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European Journal of Pharmacology 284 (1995) 77–82

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Characterization of the binding sites for [^{3}H]glibenclamide in rat liver membranes

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Received 2 February 1995; revised 13 June 1995; accepted 16 June 1995

Abstract

The specific binding sites for sulfonylureas in the rat liver membrane fraction were demonstrated and characterized. [^{3}H]Glibenclamide binding to the liver membrane was specific, time-and temperature-dependent, and reversible. Scatchard analysis showed a single class binding site. The dissociation constant (K_d) for glibenclamide was 1.1 μM and the binding capacity (B_{max}) was 50 pmol/mg protein. [^{3}H]Glibenclamide binding could be displaced by other sulfonylureas. Half-maximal inhibition of binding (IC_{50}) for glimepiride, gliclazide, acetohexamide, tolbutamide and chlorpropamide was 4.2 μM, 74 μM, 0.33 mM, 0.60 mM, 1.2 mM, respectively. Each value is close to the reported blood concentration when a therapeutic dose of each drug is administered orally. The order of IC_{50} values is coincident with the order of potency of the clinical hypoglycemic effect of these drugs. We had shown that these concentrations of sulfonylureas stimulate 6-phosphofructo-2-kinase in the liver or hepatocytes and inhibit phosphoenolpyruvate carboxykinase in the hepatoma cells. The specific binding sites demonstrated here may play some roles when sulfonylureas affect carbohydrate metabolism in the liver.

Keywords: Binding site; Glibenclamide (Glyburide); Hypoglycemic agent; Liver; Sulfonylurea compound

1. Introduction

Sulfonylureas are hypoglycemic agents that have been used for more than three decades in the treatment of non-insulin-dependent diabetes mellitus (Groop, 1992). The main action of the agents is to stimulate insulin release from the pancreatic β cells (Blackward and Nelson, 1971; Colwell and Zuckerman, 1972; Grodsky et al., 1977). This insulin secretory effect is, however, controversial when these agents are given chronically to non-insulin-dependent diabetics (Reaven and Dray, 1967; Duckworth et al., 1972; Feinglos and Lebovitz, 1980; Greenfield et al., 1982; Best et al., 1982), and extrapancreatic actions of sulfonylureas have been much discussed in the last decade. The liver (Salhanick et al., 1983; Fleig et al., 1984; Matsutani et al., 1984; Kaku et al., 1986; Patel, 1986; Davidson and Sladen, 1987), muscle (Rogers et al., 1987; Johnson et al., 1991) or adipose tissue (Maloff and Lockwood, 1981; Matsuda et al., 1991) has been examined as a target organ and sulfonylureas have been demonstrated to have a number of metabolic actions in these tissues.

Specific binding sites for sulfonylurea were first demonstrated in pancreatic β cell tumor, and then further described in cerebral cortex (Kaubish et al., 1982; Lupo and Bataille, 1987; Bernardi et al., 1988), pancreatic β cells (Kaubish et al., 1982; Geisen et al., 1985; Gaines et al., 1988; Siconolfi-Baez et al., 1990; Verspohl et al., 1990), heart (Fosset et al., 1988; French et al., 1991) and adipocytes (Martz et al., 1989). Furthermore, the relationship between sulfonylurea receptor and potassium channel has been discussed vigorously because sulfonylureas close ATP-sensitive K^+ channels (Sturgess et al., 1985; Schmid-Antomarchi et al., 1987; Fosset et al., 1988; Niki et al., 1989). Specific binding sites in the liver have not been described whereas a variety of metabolic actions by sulfonylureas have been demonstrated. In the present study, we attempted to demonstrate specific binding sites for sulfonylureas in the rat liver.

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SSDI 0014-2999(95)00368-1

2. Materials and methods

2.1. Materials

[^{3}H]Glibenclamide (1.88 TBq or 50.9 Ci/mmol) and Aquasol-2 were purchased from NEN (Boston, MA, USA). Glibenclamide (Glyburide), glimepiride (HOE-490), tolbutamide and carboxytolbutamide were provided by courtesy of Hoechst Japan (Tokyo, Japan). Acetohexamide, chlorpropamide, gliclazide and buformin hydrochloride were obtained by courtesy of Shionogi Pharmaceutical Co. (Osaka, Japan), Pfizer Pharmaceuticals (Tokyo, Japan), Dainippon Pharmaceutical Co. (Osaka, Japan) and Kodama (Tokyo, Japan), respectively. Leupeptin, iodoacetamide, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor (type II-S) and Percoll were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dithiothreitol was obtained from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade.

2.2. Membrane preparation

Crude membrane

Crude liver membrane fraction was prepared by the method of Toda et al. (1975) with a modification. Livers from two overnight-fasted male Wistar rats weighing 200 g were homogenized in 40 ml of solution A (250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg/ml trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride) with a Dounce homogenizer. Red blood cells and nuclei were removed by centrifugation at $270 \times g$ for 5 min and membranes were pelleted by centrifugation at $1500 \times g$ for 10 min. This step was repeated 3 times. The final pellet was resuspended in solution A supplemented with 10 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride at a protein concentration of 5–10 mg/ml.

Partially purified membranes

In some experiments, crude membranes were further purified by centrifugation at $10\,000 \times g$ for 60 min in solution B (250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 2 mM EGTA, 2 mM $MgCl_2$) supplemented with 17.5% v/v Percoll. Plasma membranes were washed in solution B 3 times to remove Percoll. The final pellet was resuspended in solution B supplemented with 50 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM iodoacetamide and 1 mM phenylmethylsulfonyl fluoride at a protein concentration of 1 mg/ml. The activity of 5'-nucleotidase/mg protein in this purified membrane fraction, determined by the method of Valentine et al. (1974), was 5- to 7-fold higher than that of the liver whole homogenate. Membranes were stored in small quantities in liquid nitrogen until the experiments were done.

2.3. [^{3}H]Glibenclamide binding assay

The [^{3}H]glibenclamide binding assays were performed in solution A in a final volume of 0.5 ml. In a typical experiment, each assay tube contained either 300 μg of crude membrane or 100 μg of partially purified membrane, 1 nM [^{3}H]glibenclamide (about 1 kBq or 0.025 μCi) and the desired concentrations of unlabeled agents. Glibenclamide was dissolved in 0.5 N NaOH first, then almost neutralized (pH 7.5) with 1 N HCl. The final concentration of glibenclamide stock solution was 1 mM. The samples were incubated for 120 min at 4°C unless otherwise indicated. The reaction was terminated by dilution with 2 ml of ice-cold solution A and vacuum filtration on Whatman GF/C filters followed by quick washing of the filters twice with 5 ml of ice-cold distilled water. The filters were submerged in 5 ml of Aquasol-2 and the radioactivity was measured by liquid scintillation spectroscopy. Specific binding was defined as the difference between that in the absence and that in the presence of 10 μM unlabeled glibenclamide. All incubations were carried out in triplicate and each experiment was repeated 3 or more times with similar results. The data shown in figures were the means ± S.D. for representative experiments. K_d and B_{max} values were determined with the LIGAND program (Munson and Rodbard, 1980).

3. Results

3.1. Protein dependence of [^{3}H]glibenclamide binding

Protein dependence was studied by incubating various amounts of crude rat liver membranes with 0.5 nM [^{3}H]glibenclamide for 120 min at 4°C. The extent of binding was proportional to the protein concentration between 100–1000 μg/tube (Fig. 1). For all subsequent experiments using crude membrane, protein concentrations were in the linear range (300 μg/tube).

3.2. Time and temperature dependence of binding

The binding was time-and temperature-dependent. When membranes were incubated with 1 nM [^{3}H]glibenclamide for various periods at 4°C (Fig. 2A), the binding increased gradually and reached its maximal level at 120 min. On the other hand, binding reached the maximal level in 30 min and then decreased gradually with time when the incubation was performed at 20°C (Fig. 2B). Specific binding was higher when membranes were incubated at 4°C rather than 20°C, while non-specific binding was essentially constant. Accordingly, the incubation was performed at 4°C for 120 min in routine experiments.

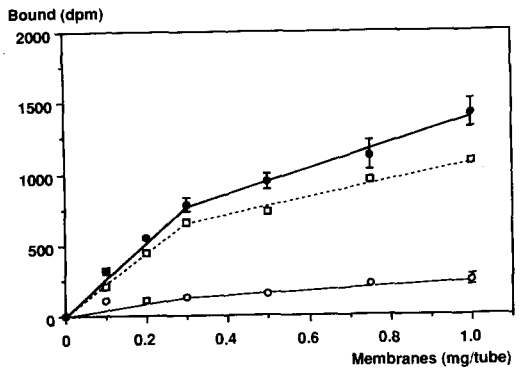

Fig. 1. Protein dependence of [^{3}H]glibenclamide binding to membranes. Various concentrations of rat crude liver membranes (0–1 mg/tube) were incubated with 0.5 nM [^{3}H]glibenclamide in the presence or absence of 10 μM unlabeled glibenclamide at 4°C for 120 min. •: total binding, ○: non-specific binding, □: specific binding.

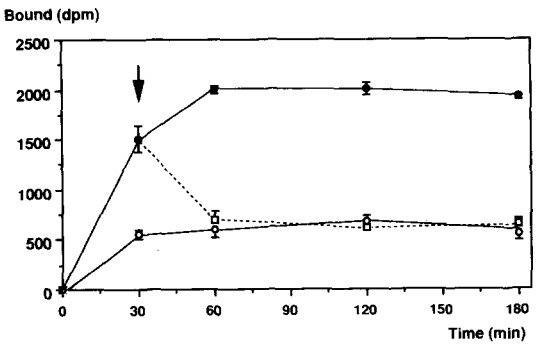

Fig. 3. Reversibility of [^{3}H]glibenclamide binding to membranes. Rat crude liver membranes (300 μg/tube) were incubated with 1 nM [^{3}H]glibenclamide in the presence or absence of 10 μM unlabeled glibenclamide at 4°C. Addition of 10 μM unlabeled glibenclamide (indicated by arrow) displaced the specific binding (dot line, □). •: total binding, ○: non-specific binding.

3.3. Reversibility of binding

[^{3}H]Glibenclamide binding to rat liver membranes was reversed when 10 μM unlabeled glibenclamide was added to the reaction mixture 30 min after the start of the reaction. Fig. 3 shows the association and dissociation of [^{3}H]glibenclamide to membranes. Addition of a large excess of unlabeled glibenclamide dissociated [^{3}H]glibenclamide bound to the membranes completely in 30 min.

3.4. Association and dissociation kinetics of [^{3}H]-glibenclamide binding

Fig. 4 shows the binding of increasing concentrations of [^{3}H]glibenclamide to the crude preparation of rat liver membranes at 4°C. As the concentration of [^{3}H]glibenclamide was increased from 1 nM to 300 nM, specific binding to membrane protein increased. However, the binding was not saturated in this range of [^{3}H]glibenclamide concentrations.

Tracer binding was inhibited by incubation of the membranes with increasing concentrations of unlabeled glibenclamide (1 nM to 100 μM) for 2 h at 4°C. Non-specific binding was defined as the binding in the presence of 10 μM unlabeled glibenclamide since no more displacement was observed in the presence of 30 μM or higher concentrations of cold glibenclamide. Non-specific binding was approximately 20–25% of the total binding (Fig. 6). Scatchard analysis revealed a single class of binding site, and the calculated K_d value was 1.8 μM and B_{max} was 45 pmol/mg protein. A similar experiment was performed using 100 μg/tube of the partially purified membrane preparation. The inhibition curve for specific binding was nearly equal to

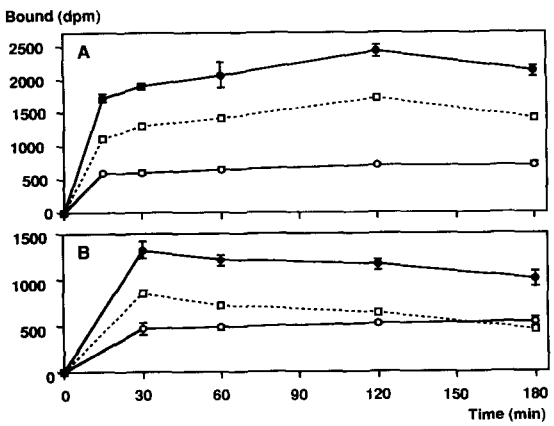

Fig. 2. Time course of [^{3}H]glibenclamide binding to membranes. Rat crude liver membranes (300 μg/tube) were incubated with 1 nM [^{3}H]glibenclamide in the presence or absence of 10 μM unlabeled glibenclamide at 4°C (A) or 20°C (B) for the time indicated. •: total binding, ○: non-specific binding, □: specific binding.

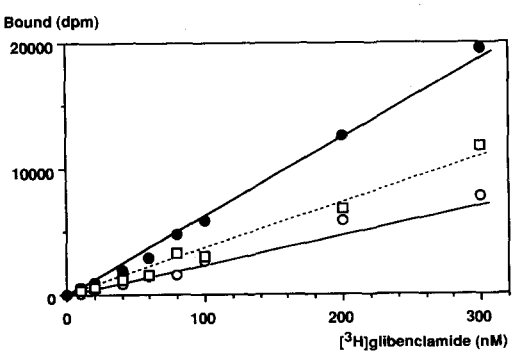

Fig. 4. Influence of tracer concentration on [^{3}H]glibenclamide binding to membranes. Rat crude liver membranes (300 μg/tube) were incubated with increasing concentrations of [^{3}H]glibenclamide (0–300 nM) in the presence or absence of 10 μM unlabeled glibenclamide at 4°C for 120 min. •: total binding, ○: non-specific binding, □: specific binding.

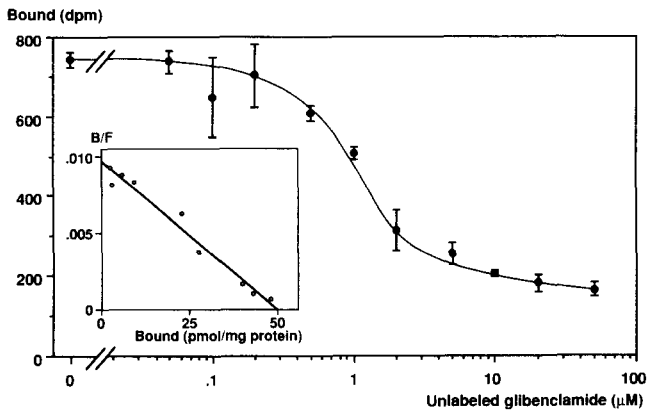

Fig. 5. Inhibition of total [^{3}H]glibenclamide binding to membranes by unlabeled glibenclamide. Rat partially purified liver membranes (100 μg/tube) were incubated with 1 nM [^{3}H]glibenclamide in the presence of increasing doses of unlabeled glibenclamide at 4°C for 120 min. Inset: Scatchard plot of the data.

that for the crude membrane preparations (Fig. 5). Scatchard analysis showed similar K_d and B_{max} values; 1.1 μM and 50 pmol/mg protein, respectively.

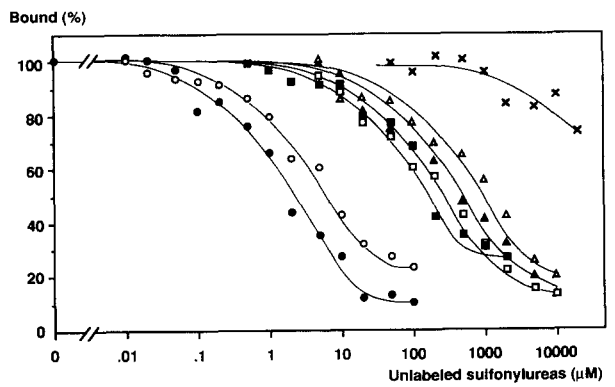

Fig. 6. Inhibition of total [^{3}H]glibenclamide binding to membranes by various unlabeled sulfonylureas. Rat crude liver membranes (300 μg/tube) were incubated with 1 nM [^{3}H]glibenclamide at 4°C for 120 min in the presence of increasing concentrations of unlabeled glibenclamide (●), glimepiride (○), gliclazide (■), acetohexamide (□), tolbutamide (▲), chlorpropamide (△) or carboxytolbutamide (×).

Table 1
Comparison of K_d or IC_{50} values for various sulfonylureas with therapeutic doses of these agents in the human

Sulfonylureas	K_D or IC_{50} (μM)	Therapeutic dose [a] (mg/day)
Glibenclamide	1.1	1.25–10
Glimepiride	4.2	2–6
Gliclazide	74	40–160
Acetohexamide	330	250–750
Tolbutamide	600	500–1500
Chlorpropamide	1200	125–500

[a] Groop (1992) with modification.

3.5. Inhibition of [^{3}H]glibenclamide binding by hypoglycemic agents

Several oral antidiabetic agents were investigated for competition with binding of [^{3}H]glibenclamide (Fig. 6). The concentrations for half-maximal inhibition (IC_{50} values) were determined with the LIGAND program (Table 1). Carboxytolbutamide, inactive metabolite of tolbutamide, displaced the binding only partially. The biguanide drug, buformin, did not displace binding in the range of 0.1 mM and 100 mM (data not shown).

4. Discussion

The current studies characterized for the first time sulfonylurea binding sites in the rat liver membrane fraction. [^{3}H]Glibenclamide binding to membranes from the rat liver was found to be specific, time-and temperature-dependent and reversible. Saturation of the binding sites by [^{3}H]glibenclamide could not be demonstrated in our experimental conditions because the calculated K_d value was higher than 300 nM, which was the maximum concentration of tracer we used. Use of concentrations higher than 300 nM of the tracer was impossible because the concentration of [^{3}H]glibenclamide in the original container was 10–20 μM in ethanol.

The affinity of the binding sites for glibenclamide in the liver was relatively low. High-affinity binding sites for sulfonylureas were originally demonstrated in rat brain membranes and rat pancreatic β cell tumor membranes (Kaubish et al., 1982). The characteristics of the binding sites have been further described for pancreatic β cell tumor membranes (Fleig et al., 1984; Geisen et al., 1985; Schmid-Antomarchi et al., 1987; Gaines et al., 1988; Niki et al., 1989; Siconolfi-Baez et al., 1990; Verspohl et al., 1990; French et al., 1991; Ronner et al., 1993), cerebral cortex (Geisen et al., 1985; Lupo and Bataille, 1987; Bernardi et al., 1988) or cardiac membranes (Fosset et al., 1988). Reported K_d values for glibenclamide are generally in a low nanomolar range (0.05–7 nM). These values are in good agreement with those found for stimulation of insulin release or for inhibition of ATP-sensitive K currents in pancreatic β cells. Aguilar-Bryan et al. (1990) and Nelson et al. (1992) recently detected and partially purified a 140000 Da protein as the high-affinity sulfonylurea receptor in HIT cells using [^{125}I]glibenclamide. During preparation of this paper, the sulfonylurea receptor was cloned by the same group (Aguilar-Bryan et al., 1995), who showes that the sulfonylurea receptor has a molecular weight of 177000 Da and seems to be a member of the ATP-binding cassette or traffic ATPase superfamily.

We could not detect high-affinity binding sites in the present study. However, a variety of metabolic effects of sulfonylureas on the liver or hepatocytes have been observed at concentrations of sulfonylureas higher than those that induce insulin secretion from pancreatic β cells. For example, stimulatory effects on glycolysis (Matsutani et al., 1984; Kaku et al., 1986; Patel, 1986), glycogenesis (Fleig et al., 1984; Davidson and Sladen, 1987), lipogenesis (Salhanick et al., 1983) or inhibitory effects on glycogenolysis (Davidson and Sladen, 1987), ketogenesis (Patel, 1986) were induced by a low micromolar range of glibenclamide concentrations (0.5–10 μM) or by a low milimolar range of tolbutamide or tolazamide (0.5–2 mM) concentrations. We have recently reported that 2–10 mM tolbutamide inhibits the gluconeogenetic key enzyme, phosphoenolpyruvate carboxykinase, in rat hepatoma cells (Emoto et al., 1993). Reported maximum blood concentrations of these sulfonylureas, when therapeutic doses were administered orally, were also in these ranges, i.e. tolbutamide, 0.3–0.7 mM; chlorpropamide, 0.4–0.7 mM; acetohexamide, 60–90 μM; gliclazide, 50–60 μM; glibenclamide, 0.04–0.1 μM (Baird and Dunchan, 1957; Bergman et al., 1980; Maha et al., 1962; Miyazaki et al., 1983).

In adipose tissue, 1 μM glibenclamide or 1 mM tolbutamide stimulates insulin-induced acetyl coenzyme A carboxylase (Matsuda et al., 1991) and $\sim$1 mM tolazamide facilitates glucose uptake in an insulin-independent manner (Maloff and Lockwood, 1981). Martz et al. (1989) have demonstrated a specific receptor for glibenclamide in rat adipocytes. The reported K_d value is 1.8 μM and the binding capacity is 220 pmol/mg protein. The latter authors emphasized that this K_d value was consistent with the activation constant (K_a) of glibenclamide to potentiate insulin-stimulated hexose transport in adipocytes. This K_d value is close to that we found. We also would like to emphasize that a low micromolar range (not a low nanomolar range) of glibenclamide induces a variety of metabolic actions in the liver or the hepatocytes as shown above. These data may suggest that specific binding sites for glibenclamide with a micromolar K_d value should be relevant to the production of a variety of extra-pancreatic actions in the liver.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Science and Culture in Japan, Nos. 0467148 and 03771826 (Y.I.).

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