Biochimica et Biophysica Acta 820 (1985) 199–206
Elsevier

BBA 72781

Saponins can cause the agglutination of phospholipid vesicles

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(Received May 28th, 1985)

Key words: Membrane vesicle agglutination; Phospholipid vesicle; Saponin; Ginsenoside; Ophiopogonin

The interaction of saponins with phospholipid vesicles was investigated by means of liposomal agglutination or a precipitation assay. Ginsenoside-Rc, which has an α-L-arabinofuranose residue at the non-reducing terminus, exhibited remarkable agglutinability toward egg yolk phosphatidylcholine vesicles, while other saponins lacking this characteristic sugar residue showed less or no agglutinability. The molar ratio of ginsenoside-Rc to egg phosphatidylcholine in the aggregates was estimated to be 0.4–0.5 by a precipitation assay using ^{14}C-labeled egg phosphatidylcholine vesicles. The agglutination was inhibited by *p*-nitrophenyl α-L-arabinofuranoside but not by *p*-nitrophenyl β-D-glucopyranoside or arabinogalactan. The results indicated that the α-L-arabinofuranose residue in ginsenoside-Rc should be important for the expression of the agglutinability. The agglutinability of ginsenoside-Rc toward lipid vesicles depended on both the polar head groups and fatty acyl chains of phospholipids. Egg yolk phosphatidylcholine vesicles were strongly agglutinated by ginsenoside-Rc, although sphingomyelin, phosphatidylethanolamine, phosphatidic acid and phosphatidylserine were less agglutinated. The agglutinability of ginsenoside-Rc was effective for phosphatidylcholines with short or unsaturated fatty acyl chains. The results suggested that the interaction of ginsenoside-Rc with phospholipid membranes should be affected not only by the chemical structure of the phospholipid but also by the membrane fluidity.

Introduction

Agglutination of phospholipid vesicles is an interesting phenomenon in relation to cell-cell interactions, since it is an important step in membrane fusion, adhesion and cellular communication. Liposomal agglutination has been reported to be mediated by the following substances: lectins [1], polysaccharides [2,3], and mono- [4] and divalent cations [5]. The molecular mechanism of the agglutination depends on the kind of substance. Lectins can bind to glycoconjugates in lipid vesicles and form vesicular aggregates, and so can divalent cations to acid phospholipids in lipid vesicles. Hepatic cells may also adhere to each other through the interaction of glycoconjugates [6]. Earlier work suggested that phospholipid vesicles can aggregate through various kinds of interactions, although the mechanism remains obscure.

Saponins are known to have various physiological activities, depending on their chemical structures [7,8]. Steroid and triterpene saponins with a single sugar chain (monodesmosides) have a strong hemolytic activity, whereas those with two sugar

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Abbreviations: Phosphate-buffered saline, sodium phosphate buffer (0.02 M, pH 7.2) containing 0.76% NaCl; DMSO, dimethyl sulfoxide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PS, phosphatidylserine; Sph, sphingomyelin; DLaPC, dilauroylphosphatidylcholine; DLiPC, dilinoleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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chains (bisdesmosides) show less activity [9,10]. The interaction of saponins with cholesterol in erythrocyte membranes has been postulated to be involved in the hemolysis [9]. Many saponins can in fact form tightly-bound complexes with cholesterol [11]. However, the interaction between saponins and membrane lipids seems to be very complicated, and other components of the membranes may affect the interactions of saponins [12].

Recently, we reported that some saponins, that is, some ophiopogonins and ginsenosides, showed hemagglutinability toward human, rabbit and sheep erythrocytes, although they had no hemolytic activity [13]. The agglutinability of ginsenosides was also confirmed with vesicles composed of the total lipids of erythrocyte membranes [13], which suggests that ginsenosides can bind to the membrane lipids of erythrocytes and form bridges between the cells.

In order to understand the interaction of saponins with biomembranes at a molecular level, we investigated, in this study, the agglutinability of some saponins, six ginsenosides and two ophiopogonins, toward phospholipid vesicles. The roles of the sugar moiety of saponins, the polar groups of phospholipids and the membrane-fluidity were also discussed.

Materials and Methods

Materials. Ginsenosides-Rb_1, Rb_2, Rc, Rd, Re and Rg_2 were obtained from *Panax ginseng* (C.A. Meyer) as reported previously [14]. Ophiopogonin-B and -D were isolated from *Ophiopogon japonicus* (Ker-Gawler) [15]. The structures of these saponins are shown in Fig. 1. Egg yolk lecithin, phosphatidylethanolamine and phosphatidic acid were prepared from hen eggs in our laboratory. [^{14}C]Phosphatidylcholine was synthesized by methylation of N-dimethyl phosphatidylethanolamine with [^{14}C]CH_3I [16]. Sphingomyelin and phosphatidylserine from bovine brain, *p*-nitrophenyl α-L-arabinofuranoside, phosphorylcholine chloride, L-α-glycerophosphorylcholine cadmium chloride complex and arabinogalactan from larch wood were obtained from Sigma Chemical Co. Other chemicals were obtained commercially. All lipids showed a single spot on thin-layer chromatography with a solvent system of $CHCl_3$/MeOH/H_2O (65 : 25 : 4, v/v/v).

Agglutination assays with egg phosphatidylcholine vesicles. Multilamellar liposomes containing the phospholipid and dicetyl phosphate (molar ratio, 1 : 0.1) were prepared as described previously [17]. Sonicated vesicles were prepared by treatment of multilamellar liposomes in a probe-type sonicator (cell disruptor 200, Bransonic) for 15 min at 0°C under an argon atmosphere. The agglutinability of saponins toward phospholipid vesicles was assayed by the following two methods. (i) The aggregate-formation on a plastic microtiter plate: Each saponin solution was mixed with egg phosphatidylcholine liposomal suspension in the each well. After 1 h at room temperature (23°C), the aggregates formed were observed visually. (ii) The turbidity-change caused by the agglutination: Each saponin solution was mixed with sonicated egg phosphatidylcholine vesicles in phosphate-buffered saline. The absorbances of the mixtures at 450 nm were measured at various times. The absorbance has been reported to be due to liposomal agglutination [1].

Stoichiometric studies on agglutination of ginsenoside-Rc toward phosphatidylcholine vesicles. (i) Precipitation assay with [^{14}C]phosphatidylcholine: Various amounts of ginsenoside-Rc were mixed with sonicated vesicles composed of 0.2 μmol egg phosphatidylcholine, a trace of ^{14}C-labeled egg phosphatidylcholine (final specific activity, 31 200 cpm/μmol) and 0.02 μmol dicetyl phosphate in phosphate-buffered saline. After centrifugation (15 000 rpm, 10 min), the radioactivity of the clear supernatant was counted with an Aloka Scintillation Counter Model 671. Only vesicles aggregated with the saponin were precipitated under the above conditions. The amount of non-aggregated egg phosphatidylcholine vesicles was determined from the recovery of [^{14}C]phosphatidylcholine in the supernatant. (ii) Precipitation assay with a saponin: various amounts of sonicated egg phosphatidylcholine vesicles were mixed with a constant amount of ginsenoside-Rc (100 μg, 0.093 μmol) in phosphate-buffered saline and left for 1 h at room temperature. After centrifugation, non-aggregated saponin in each supernatant was determined by the phenol-H_2SO_4 method (absorbance at 490 nm) [18].

Agglutination of various phospholipid vesicles. Ginsenoside-Rc (50 μg) was mixed with sonicated vesicles containing various kinds of phospholipids (0.1 μmol) and dicetyl phosphate (0.01 μmol) in phosphate-buffered saline (80 μl). After 1 h at room temperature, each mixture was centrifuged at 15000 rpm for 10 min. The amount of non-aggregated saponin in the clear supernatant (50 μl) was determined by the phenol-H_2SO_4 method. The amount of aggregated saponin was determined by subtracting the amount of non-aggregated saponin from that of total saponin.

Inhibition tests. Inhibition was examined on the basis of the turbidity-change due to liposomal agglutination in the presence of each of the following inhibitors: *p*-nitrophenyl α-L-arabinofuranoside, *p*-nitrophenyl β-D-glucopyranoside, arabinogalactan and phosphorylcholine. These inhibitors were dissolved in phosphate-buffered saline containing 16.7% DMSO. Each inhibitor solution was mixed with sonicated egg phosphatidylcholine vesicle solution in phosphate-buffered saline, and then ginsenoside-Rc solution was added to the mixture. After mixing, the turbidity was measured as to the absorbance at 450 nm at various times. The control was assayed without an inhibitor.

The inhibition test for L-α-glycerophosphorylcholine was carried out by aggregate-formation on a microtiter plate. L-α-Glycerophosphorylcholine was dissolved in phosphate-buffered saline containing 18.8% DMSO (75 mM). 30 μl the inhibitor solution was added to each well in serial 2-fold dilutions. Then, 10 μl ginsenoside-Rc solution (50 μg) was added to each well. After 30 min, 10 μl egg phosphatidylcholine vesicles (10 mM) was added to each well. After standing for 1 h, the aggregates formed were observed visually.

Results

Agglutination of saponins toward egg phosphatidylcholine vesicles

The agglutination of eight saponins (Fig. 1) toward egg phosphatidylcholine vesicles was investigated by means of aggregate formation on a microtiter plate. As shown in Fig. 2, only ginsenoside-Rc exhibited remarkable agglutinability toward egg phosphatidylcholine vesicles. Aggregates

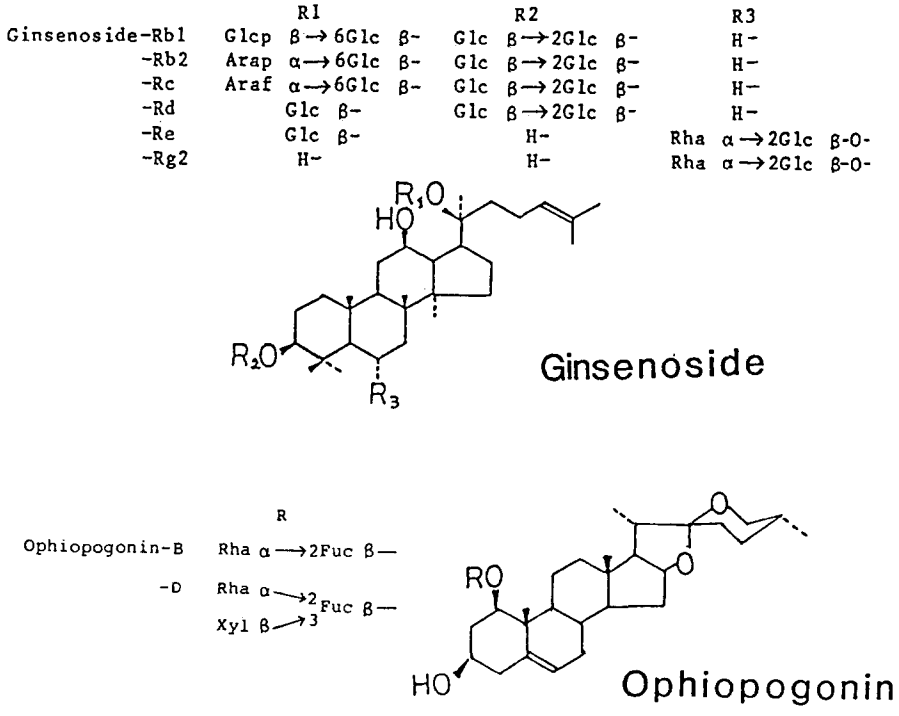

Fig. 1. Structures of saponins employed in this study.

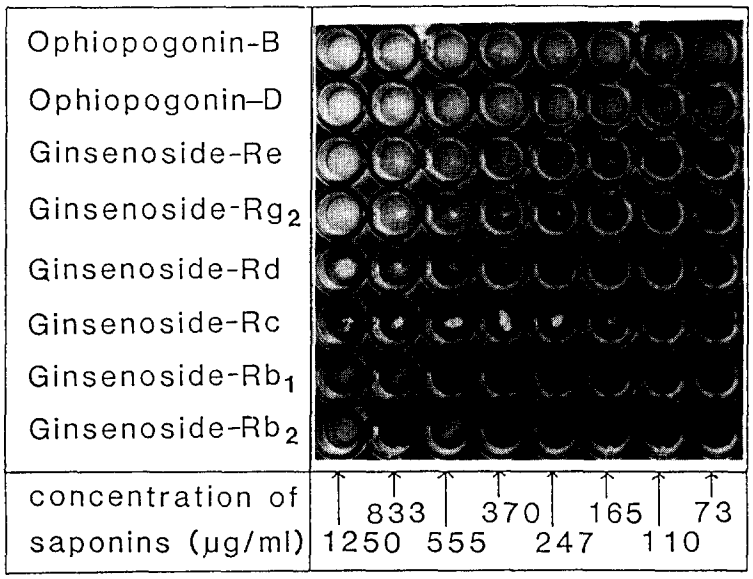

Fig. 2. Agglutination of saponins toward egg phosphatidylcholine vesicles on a microtiter plate. 25 μl of each saponin solution (0.15–2.5 mg/ml phosphate-buffered saline containing 1% DMSO) was mixed with 25 μl of egg phosphatidylcholine vesicle suspension (5 mM) containing dicetyl phosphate (0.5 mM) in each well. After 1 h at room temperature, the aggregates formed were observed visually.

were observed at concentrations of more than 165 μg/ml. Ginsenoside-Rd, which is devoid of the arabinofuranose residue in ginsenoside-Rc, exhibited faint activity only at a high concentration. No appreciable agglutinability was observed for ginsenosides-Rb_1 and Rb_2, which have glucopyranose and arabinopyranose, respectively, instead of the arabinofuranose residue in ginsenoside-Rc. Ophiopogonin-B and -D, and ginsenosides-Re and Rg_2 showed no activities.

The time-courses of the change in agglutination due to these saponins were also investigated quantitatively by means of the turbidity change caused by agglutination of sonicated egg phosphatidylcholine vesicles. As shown in Fig. 3, only ginsenoside-Rc caused a remarkable increase in turbidity. Under these conditions, the agglutination due to ginsenoside-Rc occurred almost completely within five minutes. The velocity was as fast as that of agglutination of lectin and phospholipid vesicles containing glycolipids (Honma, K., Utsumi, H. and Hamada, A., manuscript in

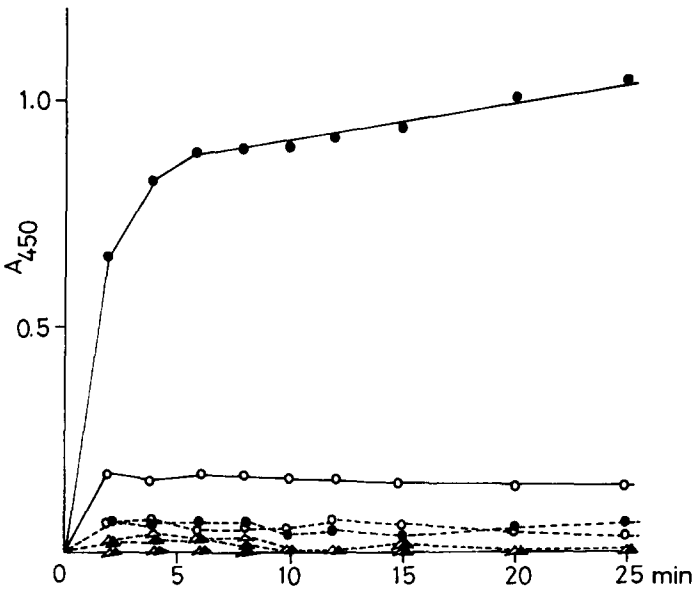

Fig. 3. Agglutination of saponins toward sonicated egg phosphatidylcholine vesicles assayed by estimating the turbidity. 30 μl of each saponin solution (75 μg) was mixed with sonicated vesicles (0.25 μmol egg phosphatidylcholine and 0.025 μmol dicetyl phosphate) in phosphate-buffered saline (0.5 ml). The turbidity was measured as to the absorbance at 450 nm. ●———●, Ginsenoside-Rc; ○———○, ophiopogonin B; ●— — —●, ginsenoside-Rb_2; ○— — —○, ophiopogonin D; ▲— — —▲, ginsenoside-Rb_1; △— — —△, ginsenoside-Rg_2; ▲———▲, ginsenoside-Re; △———△, ginsenoside-Rd.

preparation). No appreciable increase in turbidity was observed for the other saponins except ophiopogonin B which showed a slight increase in turbidity.

Stoichiometric studies on agglutination of ginsenoside-Rc toward egg phosphatidylcholine vesicles

Two different precipitation assays were performed to determine the stoichiometry of the agglutination of ginsenoside-Rc toward egg phosphatidylcholine vesicles. A constant amount (0.2 μmol egg phosphatidylcholine) of sonicated vesicles containing a trace of ^{14}C-labeled egg phosphatidylcholine was mixed with ginsenoside-Rc at various concentrations. Aggregates of phosphatidylcholine vesicles with ginsenoside-Rc were precipitated by centrifugation, and the radioactivity in each supernatant was determined for non-aggregated phosphatidylcholine (Fig. 4, solid line). The amount of non-aggregated phosphatidylcholine decreased with increasing amounts of ginsenoside-Rc up to 100 μg (0.093 μmol). When 100 μg ginsenoside-Rc was added to 0.2 μmol ^{14}C-labeled egg phosphatidylcholine vesicles (molar ratio of ginsenoside-Rc to PC, 0.47), the amount of non-aggregated phosphatidylcholine reached the minimum level and no phosphatidylcholine vesicles were recovered in the supernatant. Addition of more than 100 μg of ginsenoside-Rc caused an increase in the amount of non-aggregated phosphatidylcholine.

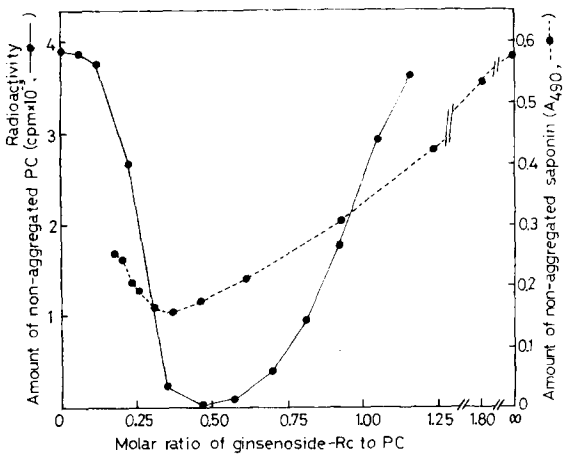

Fig. 4. Stoichiometry of agglutination of ginsenoside-Rc toward egg phosphatidylcholine vesicles. ●——●, a constant amount of sonicated vesicles (0.2 μmol ^{14}C-labelled egg phosphatidylcholine and 0.02 μmol dicetyl phosphate) was mixed with various amounts of ginsenoside-Rc (0–250 μg, 0.23 μmol) in phosphate-buffered saline (160 μl). After standing for 1 h at room temperature, centrifugation was carried out at 15000 rpm for 10 min. The amount of non-aggregated phosphatidylcholine in each supernatant (100 μl) was determined by estimating the radioactivity. ●— — —●, a constant amount of ginsenoside-Rc (100 μg, 0.093 μmol) was mixed with various amounts of sonicated egg phosphatidylcholine vesicles (0–0.5 μmol) containing dicetyl phosphate (0–0.05 μmol) in phosphate-buffered saline (160 μl). After centrifugation, the amount of non-aggregated saponin in each supernatant (100 μl) was determined by the phenol-H_2SO_4 method (*A* 490 nm). The amounts of non-aggregated phosphatidylcholine or saponin were plotted against the molar ratio of ginsenoside-Rc to PC.

Also, a constant amount of ginsenoside-Rc (100 μg, 0.093 μmol) was mixed with various amounts of sonicated egg phosphatidylcholine vesicles, and non-aggregated saponin recovered in each supernatant was determined by the phenol-H_2SO_4 method (*A* 490 nm). Fig. 4 (dotted line) shows the amount of non-aggregated saponin in the supernatant against the molar ratio of ginsenoside-Rc to phosphatidylcholine (PC). When 0.25 μmol phosphatidylcholine vesicles was added to 100 μg ginsenoside-Rc (molar ratio of ginsenoside-Rc to PC, 0.37), the amount of non-aggregated saponin was minimum, but some of the ginsenoside-Rc still remained in the supernatant (Fig. 4, dotted line). From these results, it could be concluded that the molar ratio of ginsenoside-Rc to phosphatidylcholine in the aggregates should be close to 0.4–0.5.

Specificity of phospholipids as to the agglutinability of ginsenoside-Rc

As described above, ginsenoside-Rc showed remarkable agglutinability toward egg phosphatidylcholine vesicles. To determine whether ginsenoside-Rc recognizes the polar head groups of phospholipids or not, we investigated the agglutination with other phospholipids which have different polar head groups. Table IA shows the relative amounts of the saponin in saponin-phospholipid aggregates. Ginsenoside-Rc showed no or less aggregation with sonicated vesicles containing egg phosphatidylethanolamine, egg phosphatidic acid or phosphatidylserine from bovine brain. This indicated the requirement of the choline moiety in the phospholipid for the agglutination. Sphingomyelin from bovine brain, which had phosphorylcholine in the polar head group as in the

TABLE I

SPECIFICITY OF PHOSPHOLIPIDS AS TO AGGLUTINATION OF GINSENOSIDE-Rc

The agglutinability is expressed as the relative amount of aggregated saponin.

	Phospholipids	Relative amount of aggregated saponin (%)
(A)	Egg PC	100.0
	Egg PE	2.8
	Egg PA	9.3
	Bovine PS	1.0
	Bovine Sph	2.8
(B)	Egg PC	100.0
	DLaPC (C 12:0)	101.0
	DMPC (C 14:0)	50.0
	DPPC (C 16:0)	5.6
	DLiPC (C 18:2)	96.4
	Soya-bean PC	102.9
	Hydrogenized soya-bean PC	5.9

case of phosphatidylcholine, was less reactive with ginsenoside-Rc. The predominant reactivity of phosphatidylcholines was also confirmed by the aggregate-formation test on a microtiter plate. These results suggested that ginsenoside-Rc should recognize not only phosphorylcholine but also the glycerol backbone of phospholipids.

The fluidity of membranes may have an important role from many physiological aspects. To investigate the effect of membrane-fluidity, ginsenoside-Rc was incubated with phosphatidylcholine vesicles having different fatty acyl chains. As shown in Table IB, dilauroyl(C12:0)-, dilinoleoyl(C 18:2)- and soya-bean phosphatidylcholine vesicles formed almost the same amount of aggregates as that in the case of egg yolk phosphatidylcholine vesicles, while dipalmitoyl(C 16:0)- and hydrogenized soya-bean phosphatidylcholine, which have higher phase transition temperatures (T_c (DPPC) = 41.5°C) than room temperature, formed much less aggregates. In the case of dimyristoyl(C 14:0) phosphatidylcholine, which has a phase transition temperature near the experimental temperature, half of the saponin was found in the aggregates.

Inhibition tests

Ginsenoside-Rc has a characteristic sugar residue, an α-L-arabinofluranose residue, and only this saponin showed strong agglutinability toward lecithin (Figs. 2, 3). Accordingly, *p*-nitrophenyl α-L-arabinofuranoside, *p*-nitrophenyl β-D-glucopyranoside and arabinogalactan were used as inhibitors to investigate the roles of the α-L-arabinofuranose and β-D-glucopyranose residues in ginsenoside-Rc. Arabinogalactan has been reported to have β-L-arabinofuranose residues at the non-reducing termini [19]. Fig. 5 shows the time-course of the change in turbidity of mixtures of ginsenoside-Rc, sonicated egg phosphatidylcholine vesicles and various inhibitors. No appreciable

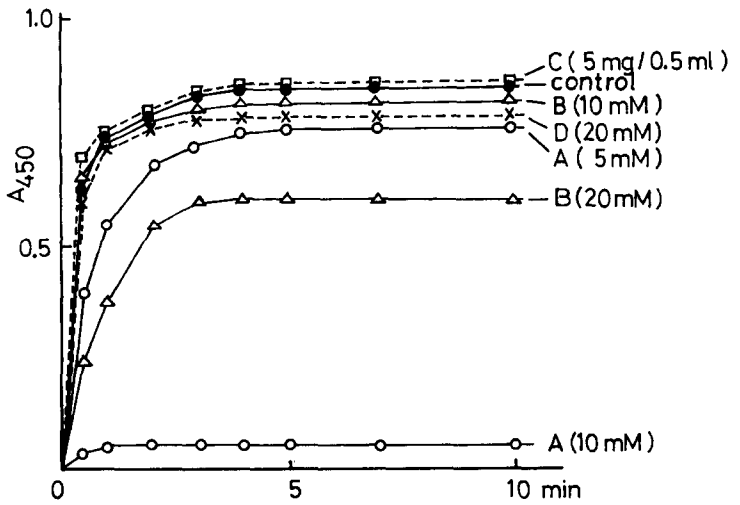

Fig. 5. Inhibitory effect on agglutination of ginsenoside-Rc toward sonicated egg phosphatidylcholine vesicles assayed by estimating the turbidity. Each inhibitor dissolved in phosphate-buffered saline containing 16.7% DMSO (100 μl) was mixed with sonicated vesicles (0.25 μmol egg phosphatidylcholine and 0.025 μmol dicetyl phosphate) in phosphate-buffered saline (0.4 ml), and then 70 μl of ginsenoside-Rc solution (175 μg) was added to the mixture. After mixing, the turbidity was measured as to the absorbance at 450 nm. The control (●———●) was assayed using the same buffer but without an inhibitor. A, *p*-nitrophenyl α-L-arabinofuranoside (○———○); B, *p*-nitrophenyl β-D-glucopyranoside (△———△); C, arabinogalactan (□— — —□); D, phosphorylcholine (×— — —×).

turbidity could be observed in the presence of *p*-nitrophenyl α-L-arabinofuranoside at a final concentration of 10 mM. The addition of arabinogalactan (5 mg/0.5 ml) did not have any inhibitory effect on the change in turbidity, while *p*-nitrophenyl β-D-glucopyranoside did a little at a high concentration (20 mM). These results indicated the importance of α-L-arabinofuranose in the reaction between ginsenoside-Rc and phosphatidylcholine vesicles.

Phosphorylcholine and L-α-glycerophosphorylcholine were also used as inhibitors to investigate the role of the polar head group of egg phosphatidylcholine vesicles. Phosphorylcholine had no inhibitory effect on the agglutination even at the final concentration of 20 mM (Fig. 5). The effect of L-α-glycerophosphorylcholine could not be examined as to the change in turbidity, as shown in Fig. 5, because it becomes an emulsion itself. On a microtiter plate, L-α-glycerophosphorylcholine did not show an inhibitory effect.

Discussion

In the present work, we found agglutinability of saponins toward phosphatidylcholine vesicles. Among eight saponins, two ophiopogonins and six ginsenosides, only ginsenoside-Rc showed strong agglutinability. Stoichiometric studies indicated that ginsenoside-Rc agglutinated egg phosphatidylcholine vesicles in the molar ratio of approximately 0.4–0.5. Cholesterol has been reported to associate with phospholipids with a stoichiometry of 1 : 2 in addition to 1 : 1 [20]. The molar ratio of cholesterol to phospholipid is quite similar to that of ginsenoside-Rc to phosphatidylcholine in aggregates, indicating that the triterpenoid backbone of ginsenoside-Rc may behave like cholesterol in phospholipid vesicles.

The carbohydrate moiety of ginsenoside-Rc, particularly the α-L-arabinofuranose residue, seems to be important for expression of the agglutinability, because other ginsenosides lacking this sugar residue showed no agglutinability. The agglutinability decreased drastically on substitution of the arabinofuranose residue by a glucopyranose or arabinopyranose residue. The role of the α-anomeric sugar residue was also supported by the result of the inhibition test. *p*-Nitrophenyl α-L-arabinofuranoside inhibited the agglutination, but arabinogalactan having β-L-arabinofuranose residues at the non-reducing termini [19] did not. On the other hand, phosphorylcholine and L-α-glycerophosphorylcholine which have the same polar head group moiety as egg phosphatidylcholine did not exhibit an inhibitory effect. The results indicated that the mechanism of agglutination by the saponin was different from that of either C-reactive protein [21] or PC-binding protein detected in rat serum [22], because their binding activities were reported to be inhibited by phosphorylcholine [21,22].

Agglutination of ginsenoside-Rc was specific for phosphatidylcholine vesicles, since ginsenoside-Rc showed less agglutination of sphingomyelin, phosphatidylethanolamine, phosphatidylserine or phosphatidic acid vesicles. The results indicated the importance of the glycerophosphorylcholine moiety in the reaction between ginsenoside-Rc and phospholipids, even if the hexagonal II phase appears in addition to the lamellar phase [23]. Fatty acyl chains of phosphatidylcholines had an important influence on the agglutinability. Phosphatidylcholines with short (DLaPC) or unsaturated (DLiPC) fatty acyl chains showed remarkable agglutinability at room temperature, as much as egg yolk or soya-bean phosphatidylcholine, while dipalmitoylphosphatidylcholine (DPPC) or hydrogenized soya-bean phosphatidylcholine showed no agglutinability. The former group of phosphatidylcholines should be in the liquid-crystalline phase, whereas the latter should be in the gel state (T_c; DLaPC, 0°C; DLiPC, < -20°C; DMPC, 23.0°C; DPPC, 41.5°C) [24]. These findings indicate that the interaction of ginsenoside-Rc with phosphatidylcholine membranes should be controlled by the membrane-fluidity. The membrane-fluidity may be required both for the insertion of ginsenoside-Rc into lipid bilayers and for the reaction with other vesicles. The agglutination of ginsenoside-Rc toward phosphatidylcholine is a very interesting phenomenon, since this phospholipid seems to recognize the specific sugar residue in saponins, and it should be useful for elucidating the cell-cell interactions mediated by glycoconjugates in membranes.

Acknowledgements

This research was supported by a grant from the Ministry of Education, Science and Culture of Japan, and the Research Foundation for Pharmaceutical Science.

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