g., nutrients, agents affecting Ca^{2+} flux, pancreatic hormones, gastrointestinal hormones, and catecholamines, at concentrations up to the maximum listed in Table 2 and found absolutely no effect on [^{3}H]glipizide binding to the β-cell tumor plasma membranes.

TABLE 2
Agents with no effect on glipizide binding

Category	Concentrations
Nutrients	
D-Glucose	20 mM
L-Arginine	1 mM
L-Leucine	1 mM
Agents affecting Ca^{2+} flux	
Verapamil	40 μM
A 23187	10 μg/ml
Pancreatic hormones	
Insulin	2.5 μg/ml
Glucagon	100 nM
Pancreatic polypeptide	350 nM
Somatostatin	100 nM
Gastrointestinal hormones	
Gastric inhibitory peptide	100 nM
Substance P	625 nM
Gastrin	100 nM
Secretin	100 nM
Catecholamines	
Epinephrine	10 μM
Isoproterenol	10 μM

[^{3}H]glipizide displacement from β-cell tumor plasma membranes was tested with the binding assay and varying concentrations of these agents. The maximum concentration tested is listed.

TABLE 3
Studies demonstrating sulfonylurea plasma membrane binding sites on β-cells

Ligand	Binding site	K_d(nM)	R_o(pmol/mg)	Refs.
[^{3}H]glipizide	Syrian hamster tumor crude membranes	17,900	204	5
[^{3}H]glipizide	Rat insulinoma purified plasma membranes	7.0	0.93	6,7
[^{3}H]glyburide	RINm5F cell microsomes	0.3	0.15	8
[^{3}H]glyburide	HIT cell membrane pellet	0.76	1.09	9
[^{3}H]glyburide	Rat insulinoma crude membranes	0.01–0.05	0.29–0.52	10
		0.24–0.32	0.75–1.29	

R_o, number of binding sites.

DISCUSSION

The presence of a specific sulfonylurea binding site on β-cells has been described by several groups of investigators. Table 3 summarizes the results of those studies. All used tumor cells as the source of β-cells, and the radiolabeled ligand has predominantly been glipizide or glyburide (5–10). The studies of Duran-Garcia et al. (5) were done with crude membranes from a Syrian hamster tumor that secreted relatively little insulin and was probably composed of a very small percentage of β-cells, so the data are uninterpretable. A single high-affinity low-capacity binding site was found by Gaines et al. (9), whereas Geisen et al. (10) found two binding sites, one with a somewhat higher affinity than the other. The quantity of binding sites in the studies varied from 0.15 to 1.81 pmol/mg. Obviously, the quantity of binding sites per milligram of membrane will vary depending on the source and purity of the membranes.

Major proof of the importance of sulfonylurea binding to the β-cell plasma membrane to its antidiabetic actions would be a strong correlation between binding affinity and hypoglycemic activity for all compounds studied. Our investigations show such a tight correlation, as do those of Gaines et al. (9). However, Geisen et al. (10) studied many chemical compounds structurally related to glyburide and found many in which there was no correlation, i.e., compounds that showed high-affinity K_ds (10^{-7}–10^{-8} M) and had no hypoglycemic activity and compounds with K_ds comparable to glyburide but with significantly lower hypoglycemic activity. Thus, this issue of correlation of binding affinity with hypoglycemic activity needs further resolution.

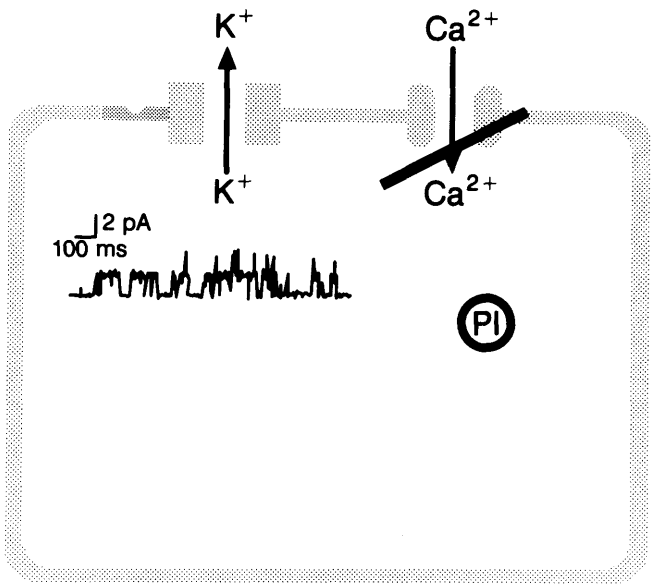

FIG. 7. Schema of resting β-cell. ATP-sensitive K^+ channel is open, and voltage-dependent Ca^{2+} channel is closed. Insulin granules containing proinsulin (PI) are being extruded very slowly. Typical voltage pattern from patch clamp is illustrated (voltage recording from Schmid-Antomarchi et al. [8]).

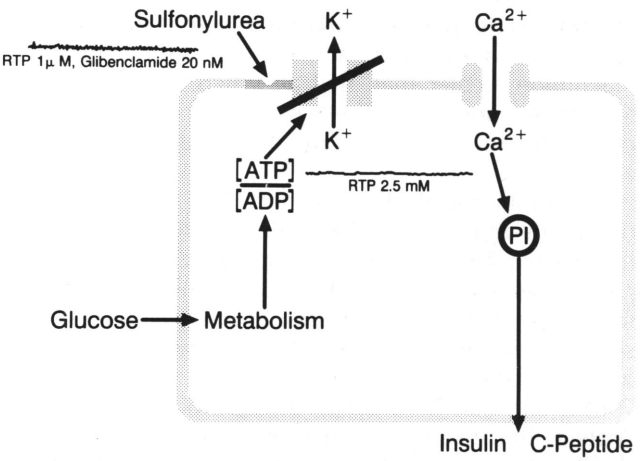

FIG. 8. Schema of stimulated β-cell. Sulfonylureas stimulate by binding to plasma membrane receptor that is coupled to ATP-dependent K^+ channel. This inhibits efflux of K^+, causing depolarization of plasma membrane. As consequence, voltage-dependent Ca^{2+} channel opens, causing influx of Ca^{2+} into cytosol, which stimulates extrusion of both mature and immature insulin granules. ATP-dependent K^+ channel can also be inhibited by intracellular metabolic events that increase ATP-ADP ratio. Typical recorded transmembrane voltage patterns (RTP) from patch clamps from Schmid-Antomarchi et al. (8) are illustrated. PI, proinsulin.

TABLE 4
Studies demonstrating sulfonylurea binding sites in brain

Ligand	Binding site	K_d(nM)	R_o(pmol/mg)	Ref.
[^{3}H]gliquidone	Rat brain crude membrane preparation	0.9		18
[^{3}H]glipizide	Rat cerebral cortex membranes	1.5	0.11	19
[^{3}H]glyburide	Rat cerebral cortex membranes	0.04–0.01	0.013–0.024	10
		0.05–0.4	0.030–0.034	

The consequences of sulfonylurea binding to the β-cell are changes in the ionic environment. Several studies indicate that K^+ efflux from the β-cell is regulated in part by an ATP-sensitive K^+ channel (8,11–14). In the basal state (Fig. 7), K^+ is extruded through the plasma membrane, and a basal membrane potential is maintained that keeps a voltage-dependent membrane Ca^{2+} channel relatively closed. Binding of a sulfonylurea to the sulfonylurea binding site inhibits the ATP-sensitive K^+ channel, which leads to depolarization of the membrane and opens the voltage-dependent Ca^{2+} channel (Fig. 8). Increased insulin secretion follows the increase in cytosolic Ca^{2+} concentration. The sulfonylurea binding site of the β-cell plasma membrane is closely associated with the ATP-sensitive K^+ channel. The demonstration of direct photoaffinity labeling of [^{3}H]glyburide to a specific 140,000-M_r polypeptide of rat β-cell tumor plasma membrane provides a new tool to identify and characterize the binding site (15).

Although the concept that sulfonylurea action on β-cells is mediated by a plasma membrane sulfonylurea receptor is supported by considerable data, there are some studies that question this concept. Deleers and Malaisse (16) have shown that hypoglycemic sulfonylureas bind to multilamellar liposomes with criteria that are somewhat characteristic of membrane receptors. Studies with glyburide in isolated islets from *ob/ob* mice indicate that glyburide, in contrast to all other sulfonylureas, enters the β-cell and is concentrated (17). In isolated rat islets incubated with [^{3}H]glyburide, only 10–15% is bound to the plasma membrane, and ~75% is internalized and binds to the β-cell granules (18).

Because sulfonylureas exert many extrapancreatic actions on liver, muscle, and adipose tissue, sulfonylurea receptors on these tissues have been sought. So far, there are no convincing data to indicate that such receptors exist, and it is unlikely that such receptors mediate extrapancreatic actions. Surprisingly, however, sulfonylurea plasma membrane binding sites on cerebral cortical tissue have been identified and characterized by several investigators (10,19,20; Table 4). The dissociation constant is similar to that noted in β-cells, but the receptor concentration is only ~10% that in β-cells. The brain sulfonylurea binding site has since been solubilized and purified 2500-fold. It appears to be a single polypeptide chain with an M_r of 150,000 ± 10,000 (21). The significance of the sulfonylurea plasma membrane binding site in cerebral cortical tissue is not known, because there is no evidence that sulfonylureas affect central nervous system function.

The demonstration of a sulfonylurea binding site on the β-cell raises many important questions. Is the sulfonylurea binding site a true receptor, and does it exist on normal β-cells? All studies to date have demonstrated it on tumor β-cells. What is the natural ligand? Because sulfonylureas are not natural products, there is no reason for plasma membrane sulfonylurea receptors, and therefore, they must be there to bind some naturally occurring material that presumably regulates β-cell function. As noted in Table 2, various candidates were examined, but none are the natural ligand. A corollary to questions about the natural ligand and its function regard how the sulfonylurea receptor is regulated. Are alterations in the sulfonylurea receptor or its natural ligand important in the pathogenesis of non-insulin-dependent diabetes mellitus? The identification of specific plasma membrane binding sites for sulfonylureas on β-cells opens up many new approaches to understanding the pathogenesis and development of new therapies in non-insulin-dependent diabetes mellitus.

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