Inhibitory Effects of Some Natural Products on the Activation of Hyaluronidase and Their Anti-allergic Actions

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The anti-allergic drugs disodium cromoglycate (DSCG) and tranilast are strong inhibitors of hyaluronidase. In the development of new anti-allergic drugs, we studied the inhibitory effects of some natural products on the activation of hyaluronidase. Among the compounds tested, liquiritigenin, isoliquiritigenin and baicalein showed dose-related inhibitory effects. Anti-allergic activities of these compounds were evident from the facts that they inhibited the histamine release from rat peritoneal exudate cells induced by antigen, compound 48/80 and calcium ionophore A-23187, and from their inhibitory effect on Shultz–Dale reaction using sensitized guinea pig ileum.

Keywords hyaluronidase; hyaluronidase-inhibitory effect; anti-allergic activity; isoliquiritigenin; liquiritigenin; baicalein

Allergic responses can be divided into four general categories, based on the mechanism of immunological involvement.[1] A type I allergy which produces various symptoms of asthma, rhinitis, atopic dermatitis and anaphylactic shock is the most common. The pathological mechanism of type I allergy has been explained as the degranulation of mast cells and the release of chemical such mediators as histamine, leucotrienes and prostaglandins from these cells.[2-5] Mast cell degranulation occurs in response to immunological stimuli in which the antigen–immunoglobulin E (IgE) antibody reaction on the cell membrane predominates. Disodium cromoglycate (DSCG)[6] and tranilast[7] which are clinically used as anti-allergic drugs, inhibit the release of chemical mediators from mast cells induced by the antigen–IgE antibody reaction. Compound 48/80,[8] a polycondensate of *N*-methyl-*p*-methoxyphenethylamine and formaldehyde, and calcium ionophore A-23187[9] are inflammatory substances which are known to release histamine, inducing an extensive degranulation of mast cells without mediation by the antigen–IgE antibody reaction.

Hyaluronidase is one of the mucopolysaccharide-splitting enzymes, and is involved in the permeability of the vascular system[10,11] and inflammation.[12,13] We reported that anti-allergic drugs such as DSCG and tranilast had a strong inhibitory effect on the activation of hyaluronidase,[14,15] and also showed that hydrangenol derivatives inhibited not only the activation of hyaluronidase but also the release of histamine from rat peritoneal exudate cells induced by antigen–IgE antibody reaction and compound 48/80.[16] Koda and his colleagues reported that DSCG and disodium baicalein 6-phosphate, which suppressed the increase in capillary permeability caused by 48 h homologous passive cutaneous anaphylaxis (PCA) in rats, inhibited not only hyaluronidase activity *in vitro* but also the activation of hyaluronidase induced by PCA.[17] These results seemed to indicate that potent hyaluronidase inhibitory substances might have anti-allergic effects, and could become leading compounds in the development of new anti-allergic drugs.

The Chinese crude drugs *Glycyrrhiza glabra*, *Scutellaria baicalensis* and *Paeonia albiflora* have been used in the clinical treatment of allergosis in Japan. In the present study, to find novel anti-allergic substances from natural products, we investigated the inhibitory effects of some natural components extracted from these Chinese crude drugs on the activation of hyaluronidase, and tested their anti-allergic activities in relation to their strong inhibition of this activation.

Results and Discussion

The inhibitory effects of some natural products and anti-allergic drugs on the activation of hyaluronidase were examined and the results are shown in Fig. 1. The compounds tested were liquiritigenin, liquiritin, isoliquiritigenin and isoliquiritin in *Glycyrrhiza glabra*, baicalein in *Scutellaria baicalensis*, and paeoniflorin in *Paeonia albiflora*. Liquiritigenin, isoliquiritigenin and baicalein were found to inhibit the activation of hyaluronidase dose-relatedly. The inhibitory effect of isoliquiritigenin was the strongest among the natural compounds tested and was stronger than those of tranilast and traxanox,[18] but was weaker than that of DSCG. The inhibitory effects of baicalein was weaker than that of isoliquiritigeinin, but was stronger than those of liquiritigenin and tranilast. Baicalein and its derivativs are known to have anti-allergic activities with the inhibition of release of chemical mediators from mast cells induced by antigen–antibody reactions.[19] The inhibitory effect of liquiritigenin was weaker than that of tranilast. IC_{50} values (μM) of isoliquiritigenin, baicalein, liquiritigenin, DSCG, tranilast and traxanox were 64, 165, 740, 29, 350 and 85, respectively. These results seem to suggest that acidic groups such as carboxyl, phenolic and tetrazoyl groups are required for the development of the inhibitory activity.

Liquiritin and isoliquiritin, which are 4-O-glucosides of liquiritigenin and isoliquiritigenin, respectively, were non-

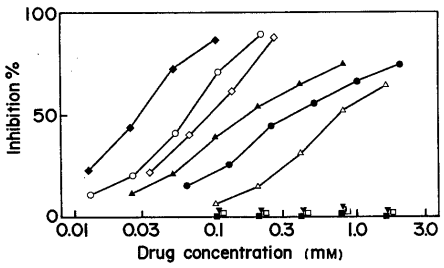

Fig. 1. Inhibitory Effects of Natural Products, DSCG, Tranilast and Traxanox on the Activation of Hyaluronidase

▼, liquiritin; △, liquiritigenin; □, isoliquiritin; ○, isoliquiritigenin; ▲, baicalein; ■, paeoniflorin; ◆, DSCG; ●, tranilast; ◇, traxanox. Each value is the mean of 3 observations.

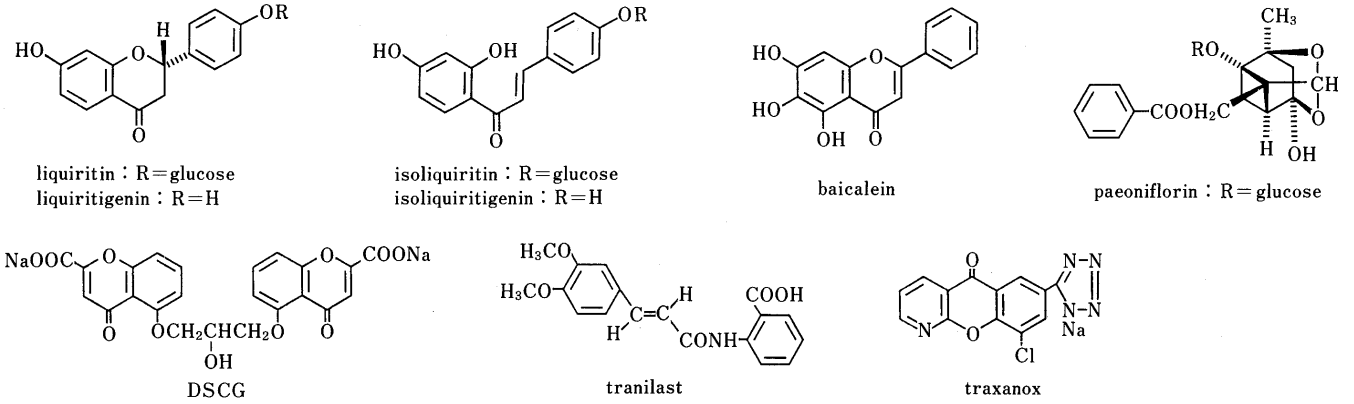

Chart 1. Chemical Structures of the Natural Products and Anti-allergic Drugs Tested

TABLE I. Inhibitory Effects of Some Natural Products and Tranilast on Histamine Release from Rat Peritoneal Exudate Cells Induced by Antigen, Compound 48/80 and Calcium Ionophore A-23187

Treatment	IC_{50} (μM)		
	Antigen	Compound 48/80	A-23187
Liquiritin	>1000	>1000	>1000
Liquiritigenin	724.7±32.2	863.8±33.8	744.5±40.8
Isoliquiritin	>1000	>1000	>1000
Isoliquiritigenin	27.6± 8.8	168.5±12.3	124.7±14.2
Baicalein	163.2±18.0	246.8±20.2	118.7±13.3
Paeoniflorin	ND	ND	ND
Tranilast	682.4±55.6	>1000	>1000

Histamine releases (%) induced by antigen (egg albumin), compound 48/80 and calcium ionophore A-23187 were 58.4±4.0, 87.4±3.2 and 64.2±3.2, respectively. Spontaneous histamine releases (%) were 11.8±3.7, 5.6±2.9 and 7.8±2.7, respectively. The reaction mixture in a total volume of 1.0 ml contained 1×10^5 mast cells. All values are means±S.E.M., n=4—5. ND: not determined.

inhibitors. We previously reported that the inhibitory effects of hydrangenol glucoside and its derivatives were very minimal due to the presence of their 2-O-glucosyl moieties, and speculated that the actions of the carboxyl groups of the compounds toward hyaluronidase might be masked by these moieties.[16] In agreement with these previous findings, the inhibitory effects of liquiritin and isoliquiritin became less potent than their corresponding aglycones due to the presence of the 4-O-glucoside moieties. Paeoniflorin showed no inhibitory effect.

To find out whether the hyaluronidase-inhibitory natural compounds possess anti-allergic activity, we then examined the inhibitory effects of natural products on the histamine release from rat peritoneal exudate cells induced by antigen–antibody reaction, compound 48/80 and calcium ionophore A-23187 (Table I). Among the compounds tested, isoliquiritigenin had the strongest inhibitory action on the histamine release induced by antigen–antibody reaction. In this case, the inhibitory effect of baicalein was stronger than that of tranilast, while that of liquiritigenin was as potent as that of tranilast. Liquiritin and isoliquiritin exhibited very little inhibitory effect, and also paeoniflorin did not possess inhibitory activity. These results were concerned with their anti-hyaluronidase activities.

Furthermore, isoliquiritigenin, baicalein and liquiritigenin inhibited histamine release from rat peritoneal exudate cells induced by compound 48/80 in a dose-related manner. In this case, isoliquiritigenin and baicalein were approximately equal in inhibitory activity. Inhibitory effect of liquiritigenin was weaker than isoliquiritigenin and baicalein, but was stronger than tranilast, while the effects of liquiritin, isoliquiritin and tranilast were very weak.

In addition, isoliquiritigenin, baicalein and liquiritigenin demonstrated a dose-related inhibitory effect on the histamine release induced by A-23187. The inhibitory effects of liquiritin, isoliquiritin and tranilast were minimal.

In both cases, tranilast did not show an obvious inhibitory effect in contrast to its anti-hyaluronidase activity. Ujiie *et al.*[20] demonstrated that tranilast (100 μM) had significant inhibitory effect on histamine release from rat peritoneal exudate cells induced by 9.6×10^{-8} M of A-23187, whereas it had not effect on release by 3.8×10^{-7} M of A-23187, and also indicated that tranilast could not inhibit histamine release induced by a high concentration of A-23187, which has been thought to produce cytolytic response.[21] In the present case, the concentration of A-23187 used was 1×10^{-6} M, which might be the reason tranilast exhibited no marked inhibitory effect. Unlike tranilast, on the other hand, isoliquiritigenin and baicalein clearly inhibited histamine release induced by a high concentration of A-23187, suggesting that their inhibition might be due to protection of the cytolytic response by A-23187.

The histamine release from mast cells is an essential step in the pathological processes of type I allergy.[2-5] The histamine release from mast cells induced by antigen–IgE antibody reaction in higher animals causes various anaphylatic diseases, while the histamine releases from mast cells induced by compound 48/80 and calcium ionophore A-23187 are non-immunological phenomena. Isoliquiritigenin, baicalein and liquiritigenin inhibited the histamine release from rat peritoneal exudate cells induced by not only immunological reaction but also severe non-immunological histamine releasers such as compound 48/80 and calcium ionophore A-23187. In addition, these natural products protect red blood cell membrane against the insults of various agents (T. Satoh, unpublished data). These facts seem to suggest that these compounds may possess cell membrane stabilizing effects. Further investigations on the cell membrane stabilizing actions of these compounds are now in progress.

To confirm the anti-allergic actions of isoliquiritigenin

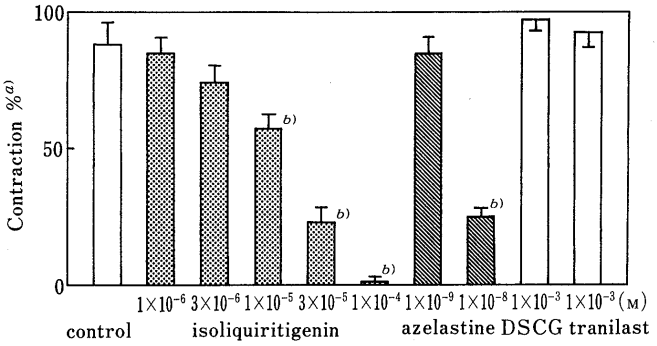

Fig. 2. Effects of Isoliquiritigenin, Azelastine, DSCG and Tranilast on the Schultz–Dale Reaction in Guinea Pig Ileum

a) Responses to the test compounds were expressed as percentages of the maximal response to histamine at 1×10^{-6} M. All values are means±S.E.M., $n=4$. *b*) Significantly different from control at $p<0.01$ (Student's *t*-test).

which exhibited the strongest inhibitory action on histamine release among the natural compounds tested, we examined the effect of isoliquiritigenin on the Shultz–Dale reaction using sensitized guinea pig ileum (Fig. 2). The contraction induced by specific antigen (egg albumin) was 97.5±8.1% when the contraction induced by histamine (1×10^{-6} M) was regarded as 100%. Isoliquiritigenin inhibited dose-relatedly the contraction of guinea pig ileum induced by antigen in a range of 1×10^{-6} to 1×10^{-4} M. The contractions induced by antigen with 1×10^{-6}, 3×10^{-6}, 1×10^{-5}, 3×10^{-5} and 1×10^{-4} M of isoliquiritigeinin were 94.0±5.5, 82.3±6.2, 63.5±5.8, 25.5±6.0 and 1.2±2.3%, respectively. DSCG and tranilast did not show an inhibitory effect in the present experimental system. Baicalein had been demonstrated to inhibit the Schultz–Dale reaction in sensitized guinea pig ileum,[19] its effect being about 3 times weaker than that of isoliquiritigenin. This suggests that the action of isoliquiritigenin is perhaps similar to that of baicalein. On the other hand, azelastine,[22] a basic anti-allergic drug which has strong anti-histamine action, showed a significant inhibition of the contraction induced by antigen at a dose of 1×10^{-8} M. The contractions with 1×10^{-9} and 1×10^{-8} M of azelastine were 93.9±6.6 and 27.4±3.7%, respectively. The excellent inhibitory activity of azelastine on the Schultz–Dale reaction was believed attributable to its strong anti-histamine action.

To determine whether the inhibitory effect of isoliquiritigenin on the Schultz–Dale reaction resulted from its anti-histamine action, anti-histamine activities of this compound and azelastine were investigated. It was found that the inhibitory effect of isoliquiritigenin on the Schultz–Dale reaction was evident at a concentration (3×10^{-5} M) which did not cause a singificant anti-histamine action. The contractions induced by histamine (1×10^{-6} M) with 1×10^{-5}, 3×10^{-5} and 1×10^{-4} M of isoliquiritigenin were 98.3±4.6, 92.5±4.2 and 67.9±5.2%, respectively. The contractions with 1×10^{-9} and 1×10^{-8} M of azelastine were 35.3±5.4 and 2.7±4.2%, respectively. Thus, the inhibitory effect of isoliquiritigenin on the Schultz–Dale reaction was not relevant to its antihistamine action.

In summary, in the present study isoliquiritigenin and liquiritigenin in *Glyzyrrhiza glabra*, and baicalein in *Scutellaria baicalensis* demonstrated an inhibitory effect on activation of hyaluronidase and histamine release from mast cells induced by both immunological and non-immunological stimuli. Furthermore, among the natural compounds tested isoliquiritigenin, unlike the anti-allergic drugs DSCG and tranilast, exhibited the inhibitory effect on the Schultz–Dale reaction in sensitized guinea pig ileum, suggesting that it may have an extensive anti-allergic property. Isoliquiritigenin appears to be a useful compound in the development of novel anti-allergic drugs.

Experimental

Materials Hyaluronidase (from bovine testis, type IV-S), compound 48/80, calcium ionophore A-23187, egg albumin and phosphatidyl-L-serine (from bovine brain) were purchased from Sigma Chemical Co., St. Louis; hyaluronic acid potassium salt, *o*-phthalaldehyde, trichloracetic acid, histamine 2HCl, *Bordetella pertussis* vaccine and Freund's complete adjuvant (FCA) were from Wako Pure Chemical Co., Osaka. Liquiritin, liquiritigenin, isoliquiritin, isoliquiritigenin, baicalein and paeoniflorin were donated by the Kanebo Co., Ltd., Osaka. Tranilast was synthesized in our laboratory according to the method described elsewhere.[23] Traxanox was a gift from Yoshitomi Pharmaceutical Industries, Ltd., Osaka. DSCG was generously supplied by the pharmacology department of this university.

Assay of Hyaluronidase Activity Hyaluronidase activity was determined by the Morgan–Elson method[24] as modified by Davidson and Aronson.[25]: hyaluronidase (340 NF unit/ml) with hyaluronic acid potassium salt (0.6 mg/ml) was incubated at 37 °C for 40 min in 0.1 M acetate buffer, pH 3.5. Calcium chloride (2.5 mM) was used as the activator of hyaluronidase.

Inhibitory Effects on the Activation of Hyaluronidase These effects were determined by the method described above. It was followed by incubation at 37 °C for 20 min in an acetate buffer containing the activator and hyaluronidase which had been preincubated with test substances at 37 °C for 20 min in the same buffer. Test substances were replaced by the buffer solution for control. The percent inhibition was calculated as follows:

$$\text{inhibition (\%)}=\frac{\text{control OD}-\text{sample OD}}{\text{control OD}}\times100$$

Isolation of Rat Peritoneal Exudate Cells Male Wistar rats weighing 200—250 g were exsanguinated and injected intraperitoneally with 20 ml of physiological solution consisting of 137 mM NaCl, 2.7 mM $CaCl_2$, 1.0 mM $MgCl_2\cdot6H_2O$, 5.6 mM glucose, 1 unit of heparin/ml and 5 mM phosphate buffer, pH 7.2. The abdominal region was gently massaged for 2 min and then the peritoneal exudate was collected in a siliconized glass vessel. The cell suspension was centrifuged ($300\times g$ at 4 °C for 5 min) and washed several times with the physiological solution. The cells obtained from 15 animals were then pooled (5×10^4 cells/ml).

Preparation of Sensitized Rat Peritoneal Exudate Cells A dose of 0.5 ml of *Bordetella pertussis* vaccine (containing 2×10^{10} heat-killed *Bordetella* organisms/ml) containing egg albumin (2 mg/ml) as antigen and aluminum hydroxide gel (40 mg/ml) was injected subcutaneously into the footpads of male Wistar rats weighing 180—220 g according to the method of Stotland and Share.[26] Two weeks later, peritoneal exudate cells were obtained from the sensitized rats and separated as described above.

Assay of Histamine Release from Sensitized Rat Peritoneal Exudate Cells Induced by Antigen) The peritoneal exudate cell suspension (2.5 ml) prepared was mixed with 0.3 ml of the physiological solution containing various concentrations of test substances and phosphatidyl-L-serine (3×10^{-5} g/ml) in 0.35 ml of physiological solution and then the mixture was incubated at 37 °C for 5 min. As controls, test substances were replaced by the physiological solution. The preincubated exudate cell suspension (3.5 ml) with 0.35 ml of egg albumin (antigen) solution (1×10^{-3} g/ml) was incubated at 37 °C for a further 10 min. The control solution without egg albumin was treated in the same manner. The mixture was cooled to 4 °C and centrifuged at $2500\times g$ at 4 °C for 10 min. Histamine in the supernatant and residue was measured, respectively, according to the method of Shore *et al.*[27] and the percent inhibition was calculated as follows.

$$\text{histamine release (\%)}=\frac{P_s}{P_s+P_r}\times100=A$$

P_s: histamine in supernatant, P_r: residual histamine in cells.

$$\text{inhibition (\%)} = 100 - \frac{S-B}{C-B} \times 100$$

S: A obtained from test smaple, C: A obtained from control, B: A obtained from blank.

Assay of Histamine Release from Rat Peritoneal Exudate Cells Induced by Compound 48/80 The peritoneal exudate cell suspension (2.5 ml) prepared was mixed with various concentrations of test substances in 0.5 ml of each physiological solution and the mixture was preincubated at 37 °C for 5 min. The control and blank were treated in the same manner. The mixture (3.0 ml) was then mixed with 0.5 ml of compound 48/80 solution (1×10^{-5} g/ml) and incubated at 37 °C for 10 min. The physiological solution was added in place of the solution of compound 48/80 as blank. The mixture (3.5 ml) was cooled to 4 °C and centrifuged at $2500 \times g$ at 4 °C for 10 min, and the histamine released in the supernatant and residue was measured. Percent inhibition was calculated as described above.

Assay of Histamine Release from Rat Peritoneal Exudate Cells Induced by Calcium Ionophore A-23187 The peritoneal exudate cell suspension (2.5 ml) prepared was mixed with various concentrations in 1.0 ml of each physiological solution and the mixture was preincubated at 37 °C for 10 min, the control and blank were treated in the same manner. The mixture (3.5 ml) was then mixed with 0.035 ml of calcium ionophore A-23187 ethanol solution (1×10^{-4} M) and incubated at 37 °C for 10 min. Ethanol was added in place of the ethanol solution of calcium ionophore A-23187 as blank. The mixture was cooled to 4 °C and centrifuged at $2500 \times g$ at 4 °C for 10 min, and the histamine released in the supernatant and residue was measured. The percentage of inhibition was calculated as described above.

Effects on the Schultz–Dale Reaction A dose of 0.6 ml of emulsion consisting of egg albumin (10 mg/ml saline) and 1.0 ml of FCA was injected intramuscularly into male Hartley guinea pigs weighing about 300 g. Ten days later, a 0.4 ml dose of the emulsion consisting of egg albumin (4 mg/ml saline) and 1.0 ml of FCA was injected intramuscularly. Repeated intramuscular injections were performed once every week for 3 weeks. One week after the last sensitization (4 times in total), the animals were exsanguinated from the abdominal aorta, and the ilea were prepared in cold Tyrode solution. The ileal strip was then suspended in a 10 ml magnus tube (32 °C, 95% O_2 + 5% CO_2) containing the Tyrode solution, and the contractile response was recorded isotonically. Each test substance was added to the preparation 5 min before the treatment with antigen (egg albumin, 1×10^{-4} g/ml). The percent contraction was expressed as a percent of the maximal response to histamine at 1×10^{-6} M.

Anti-histamine Actions Male Hartley guinea pigs weighing about 300 g were sacrificed by exsanguination and the ilea were prepared in the cold Tyrode solution. The ileal strip prepared was then suspended in a 10 ml magnus tube (32 °C 95% O_2 + 5% CO_2) containing the Tyrode solution. Each test drug was added to the preparation 30 s before the treatment of histamine (1×10^{-6} M). The percent contraction was shown as a percent of the maximal response to histamine.

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