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Specific binding of glycyrrhetinic acid to the rat liver membrane

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Glycyrrhetinic acid bound specifically to a particulate fraction of rat liver. The binding was dependent on time, temperature and pH, equilibrium being reached after 10 min at 37 °C. The equilibrium dissociation constant and the maximal concentration of the binding site, as determined by Scatchard plot analysis, were 31 nM and 43 pmol / mg protein, respectively, indicating a single binding site entity. The binding site was highly specific for glycyrrhetinic acid, glycyrrhizin, various steroids, various fatty acids and retinoids showing no or only very low affinity. The binding was inhibited by boiling or treatment with trypsin or phospholipases. The specific activity of glycyrrhetinic acid binding was the highest in the liver, followed by in the kidney. The results suggest that glycyrrhetinic acid plays a significant role in the rat liver through its specific binding protein.

Introduction

Glycyrrhizin, extracted from the roots of licorice (*Glycyrrhiza glabra*), and its aglycone, glycyrrhetinic acid, exhibit various anti-inflammatory [1], anti-allergic [2], anti-gastric ulcer [3], anti-hepatitis [4] and anti-hepatotoxic [5] activities. The activity of a licorice extract is believed to be due to the aglycone, glycyrrhetinic acid, which is released from glycyrrhizin through hydrolysis [6]. The structure of glycyrrhetinic acid shows a resemblance to that of hydrocortisone. Glycyrrhetinic acid has been demonstrated to have a corticoid-like action [7,8], and inhibitory effects on the cortisone acetate-induced antigranulomatous action [8] and prednisolone-induced inhibition of the growth of fibroblasts in culture [9]. However, glycyrrhetinic acid showed practically no affinity for glucocorticoid receptors in mastocytoma P-815 cells [10] and only low affinity for them in rat kidney [11]. Therefore, it is anticipated that glycyrrhetinic acid may interact with a different target(s) from steroid receptors and exhibit a variety of biological actions, including a steroid action through the target(s).

However, the site of action of glycyrrhetinic acid and the molecular mechanisms of its actions remain obscure. In an attempt to solve these problems, the identification of the target of glycyrrhetinic acid and the understanding of the mechanisms by which glycyrrhetinic acid interacts with the target to regulate intracellular metabolic events appear to be of primary importance. In this paper, we report finding of a highly specific binding site of glycyrrhetinic acid in a rat liver particulate fraction, and also describe its specificities and tissue distribution. Our results represent the first demonstration of a specific binding protein for glycyrrhetinic acid in the particulate fraction of rat liver.

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Materials and Methods

Materials

[3-^{3}H]Glycyrrhetinic acid (11.2 Ci/mmol), unlabeled glycyrrhetinic acid and glycyrrhizin were obtained from Minophagen (Tokyo, Japan). The purity of [^{3}H]glycyrrhetinic acid showed 96.9% in thin-layer chromatography. Other agents obtained and their commercial sources were as follows: corticosterone, β-estradiol, oleic acid, retinoic acid, retinol, arachidonic acid, phospholipase A_2 (*Naja naja venom*), phospholipase C (*Clostridium perfringens*), bovine pancreas ribonuclease A and bovine pancreas deoxyribonuclease I, Sigma; cholesterol, lanosterol, hydrocortisone, testosterone, progesterone, prednisolone, dexamethasone, palmitic acid, dithiothreitol and *N*-ethylmaleimide, Nacalai Tesque (Kyoto); trypsin, Difco; neuraminidase (*Clostridium perfringens*), Boehringer-Mannheim. All other chemicals were of reagent grade.

Preparation of subcellular fractions of rat liver

Male Wistar rats (body weight, 200–300 g) were killed by cervical dislocation and decapitation. All the following procedures were carried out at 0–4 °C. Livers were homogenized in 5 volumes of 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 0.25 M sucrose and 0.1 mM phenylmethylsulfonyl fluoride, using a Potter-Elvehjem glass homogenizer with a Teflon pestle. After centrifugation at $800 \times g$ for 10 min, the pellet was washed once by recentrifugation as described above in the same medium and then the combined supernatants were centrifuged at $9500 \times g$ for 10 min. The resulting supernatant was further centrifuged at $100\,000 \times g$ for 60 min. The pellet of each fraction (Table I) was resuspended in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA (buffer A) To determine the tissue distribution of [^{3}H]glycyrrhetinic acid binding activity, various tissues of rat were cut into small pieces and fractionated essentially as described above. The membrane fraction (800–$100\,000 \times g$ pellet) was used in the tissue distribution experiment. Protein concentrations were determined by the method of Lowry et al. [12] with bovine serum albumin as a standard.

[^{3}H]Glycyrrhetinic acid binding assay

The standard assay mixture contained 25 nM [^{3}H]glycyrrhetinic acid (61 500 dpm) and 50 μg of the membrane fraction in 0.1 ml of buffer A, unless stated otherwise. After incubation for 30 min at 37 °C, the reaction was stopped by the addition of 5 ml of ice-cold buffer A, after which the mixture was rapidly filtered through a Whatman GF/C glass filter (Ø 2.5 cm). The filter was then washed three times with 5 ml of ice-cold buffer A and the radioactivity associated with the filter was measured in 5 ml of Clearsol (Nacalai Tesque, Kyoto, Japan). Nonspecific binding was determined using a 1000-fold excess of unlabeled glycyrrhetinic acid in the incubation mixture. The specific binding was calculated by subtracting the nonspecific binding from the total binding. Alternatively, bound and free [^{3}H]glycyrrhetinic acids were separated by gel filtration method using columns of Sephadex G-50 (Pharmacia) in a manner similar to that described previously [13]. Both of these assay methods, glass filter and gel filtration methods, gave the same extent of [^{3}H]glycyrrhetinic acid binding. From the simplicity of preparation, [^{3}H]glycyrrhetinic acid binding assay was performed by the glass filter method.

Results

Specific [^{3}H]glycyrrhetinic acid binding to the membrane fraction of rat liver

In order to investigate the subcellular localization of glycyrrhetinic acid binding sites in rat liver, various fractions were prepared from a rat liver homogenate by successive centrifugation. Table I shows typical results obtained with membrane preparations derived from 50 g of rat liver. About 26% of the [^{3}H]glycyrrhetinic acid binding activity in the homogenate was recovered in the (9500–$100\,000) \times g$ pellet, the specific binding activity being 14.9 pmol/mg protein, which was the highest value among all the fractions. Therefore, we used the $100\,000 \times g$ pellet as the membrane fraction in the following experiments.

TABLE I

Fractionation of [^{3}H]glycyrrhetinic acid binding activity from rat liver

The homogenate obtained from 50 g of rat liver was successively centrifuged at $800 \times g$ for 10 min, at $9500 \times g$ for 10 min, and at $100\,000 \times g$ for 60 min. [^{3}H]Glycyrrhetinic acid binding activity in each fraction was determined as described under Materials and Methods.

Fraction	Specific [^{3}H]glycyrrhetinic acid binding			
	protein (mg)	activity		yield (%)
		total (pmol)	specific (pmol/mg)	
Homogenate	7567	64860	8.57	100
$800 \times g$ pellet	2660	19000	7.14	29
$9500 \times g$ pellet	1180	15300	6.48	24
$100\,000 \times g$ pellet	1120	16640	14.9	26
$100\,000 \times g$ supernatant	3105	n.d. [a]	n.d.	–

[a] n.d., not detected.

Fig. 1 shows the time course of [^{3}H]glycyrrhetinic acid binding to the membrane fraction. At 37 °C, equilibrium was reached after 10 min, the specific binding amounting to 90% of the total binding at 10 min. Nonspecific binding of [^{3}H]glycyrrhetinic acid reached equilibrium in 10 min and was relatively independent of temperature. With a decrease in the incubation

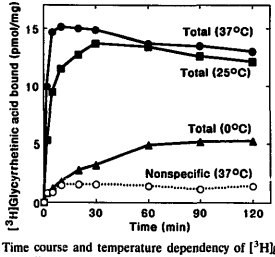

Fig. 1. Time course and temperature dependency of [^{3}H]glycyrrhetinic acid binding to the membrane fraction. The membrane fraction was incubated with 25 nM [^{3}H]glycyrrhetinic acid at 0 (▲), 25 (■) or 37 °C (●) for total binding. The nonspecific binding assay was carried out at 0, 25 or 37 °C, the results for 37 °C being shown (○). The amounts of bound [^{3}H]glycyrrhetinic acid were determined at various times as described under Materials and Methods.

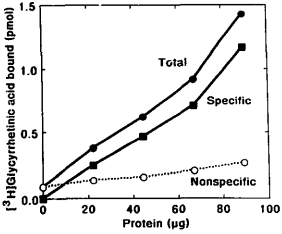

Fig. 2. Relationship between the specific binding of [^{3}H]glycyrrhetinic acid and the amount of membrane material added to the incubation medium. Incubations were carried out with the standard incubation mixture as described under Materials and Methods, except that various amounts of the membrane fraction were used. Specific binding (■) was calculated by subtracting the nonspecific binding (○) from the total binding (●).

temperature, the rate of binding decreased and equilibrium was not reached within 20 min at either 0 or 25 °C. The specific binding of [^{3}H]glycyrrhetinic acid showed a linear relationship with the amount of the membrane fraction over 90 μg, as shown in Fig. 2. [^{3}H]Glycyrrhetinic acid binding to the membrane fraction was dependent on pH, the optimal pH being 7.5 (Fig. 3A). Fig. 3B shows the effects of monovalent and divalent cations on [^{3}H]glycyrrhetinic acid binding. NaCl, KCl, $CaCl_2$ and $MgCl_2$ showed very weak inhibition, the level of which was less than 20% even at higher concentrations.

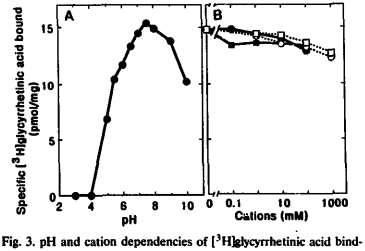

Fig. 3. pH and cation dependencies of [^{3}H]glycyrrhetinic acid binding. (A) The buffers used were 50 mM sodium acetate buffer (pH 3–5.5), 50 mM potassium phosphate buffer (pH 6–7), 50 mM Tris-HCl buffer (pH 7.5–9), and 50 mM glycine-KOH buffer (pH 10). All incubations were performed in the presence of 1 mM EDTA, 25 nM [^{3}H]glycyrrhetinic acid and 50 μg of the membrane fraction. (B) Incubations were carried out under the standard assay conditions except for the presence of various concentrations of $MgCl_2$ (●), $CaCl_2$ (■), NaCl (○) or KCl (□). After incubation for 30 min at 37 °C, the amounts of bound [^{3}H]glycyrrhetinic acid were determined as described under Materials and Methods.

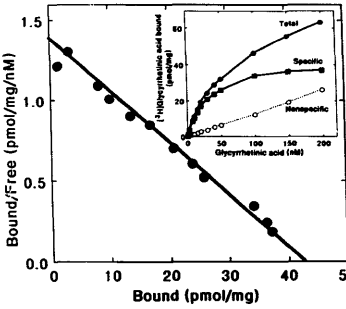

Fig. 4. Scatchard plot for glycyrrhetinic acid binding to the membrane traction. The membrane fraction (10 μg) was incubated in a larger assay volume (1.0 ml) for 30 min at 37 °C with increasing concentrations of [^{3}H]glycyrrhetinic acid (1–200 nM) in the presence (○) or absence (●) of 200 μM unlabeled glycyrrhetinic acid. Bound [^{3}H]glycyrrhetinic acid was determined as described under Materials and Methods. Specific [^{3}H]glycyrrhetinic acid binding (■) was calculated by subtraction of the nonspecific binding (○) from the total binding with the indicated concentrations of [^{3}H]glycyrrhetinic acid (inset). The Scatchard plot was transformed from the value of specific [^{3}H]glycyrrhetinic acid binding.

In order to evaluate the binding affinity of [^{3}H]glycyrrhetinic acid to the membrane fraction, we carried out Scatchard analysis of the binding. [^{3}H]Glycyrrhetinic acid specifically bound to the membrane fraction in a dose-dependent manner (1–200 nM) and became almost saturated at higher concentrations (Fig. 4, inset). The Scatchard plot analysis transformed from the saturable specific [^{3}H]glycyrrhetinic acid binding showed an apparently single high-affinity binding site (K_d = 31 nM, B_{max} = 43 pmol/mg protein), indicating a single binding site entity.

Specificity of the [^{3}H]glycyrrhetinic acid binding site

Glycyrrhetinic acid belongs to a family of steroids. To evaluate the specificity of the [^{3}H]glycyrrhetinic acid binding site, various unlabeled steroids, at different concentrations, were added to the incubation mixture. As shown in Fig. 5, specific [^{3}H]glycyrrhetinic acid binding was specifically inhibited by glycyrrhetinic acid, the K_i value of glycyrrhetinic acid being about 50 nM. On the other hand, glycyrrhizin and various steroids tested showed no or only very low affinity.

A family of fatty acid binding proteins exists in various tissues, including the liver. Therefore, we examined whether or not various fatty acids and retinoids could inhibit the binding. As shown in Fig. 6, the various fatty acids tested had no ability to inhibit the

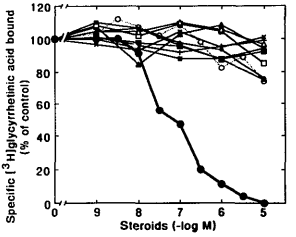

Fig. 5. Effects of unlabeled glycyrrhetinic acid, glycyrrhizin and various steroids on [^{3}H]glycyrrhetinic acid binding. The membrane fraction was incubated with 25 nM [^{3}H]glycyrrhetinic acid in the presence of various concentrations of unlabeled glycyrrhetinic acid (●), glycyrrhizin (○), cholesterol (+), lanosterol (□), hydrocortisone (■), testosterone (▲), β-estradiol (□), progesterone (△), prednisolone (◇), corticosterone (⊞) or dexamethasone (×). All values were corrected for nonspecific binding and are expressed as percentages of the control as described under Materials and Methods. The specific binding in the control was 14 pmol/mg protein.

binding. Retinol also did not affect the binding, but retinoic acid fairly inhibited it by 15% at 10 μM.

Effects of treatment with enzymes and sulfhydryl reagents on [^{3}H]glycyrrhetinic acid binding

In order to determine which membrane component and functional groups are involved in glycyrrhetinic acid binding, we treated the membrane fraction with a variety of enzymes, *N*-ethylmaleimide or dithiothreitol (Table II). The binding site for glycyrrhetinic acid appeared to be of a protein nature, since the specific

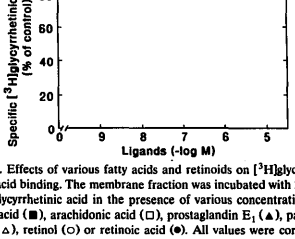

Fig. 6. Effects of various fatty acids and retinoids on [^{3}H]glycyrrhetinic acid binding. The membrane fraction was incubated with 25 nM [^{3}H]glycyrrhetinic acid in the presence of various concentrations of oleic acid (■), arachidonic acid (□), prostaglandin E_1 (▲), palmitic acid (△), retinol (○) or retinoic acid (●). All values were corrected for nonspecific binding and are expressed as percentages of the control as described under Materials and Methods. The specific binding in the control was 14 pmol/mg protein.

TABLE II

Effects of enzymes and sulfhydryl reagents on the specific binding of [^{3}H]glycyrrhetinic acid to the membrane fraction.

The membrane fraction (4 mg/ml) was preincubated for 15 min at 37 °C in the presence of various enzymes or sulfhydryl reagents. Treatment with neuraminidase and ribonuclease A was carried out at pH 5 (50 mM sodium acetate buffer), and that with other enzymes at pH 7.5 (50 mM Tris-HCl buffer). Treatment with phospholipases A_2 and C was performed in the presence of 5 mM $CaCl_2$; and for deoxyribonuclease I, 5 mM $MgCl_2$ was included in the preincubation mixture. Then, aliquots (20 μl) were further incubated in the standard assay mixture, as described under Materials and Methods. The results are expressed as percentages of the control. The control was preincubated for 15 min with 50 mM sodium acetate buffer at pH 5 or with Tris-HCl buffer at pH 7.5 without enzymes or sulfhydryl reagents. The values shown are the means ± S.E. for triplicate experiments.

Treatment	Concn.	[^{3}H]Glycyrrhetinic acid bound (% of control)
Deoxyribonuclease I	500 μg/ml	89.1 ± 0.6
Ribonuclease A	500 μg/ml	90.1 ± 1.2
Neuraminidase	0.1 U/ml	87.9 ± 5.5
Trypsin	50 μg/ml	21.9 ± 2.0
Phospholipase A_2	50 μg/ml	5.4 ± 0.3
Phospholipase C	50 μg/ml	25.9 ± 4.5
Boiled for 3 min		n.d. [a]
Dithiothreitol	1 mM	99.4 ± 3.9
N-Ethyimaleimide	1 mM	69.0 ± 3.3

[a] n.d., not detected.

binding was completely abolished on boiling of the membrane for 3 min or was markedly reduced with prior treatment with a low concentration of trypsin. Deoxyribonuclease I, ribonuclease A and neuraminidase all had no appreciable effect on the binding, whereas phospholipase C decreased the specific binding by about 26% and phospholipase A_2 drastically reduced the specific binding of the ligand. These results strongly suggest that the glycyrrhetinic acid binding site may be composed of protein, and that phospholipids are also important for the interaction of the ligand and/or the integrity of the membrane structure. The addition of proteinase inhibitors (20 μg/ml of leupeptin, pepstatin, aprotinin and 0.1 mM phenylmethylsulfonyl fluoride) to the preincubation mixtures together with the phospholipases did not attenuate the actions of the enzymes (data not shown). On the other hand, dithiothreitol had no effect on the binding, but *N*-ethylmaleimide significantly inhibited it, suggesting that one or more sulfhydryl groups partly participate in the interaction of the ligand with the binding site.

Tissue distribution of [^{3}H]glycyrrhetinic acid binding

The tissue distribution of [^{3}H]glycyrrhetinic acid binding activity in rat was investigated (Table III). The specific binding of [^{3}H]glycyrrhetinic acid was the high-

TABLE III

Tissue distribution of specific [^{3}H]glycyrrhetinic acid binding in rat

Freshly prepared membrane fractions derived from various tissues of rat were incubated with 25 nM [^{3}H]glycyrrhetinic acid for 30 min at 37 °C. The amounts of bound [^{3}H]glycyrrhetinic acid were determined as described under Materials and Methods. All values were corrected for nonspecific binding. The values shown are the means ± S.E. for triplicate experiments.

Tissue	Specific [^{3}H]glycyrrhetinic acid bound (pmol/mg)
Brain	1.20 ±0.31
Thymus	0.0370 ± 0.030
Heart	0.142 ± 0.12
Lung	1.14 ±0.89
Spleen	n.d. [a]
Liver	12.9 ±0.75
Kidney	3.86 ±0.17
Stomach	1.79 ±0.32
Small intestine	n.d.

[a] n.d., not detected.

est in liver (12.9 ± 0.75 pmol/mg protein), followed by in kidney. Low but significant activity was also detected in brain and stomach, whereas the specific binding activity in other organs appeared to be almost negligible. These results indicate that glycyrrhetinic acid binding activity is mainly located in rat liver.

Discussion

The major finding in this study was a specific binding protein for glycyrrhetinic acid in the particulate fraction prepared from rat liver. As shown in Fig. 4, the binding interaction of glycyrrhetinic acid with the membrane fraction was saturable, and Scatchard analysis of this binding at equilibrium gave a K_d value of 31 nM, indicating an apparently single high affinity binding site entity. Monovalent and divalent cations failed to influence the binding at physiological concentrations but slightly inhibited it at higher concentrations, indicating that the binding of glycyrrhetinic acid does not basically require either monovalent or divalent cations.

We next studied the specificity of the glycyrrhetinic acid binding site. Glycyrrhetinic acid contains a steroid structure. As shown in Fig. 5, various steroids tested had no or only very low affinity for the glycyrrhetinic acid binding site, indicating that the glycyrrhetinic acid binding site is neither steroid hormone receptors [14] nor sterol carrier proteins [15]. In turn, it has been shown that glycyrrhetinic acid has a low but definite affinity for mineralocorticoid receptors but has no significant affinity for glucocorticoid receptors in rat kidney [11]. Therefore, glycyrrhetinic acid binding protein and steroid hormone receptors are mutually different entities. Glycyrrhizin, the glycoside of glycyrrhetinic acid, showed very low affinity for the glycyrrhetinic acid binding site, indicating that the 3-hydroxy function is important. We previously reported that glycyrrhetinic acid, but not glycyrrhizin, inhibited the histamine release from antigen-stimulated mast cells [10]. This glycyrrhetinic acid-specific effect is well consistent with the specificity of the glycyrrhetinic acid binding site. Concerning the target of glycyrrhizin, a casein phosphorylating protein kinase prepared from the membrane fraction of rat liver was demonstrated to be inhibited by glycyrrhizin [16]. This protein kinase is not the binding site for glycyrrhetinic acid, because glycyrrhizin showed very low affinity for the binding site (Fig. 5). It has been proposed that various fatty acid binding proteins and retinoid binding proteins exist [17]. In the rat liver plasma membrane, a 40-kDa fatty acid binding protein, which binds oleic, palmitic and arachidonic acids, but has little or no affinity for cholesteryl esters, has been shown to play a role in membrane free fatty acid transport [18]. Various fatty acids, which can bind to the 40-kDa fatty acid binding protein in rat liver, and retinoids failed to inhibit the binding of glycyrrhetinic acid to the membrane fraction, excluding the possibility that the glycyrrhetinic acid binding protein is a fatty acid binding or retinoid binding protein. Recently, glycyrrhetinic acid has been shown to inhibit corticosteroid 11β-dehydrogenase of rat kidney and liver, and also inhibit cytosolic 5β-reductase and microsomal 3β-hydroxysteroid dehydrogenase [19,20]. Furthermore, it has been reported that glycyrrhetinic acid-oxidizing and 3-ketoglycyrrhetinic acid-reducing activities were detected in a microsomal fraction of rat liver [21]. Although various steroids failed to inhibit the binding of glycyrrhetinic acid, the possibility that the glycyrrhetinic acid binding sites are enzymes involved in steroid metabolism could arise. Purification and further characterization of the binding sites will be required.

The binding site for glycyrrhetinic acid in the membrane fraction may be of a protein nature, since the binding was abolished by boiling or treatment with trypsin (Table II). These properties are similar to those of receptors for prostaglandins and various neurotransmitters [22–24]. The inhibition by phospholipases suggests that membrane phospholipids may also play a role in the binding of the ligand, as reported for prostaglandin E receptors in the adrenal medulla [22] and thyroid gland [25]. These inhibitory effects appear not to be due to proteinases contaminating the commercial phospholipase preparations, since they were not affected by the presence of the mixture of proteinase inhibitors in the preincubation mixtures.

Glycyrrhetinic acid exhibits anti-hepatitis [4] and anti-hepatotoxic [5] activities. Furthermore, glycyrrhetinic acid has been clinically tested for gastric and duodenal ulcers [3]. *Glycyrrhiza* extracts have also been reported to have sodium and water retaining

properties, probably through an action on the kidney tubule [26]. Because the specific binding activity of glycyrrhetinic acid was detected in stomach and kidney, as well as liver, among various tissues (Table III), the actions of glycyrrhetinic acid in liver, stomach and kidney mentioned above, i.e., anti-hepatitis, anti-gastric ulcer and mineralocorticoid-like activities, may be mediated by the binding site for glycyrrhetinic acid. However, liver showed the highest binding activity of glycyrrhetinic acid, suggesting that the main target of glycyrrhetinic acid is located in liver and that this binding site may play a role in the function of liver.

It remains to be clarified what the function of the membrane binding protein for glycyrrhetinic acid is and how it accomplishes the function. Although purification of the binding protein and elucidation of its physiological role is now in progress in our laboratory, our present knowledge on glycyrrhetinic acid in the liver suggests that glycyrrhetinic acid binds to a specific site in the liver particulate fraction, where it exerts some regulatory function.

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