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GLYCYRRHIZIC ACID AND ITS HYDROLYSATE AS MINERALOCORTICOID AGONIST

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Summary—Mineralocorticoid activity of glycyrrhetinic acid (GR) was studied *in vivo* (electrical potential difference in rat rectum) and *in vitro* (brush border Mg^{2+}-HCO_3^- ATPase in rat small intestine, kidney cytosol binding of GR with and without RU-28362, anti-glucocorticoid compound) in order to clarify the mechanism of mineralocorticoid-like activity of GR. Scatchard analysis of [^{3}H]aldosterone showed that K_d of higher affinity site (type I) 6.0×10^{-9} M, B_{max} 1.0×10^{-14} mol/mg protein, and K_d of lower affinity site (type II) 1.6×10^{-7} M, B_{max} 7.5×10^{-14} mol/mg protein. GR competed for [^{3}H]aldosterone binding sites in kidney cytosol at the concentration of 10^4 times as that of unlabeled aldosterone. RU-28362 displaced aldosterone binding curve, whereas GR binding kinetic was not affected by this compound. Adrenalectomy caused a significant fall in brush border Mg^{2+}-HCO_3^- ATPase activity (75% reduction compared with the initial level) which was not restored by GR administration. Electrical potential differences in the adrenalecomized rats were significantly lower than those in the control rats, which did not increase after GR administration.

INTRODUCTION

It is well known that the habitual ingestion of liquorice or the long-term medication with drugs containing glycyrrhizin, an active principle of liquorice, causes hypertension accompanied by marked hypokalemia and suppressed renin–aldosterone system (pseudo-aldosteronism). As an explanation for mineralocorticoid-mimetic activity of glycyrrhizin, Tamura[1] demonstrated an inhibitory action of glycyrrhizin on 5α-reductase which may delay the metabolic clearance of aldosterone and prolong its biological half-life. On the other hand, Ulmann *et al.*[2] have shown first that glycyrrhizin and its hydrolysed compound have a measurable affinity to mineralocorticoid receptor in rat kidney cytosol *in vitro*. In support of this finding, we [3–5] have confirmed that glycyrrhetinic acid has low but definite competing activity for aldosterone binding to kidney cytosol *in vitro*, and treatment of the rat with a higher dose of glycyrrhetinic acid decreased the concentration of mineralocorticoid receptor (maximum binding, B_{max}) without affecting the dissociation constant (K_d). Later, Armanini *et al.*[6] have also shown that the affinity of glycyrrhizin and its deconjugate for mineralocorticoid receptor is sufficient to explain the pathophysiology of pseudoaldosteronism, which develops when these compounds are given in a large amount. To further study the mineralocorticoid agonistic property of glycyrrhizin, the authors attempted first to evaluate the effect of RU-28362, glucocorticoid receptor blocker, on binding of the ligands to the renal mineralocorticoid receptor, to overcome the interference from glucocorticoid receptor and second to measure the change in activities of brush border Mg^{2+}-HCO_3^--ATPase from the upper intestine of rats, given glycyrrhizin.

EXPERIMENTAL

Assay procedures for characterization of mineralocorticoid receptor in rat kidney cytosol

Male Wistar rats were decapitated and kidneys were immediately perfused with 50 ml of ice-cold physiological saline via thoracic aorta. The kidney were removed, decapsulated, cut into small pieces and washed with the ice-cold incubation buffer [10 mM Tris–HCl buffer, pH 7.4, containing 20 mM Na_2MoO_4, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol (w/v)]. The tissue were homogenized in 3 vol of incubation buffer with 5 strokes of a motor-drived Teflon–glass homogenizer. The homogenate was centrifuged for 30 min at 1000 ***g***, at 4°C, using a Hitachi 5PR Centrifuge. Kidney cytosol was obtained by subjecting the 1000-***g*** supernatant to ultracentrifugation at 105,000 ***g*** at 4°C, utilizing a 50.2-Ti rotor on a Beckman L5-50 Ultracentrifuge for 60 min. Five hundred µl of cytosol containing approximately 1 mg protein was incubated with 1.0 pmol (10^5 dpm) of D-1,2-[^{3}H]aldosterone (New England Nuclear, Boston, MA, 46.9 Ci/mmol) in 1 ml of assay mixture at 4°C for 4 h with and without non-radioactive, glycyrrhetinic acid or RU-28362. After the incubation, 1 ml of a 4% charcoal (Norit A,

Wako Pure Chemical Inc., Osaka) and 0.4% dextran in incubation buffer was added to remove unbound aldosterone. The charcoal was then sedimented by centrifugation for 15 min at 1000 **g**. Radioactivities of supernatants in 10 ml Aquasol-2 (New England Nuclear) were counted using a liquid scintillation spectrometer (Aloka LSC-671). Specific binding was calculated by subtracting the nonspecific radioactivity in the presence of 100-fold excess of nonradioactive aldosterone from the total radioactivity.

Protein concentrating in the cytosol fraction was determined by the method of Lowry *et al.*[7] using bovine serum albumin as a standard.

Enzyme assay

The small intestine samples, excised from the pylorus to the distal end of the ileum, from four adrenalectomized rats, were homogenized in 30 vol (w/v) of ice-cold 50 mM D-mannitol–2 mM Tris–HCl buffer (pH 7.1 at 4°C). Homogenates were filtered through a piece of nylon mesh, and aliquots of this homogenate were taken for the determination of enzyme activity and protein content. One-M $CaCl_2$ solution was added to residual homogenate to a final concentration of 10 mM and the mixture was stirred in ice-water for 10 min. The homogenates were centrifuged at 3000 **g** for 15 min. The supernatant was decanted and centrifuged at 43,000 **g** for 20 min and the pellet was subjected to the enzyme assay following the method described by Suzuki[8, 9]. In brief, the pellet were incubated at 37°C for 10 min with 1.0 mM $MgCl_2$, 50 mM $NaHCO_3$, 3 mM ATP and 50 mM Tris–HCl buffer (pH 9.0). The reaction was stopped by the addition of 20% trichloracetic acid and yielded inorganic phosphate, determined by the method of Allen[10]. Phosphatase activity was expressed as mol Pi/mg protein/h. Na^+–K^+-ATPase activity was assayed by the method of Hamlyn[11].

Measurement of subtraction potential difference

Male Wistar rats weighing 220–250 g were used without anesthesia. These rats were adrenalectomized under pentobarbital anesthesia and given either vehicle alone or 1.0 ng/g of glycyrrhetinic acid intraperitoneally for 3 consecutive days. Rats received abdominal incision alone and fed normal rat chow were used as controls. The electrical potential difference across the colon wall was measured using electrodes with tapered shank which was connected to Adrenosonde (Fischerlehner & Kucera, Innsbruck). The electrode was inserted into rectum in conscious restrained rats, after the rats were defecated and washed with saline. The reference electrode was placed on the shaved skin where 0.2 ml of saline was injected subcutaneously. The buccal electric potential was measured simultaneously and the difference between rectum and buccal membrane electrical potential was used as the subtraction potential difference, which reflects active sodium transport [12].

RESULTS

Characteristics of aldosterone receptors in rat kidney cytosol

A Scatchard analysis of [^{3}H]aldosterone binding to kidney cytosol in control rats shows the apparent B_{max} of aldosterone to the higher affinity sites was 1.0×10^{-14} mol/mg cytosol protein and K_d was 6.0×10^{-9} M. The K_d for lower affinity sites was 1.6×10^{-7} M. Glycyrrhetinic acid competes for [^{3}H]aldosterone binding sites in kidney cytosol receptor at the concentration of 10^4 times as that of unlabeled aldosterone.

Effect of RU-28362 on binding of glycyrrhetinic acid to kidney cytosol

The binding of unlabled aldosterone or glycyrrhetinic acid to adrenalectomized rat renal cytosol were studied with and without RU28362, a glucocorticoid antagonist, sufficient to block binding to the Type II (glucocorticoid) receptors. The binding curve of aldosterone to the cytosol receptor was shifted to the lower level as a result of Type II receptor occupancy by RU28362. The binding curve of glycyrrhetinic acid to the rat renal cytosol was not affected by the addition of RU28362, which indicate that glycyrrhetinic acid only binds to Type I receptors.

Effect of glycyrrhetinic acid on Mg^{2+}-HCO_3^--ATPase activities in rat small intestinal mucosa

In the normal small intestinal mucosa Mg^{2+}-HCO_3^- activity was around 3000 mol Pi/mg protein per h. Adrenalectomy significantly reduced this enzyme activity ($P < 0.01$ vs normal). The administration of glycyrrhetinic acid in the adrenalectomized rats, however, did not restore Mg^{2+}-HCO_3^- activity. Using the same samples of rat small intestine. Na^+–K^+ ATPase activity was assayed. Ad-

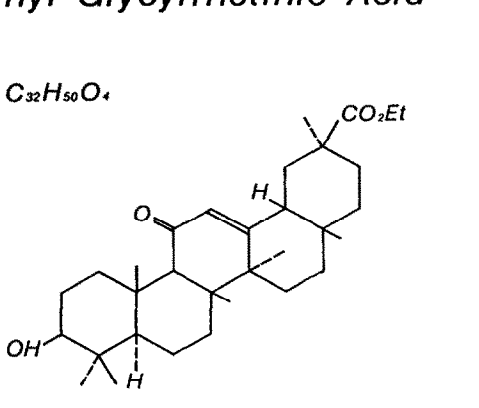

Fig. 1. Structure of ethyl glycyrrhetinic acid.

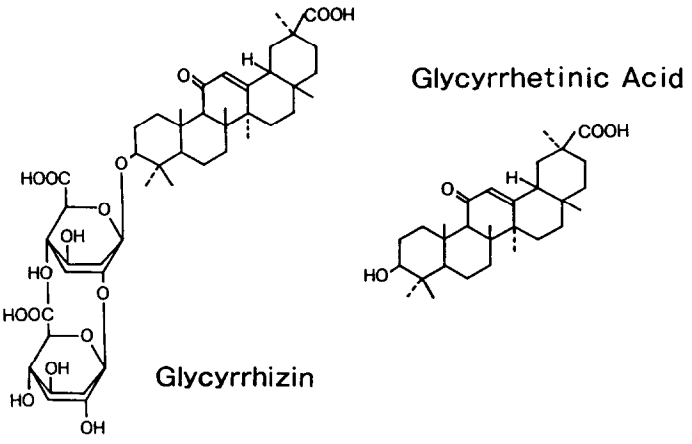

Fig. 2. Structure of glycyrrhizin and glycyrrhetinic acid.

renalectomy also decreased Na^+-K^+-ATPase activity in the rat small intestinal mucosa to approximately 75% of the initial level. The administration of glycyrrhetinic acid in the adrenalectomized rats did not increase this enzyme activity.

Effects of glycyrrhetinic acid on the subtraction potential difference

In the control rats, the subtraction potential difference ranged from 5.7 to −4.3 mV with a medium value of 0 mV. In the adrenalecomized rats given vehicle alone, the subtraction potential difference was significantly reduced, to −14.7 mV (medium value). ($P < 0.05$ vs control, nonparametric analysis.) In the adrenalectomized rats given glycyrrhetinic acid, the value tends to increase compared with those in adrenalectomized rats with vehicle alone, but still lower than those in controls.

DISCUSSION

Ulmann *et al.* first reported that glycyrrhetinic acid possesses a high affinity to mineralocorticoid receptor in rat kidney cytosol *in vitro*[2]. Later, Armanini *et al.* using a more accurate determination of affinity demonstrated that glycyrrhetinic acid as well as glycyrrhizic acid bind to mineralocorticoid receptors, suggesting that these compounds, though different in affinity, are sufficient to explain the pathogenesis of mineralocorticoid-like symptoms [6]. In keeping with these *in vitro* studies, we have previously reported the therapeutic dose of liquorice causes a syndrome of pseudohyperaldosteronism [13], and also confirmed that glycyrrhetinic acid compete with aldosterone for mineralocorticoid receptors [3]. In the present study, we intended to further clarify the binding kinetics using a highly specific glucocorticoid antagonist, RU 28362. Our results demonstrated that the presence of RU 28362 displaced aldosterone binding curve to lower level, and on the Scatchard plot lower affinity site for aldosterone was blocked. On the other hand, binding of glycyrrhetinic acid to kidney cytosol was not affected by the addition of RU 28362. These results indicate that RU 28362 has a specific blocking action for type II receptors, and glycyrrhetinic acid exclusively binds to type I receptors. These results differ from data of the previous workers, who showed that glycyrrhetinic acid binds both mineralocorticoid and glucocorticoid receptors [2]. Since we used a more accurate procedure compared with previous reports, by adding 20 mM of sodium molybdate in the assay system, which is an optimal concentration for the stability of receptors, our results may indicate the single type of receptor binding of glycyrrhetinic acid.

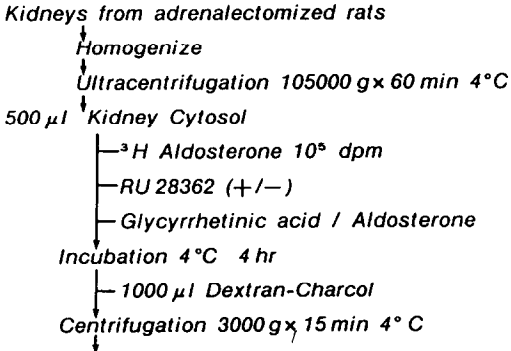

Fig. 3. Preparation procedures of kidney cytosol from adrenalectomized rats.

Recently, abundant evidence has been accumulated to support the hypothesis that aldosterone plays some physiological role in regulating the electrolyte metabolism through activating the enzyme protein synthesis in the upper small intestine, especially in the duodenum. The demonstration of specific binding receptors for aldosterone in the duodneal mucosa favors the above assumption. In fact, there seems to be a functional similarity in ion

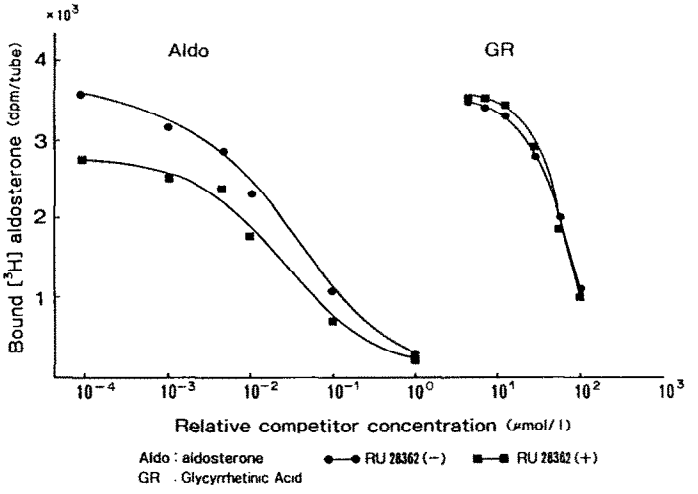

Fig. 4. Competition of aldosterone and glycyrrhetinic acid for [^{3}H]aldosterone binding sites with and without RU28362, anti-glucocorticoid compound. The competition of glycyrrhetinic acid was not affected by the addition of RU28362.

transport via the cell membrane between the renal tubules and the intestinal mucosa. The authors confirmed the previous work of Suzuki *et al.*[8], who reported that the Mg^{2+}-HCO_3^--ATPase in the upper intestinal mucosa decreased to approximately 75% of the initial level following the adrenalectomy, and we found that administration of mineralocorticoid restored this value towards basal levels in the present study. It is interesting that glycyrrhetinic acid had no replacement effect as mineralocorticoid on the decreased enzyme activities in the upper intestinal mucosa of adrenalectomized rats, although it restored the decreased mucosal electrical potential difference to some extent.

Further study is needed to clarify these discrepant results between *in vitro* and *in vivo* experiments.

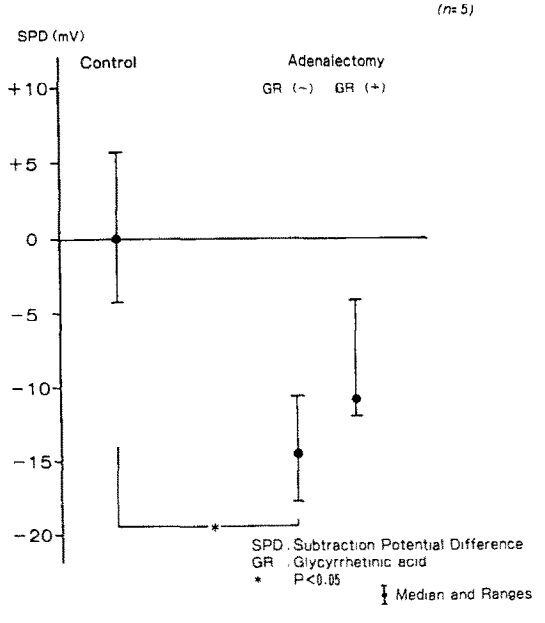

Fig. 5. Electrical potential difference in the rat rectal mucosa was significantly reduced in adrenalectomized rats compared with normal rats. Administration of glycyrrhetinic acid (1.0 ng/g intraperitoneally for 3 consecutive days) tended to restore the potential difference, but it did not come back to the levels of control rats.

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