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Specific interaction of arabinose residue in ginsenoside with egg phosphatidylcholine vesicles

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The interaction of the specific sugar residue in ginsenosides with egg phosphatidylcholine vesicles was investigated by ESR spectrometry using phosphatidic acid spin-labeled at the polar head groups. Ginsenoside-Rc, which has an α-L-arabinofuranose residue and agglutinability toward egg yolk phosphatidylcholine vesicles (Fukuda, K. et al. (1985) Biochim. Biophys. Acta 820, 199–206), caused the restriction of the segmental motion of spin-labeled phosphatidic acid in egg phosphatidylcholine vesicles, indicating that the saponin interacted with the polar head groups of vesicles. Other ginsenosides-Rb_2, Rb_1, Rd and *p*-nitrophenyl glycoside derivatives which have less or no agglutinability were also investigated in the same manner. Only ginsenoside-Rb_2 and *p*-nitrophenyl α-L-arabinofuranoside which have the specific sugar residue (arabinose) showed a strong interaction with the polar head groups of vesicles. To gain an insight into the mechanism of agglutination by ginsenoside-Rc, the interaction with the fatty acyl groups was also studied by using phosphatidylcholine spin-labeled at the fatty acyl groups. Ginsenoside-Rc increased the order parameter of the spin-labeled phosphatidylcholine, indicating that the saponin was inserted into lipid bilayers. In other saponins investigated, only ginsenoside-Rb_2 interacted with the fatty acyl part of vesicles. The process of expression of agglutination by ginsenoside-Rc was discussed on the basis of the ESR studies.

Introduction

The agglutination of phospholipid vesicles is an important phenomenon in relation to cell–cell interaction. Recently, we reported that a saponin, ginsenoside-Rc, with an α-L-arabinofuranose residue at the non-reducing terminus of its sugar chain could cause the agglutination with egg phosphatidylcholine [1,2]. This is a novel example of a phospholipid recognizing a specific sugar residue because other ginsenosides lacking this sugar residue showed less or no agglutinability.

This agglutination was specific to phosphatidylcholine with short or unsaturated fatty acyl chains and not to phosphatidylethanolamine, phosphatidylserine or phosphatidic acid. This 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.

We studied the interaction of ginsenoside-Rc

Abbreviations: phosphate-buffered saline, sodium phosphate buffer (0.02 M, pH 7.2) containing 0.76% NaCl; PC, phosphatidylcholine; SL-PC, spin-labeled phosphatidylcholine; SL-PA, spin-labeled phosphatidic acid; ESR, electron spin resonance; DMSO, dimethyl sulfoxide.

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with egg phosphatidylcholine vesicles by means of ESR spectrometry. We used phosphatidic acid spin-labeled at the polar head groups (SL-PA) to elucidate the interaction of the specific sugar residue in ginsenoside-Rc with the polar head groups of phospholipids. The interaction of other saponins and sugar derivatives with the polar head groups was also investigated and compared with ginsenoside-Rc. In order to gain an insight into the mechanism of agglutination by ginsenoside-Rc, we also used spin-labeled phosphatidylcholine carrying a nitroxide at the seventh position of tis fatty acyl chain. On the basis of the results of analyses of ESR spectra, we have discussed a process of agglutination by ginsenoside-Rc of phosphatidylcholine vesicles.

Materials and Methods

Materials. Ginsenosides-Rb_1, Rb_2, Rc and Rd were prepared from *Panax ginseng* (C.A. Meyer) as reported previously [3]. The structures of these saponins are shown in Fig. 1. Egg yolk phosphatidylcholine and phosphatidic acid were prepared from hen eggs in our laboratory. The two kinds of spin-labeled phospholipids used were synthesized as follows, and their structures are also shown in Fig. 1. A stearic acid derivative, (7-[*N*-oxyl-4,4-dimethyloxazolidine] stearic acid (7SLS) which has a nitroxide-containing ring at

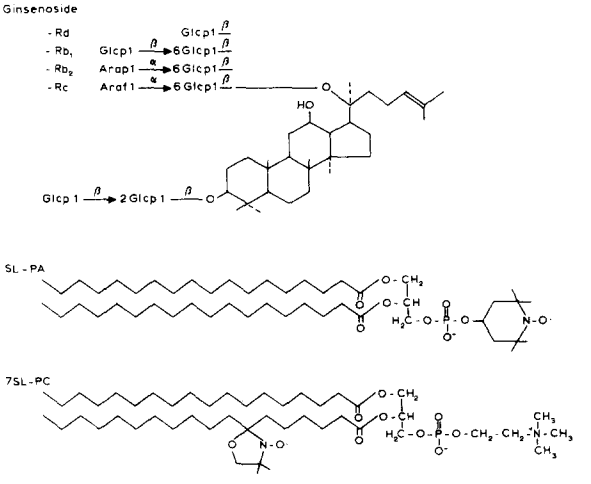

Fig. 1. Structures of ginsenosides, spin-labeled phosphatidic acid (SL-PA) and phosphatidylcholine (7SL-PC).

the C-7 position of its fatty acyl part was synthesized from 7-keto methyl stearate according to the literature [4]. Spin-labeled phosphatidylcholine (7SL-PC) was synthesized by condensation of 7SLS anhydride and egg yolk lysophosphatidylcholine [5]. Phosphatidic acid spin-labeled at the polar head group (SL-PA) was synthesized from egg phosphatidic acid according to the literature [6]. Dicetyl phosphate was obtained from Sigma Chemical Co.

Preparation of liposomes. Multilamellar vesicles containing egg PC, dicetyl phosphate and 7SL-PC (molar ratio, 1 : 0.1 : 0.05), or SL-PA (1 : 0.1 : 0.03) were prepared as described previously [7]. Sonicated vesicles were prepared by treatment of multilamellar liposomes in a probe-type sonicator as described previously [2].

ESR studies. Various amounts of ginsenoside-Rc (0–0.46 μmol/μmol PC) were exogenously mixed with multilamellar egg phosphatidylcholine vesicles incorporating 7SL-PC or SL-PA as the spin-label agent in phosphate-buffered saline (0.04 ml, final concn. 5 mM PC). In the case of phosphatidylcholine vesicles containing 7SL-PC, ginsenoside-Rc was also endogenously mixed with vesicles during their preparation. In the case of other saponins-ginsenosides-Rb_2, Rb_1 and Rd, various amounts (0–125 μg) were exogenously mixed with multilamellar egg phosphatidylcholine vesicles containing 7SL-PC or SL-PA as the spin label agent in phosphate-buffered saline (0.1 ml, final concn. 3 mM PC). *p*-Nitrophenyl glycoside derivatives (final concn. 0–20 mM) were also mixed with phosphatidylcholine vesicles in phosphate-buffered saline containing DMSO (0.1 ml, final concn. 4.15% DMSO, 3 mM PC). An aliquot of each mixture was taken up into a disposable micro-pipette (Drummond Sci. Co.). The pipette was sealed at one end with Hemat Sealer and then inserted into the ESR cavity. Electron spin resonance spectra were measured at 25 °C with a Jeol JES-PE ESR spectrometer, with 100 kHz field modulation as described in the literature [7].

Inhibition tests. Inhibition was examined on the basis of the turbidity change due to liposomal agglutination by ginsenoside-Rc in the presence of ginsenoside-Rb_2, Rb_1 or Rd as inhibitor [2,8]. The inhibitor (25–125 μg) was mixed before or after the agglutination by ginsenoside-Rc of sonicated

egg phosphatidylcholine vesicles was caused in 0.5 ml of phosphate-buffered saline (final concn. 0.5 mM PC; Rc 150 μg, 0.28 mM).

Results

Interaction of the specific sugar residue with the polar head groups of phospholipids

Ginsenoside-Rc, which has an α-L-arabinofuranosyl residue at its non-reducing terminus, exhibited remarkable agglutinability toward egg yolk phosphatidylcholine vesicles [2]. On the other hand, ginsenosides-Rb_1, Rb_2 and Rd which have other substituted sugar residues, as shown in Fig. 1, showed less or no agglutinability. We investigated the interaction of the specific sugar residue with the egg phosphatidylcholine vesicles using a spin-label technique. As shown in Fig. 1, phosphatidic acid spin-labeled at the polar head group (SL-PA) was used to obtain information on hydrophilic regions of liposomal membranes.

The addition of ginsenoside-Rc to egg phosphatidylcholine vesicles affected the motion of phospholipids at the membrane surface. Fig. 2A shows the ESR spectra for SL-PA in egg phosphatidylcholine vesicles with or without ginsenoside-Rc. When ginsenoside-Rc was added to vesicles containing SL-PA (0.46 μmol/μmol PC), the ratios of peak heights, $h(0)/h(+1)$ and $h(0)/h(-1)$, increased greatly, suggesting that ginsenoside-Rc may restrict the segmental motion of SL-PA. Three parameters concerning the rotational correlation time, τ_B, τ_C and τ_R, and the anisotropy index, τ_B/τ_C, were calculated using the peak height ratios and the line widths as described previously [9,10].

$$\tau_B = 0.635 \times \Delta H(0) \times \left(\sqrt{h(0)/h(-1)} - \sqrt{h(0)/h(+1)}\right)$$

$$\tau_C = 0.595 \times \Delta H(0) \times \left(\sqrt{h(0)/h(+1)} + \sqrt{h(0)/h(-1)} - 2\right)$$

$$\tau_R = 0.650 \times \Delta H(0) \times \left(\sqrt{h(0)/h(-1)} - 1\right)$$

The anisotropic index, τ_B/τ_C, shows the degree of anisotropy about the rotational axis of the nitroxide moiety, which exhibits anisotropic motion above 1.0 and isotropic motion below 1.0 [9,10]. The parameter, τ_R, represents the rotational correlation time of the tumbling motion of the spin-labeled moiety [11]. When various amounts of ginsenoside-Rc were added to vesicles containing SL-PA, the three parameters, τ_B, τ_C and τ_R, increased greatly in proportion to the amount of added saponin, as shown in Fig. 3A. The anisotropy index, τ_B/τ_C, decreased greatly and became nearly 1.0. The changes in τ_R and τ_B/τ_C were in proportion to the amount of added ginsenoside-Rc. The results showed that the aniso-

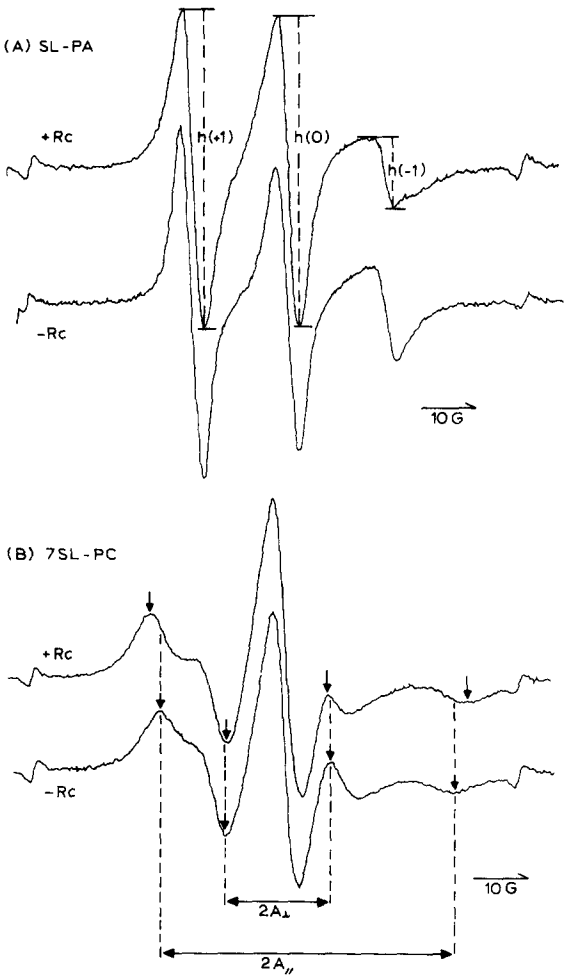

Fig. 2. ESR spectra for phosphatidylcholine vesicles containing SL-PA (A) or 7SL-PC (B) in the presence and absence of ginsenoside-Rc. The liposomes were composed of egg yolk PC, DCP and SL-PA or 7SL-PC in a molar ratio of 1:0.1:0.03 or 0.05. Ginsenoside-Rc (0.46 μmol/μmol PC) or phosphate-buffered saline was added to the liposomes (final 5 mM PC). The order parameter was calculated as follows: $(2A_{\parallel} - 2A_{\perp})/50$.

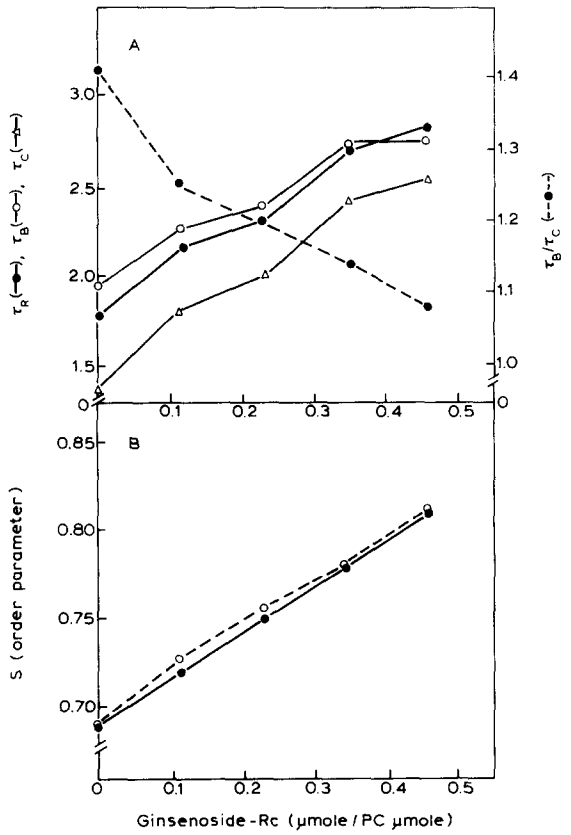

Fig. 3. (A) Changes in three parameters, τ_B, τ_C and τ_R, and anisotropy index, τ_B/τ_C, calculated from ESR spectra for SL-PA incorporated into liposomes with various ginsenoside-Rc concentrations. The liposomes were composed of egg yolk PC, DCP, and SL-PA (1 : 0.1 : 0.03). Various amounts of ginsenoside-Rc were added to preformed liposomes (final 5 mM PC). τ_B (○———○), τ_C (△———△), τ_R (●———●) and τ_B/τ_C (●-----●). (B) Changes in the order parameter (S) in ESR spectra for 7SL-PC incorporated into liposomes with various ginsenoside-Rc concentrations. The liposomes were composed of egg yolk PC, DCP and 7SL-PC in a molar ratio of 1 : 0.1 : 0.05. Various amounts of ginsenoside-Rc were added before (○------○) or after (●———●) the formation of liposomes (final 5 mM PC).

tropic motion of SL-PA gradually became near to being an isotropic one, and the motion itself was decreased by ginsenoside-Rc. This means that the segmental mobility of the nitroxide moiety in SL-PA incorporated into the lipid bilayer is restricted by the interaction with ginsenoside-Rc. These results indicated that ginsenoside-Rc could interact with the polar head groups of phosphatidylcholine and that this interaction should reduce the segmental mobility of SL-PA.

Other saponins-ginsenosides-Rb_2, Rb_1 and Rd which have less or no agglutinability were also investigated for the interaction with the polar head groups of vesicles using SL-PA as spin-label agent. As shown in Fig. 4A, only ginsenoside-Rb_2 which has an α-L-arabinopyranosyl residue showed the increase of the rotational correlation time (τ_R) of SL-PA in proportion to the amount of the saponin added (0–0.39 μmol/μmol PC). Ginsenosides-Rb_1 and Rd which have not arabinose residue showed little increase of τ_R under the addition of the saponin (0–125 μg, Rb_1 0–0.38 μmol/μmol PC, Rd 0–0.44 μmol/μmol PC).

In the previous paper [2], we reported that *p*-nitrophenyl α-L-arabinofuranoside inhibited the agglutination by ginsenoside-Rc of egg phosphatidylcholine vesicles at the final concentration of 10 mM, but *p*-nitrophenyl β-D-glucopyranoside did not inhibit at the same concentration. The changes of τ_R of SL-PA were investigated by the addition of these *p*-nitrophenyl glycoside derivatives to egg phosphatidylcholine vesicles over the final concentration of 0–20 mM (0–6.67 μmol/μmol PC). As shown in Fig. 4B, *p*-nitrophenyl α-L-arabinofuranoside showed the increase of τ_R in proportion to the amount added but the increase of τ_R by glucose derivative was negligible,

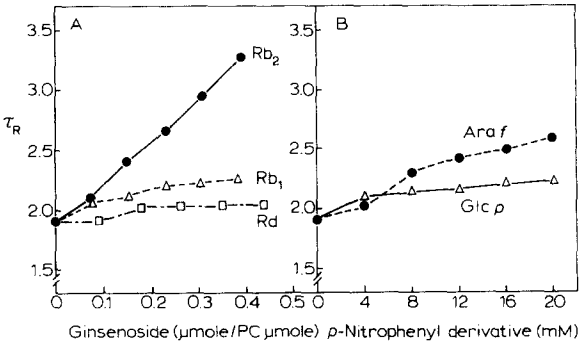

Fig. 4. Changes in the rotational correlation time (τ_R) calculated from ESR spectra for SL-PA incorporated into liposomes with other ginsenosides (A) or *p*-nitrophenyl glycoside derivatives (B). Various amounts of ginsenoside-Rb_2 (●———●), Rb_1 (△-----△), Rd (□-----□), *p*-nitrophenyl α-L-arabinofuranoside (●-----●) or β-D-glucopyranoside (△———△) were added to preformed egg PC vesicles containing SL-PA (final 3 mM PC).

although the concentration of the derivatives was 16-fold higher than that of ginsenosides. These results indicated that the specific sugar residue, arabinose, in saponins and *p*-nitrophenyl glycoside derivatives interacted with the polar head groups of phospholipids and cause the restriction of the segmental mobility of SL-PA.

Interaction of saponin with the fatty acyl chains of phospholipids

We have also studied using phosphatidylcholine spin-labeled at the fatty acyl chains (7SL-PC) to obtain information on the interaction with hydrophobic regions of liposomal membranes. Two representative ESR spectra for 7SL-PC in vesicles with or without ginsenoside-Rc are shown in Fig. 2B. The order parameter (S), which represents the degree of membrane fluidity, was calculated from the spectra as described previously [12,13]. The order parameter in the presence of ginsenoside-Rc was greater than that without ginsenoside-Rc. To obtain further precise data, various amounts of ginsenoside-Rc were exogenously added to egg phosphatidylcholine vesicles containing 7SL-PC, and then the order parameter was calculated. The order parameter increased gradually in proportion to the amount of added ginsenoside-Rc (Fig. 3B). The endogenous addition of ginsenoside-Rc gave almost the same results (Fig. 3B) as in the case of the exogenously incorporated saponin. These results suggested that ginsenoside-Rc could be inserted into the hydrophobic region of egg phosphatidylcholine vesicles and then reduced the segmental mobility of 7SL-PC.

Other saponins, ginsenosides-Rb_2, Rb_1 and Rd, which exhibit no agglutinability were investigated for the interaction with the fatty acyl chains of vesicles using 7SL-PC as spin-label agent. As shown in Fig. 5A, only ginsenoside-Rb_2 increased the order parameter of 7SL-PC to the same degree as the case of ginsenoside-Rc, indicating that ginsenoside-Rb_2 could be inserted into the lipid bilayer and interact with the fatty acyl chains of phospholipids. Whereas, ginsenosides-Rb_1 and Rd showed a little and no increase of the order parameter, respectively, suggesting that these saponins could not be inserted into the lipid bilayer. As shown in Fig. 5B, two *p*-nitrophenyl-glycoside derivatives gave no effect on the change

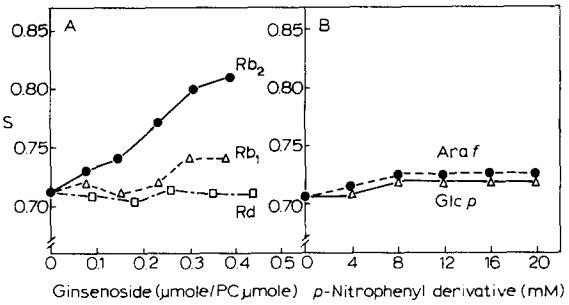

Fig. 5. Changes in the order parameter (S) calculated from ESR spectra for 7SL-PC incorporated into liposomes with other ginsenosides (A) or *p*-nitrophenyl glycoside derivatives (B). Various amounts of ginsenoside-Rb_2 (●———●), Rb_1 (△- - - - -△), Rd (□ - - - - -□), *p*-nitrophenyl α-L-arabinofuranoside (●- - - - -●) or β-D-glucopyranoside (△———△) were added to preformed egg PC vesicles containing 7SL-PC (final 3 mM PC).

of the order parameter, indicating that these derivatives could not interact with the hydrophobic regions of phospholipids.

Inhibition by other ginsenosides of the agglutination caused by ginsenoside-Rc

In the presence of other ginsenosides, inhibitions of the agglutination by ginsenoside-Rc of egg phosphatidylcholine vesicles were examined on the basis of the turbidity change. When ginsenoside-Rb_2 was simultaneously added to the reaction mixture as the inhibitor, the agglutination by ginsenoside-Rc was suppressed to approx. 80% and 0% by the additions of ginsenoside-Rb_2 (50 μg and 100 μg), respectively (Fig. 6). Whereas, ginsenosides-Rb_1 and Rd showed a little and no suppression, respectively, by the additions of 50 μg of each saponin (Fig. 6).

After the agglutination by ginsenoside-Rc was caused without the inhibitor, ginsenoside-Rb_2, Rb_1 or Rd was added to the reaction mixture which showed a high turbidity. Addition of ginsenoside-Rb_2 (125 μg) completely suppressed the agglutination by ginsenoside-Rc and the turbidity entirely disappeared (Fig. 6). Addition of ginsenoside-Rb_1 or Rd (125 μg) also suppressed the agglutination to 66% and 89%, respectively (Table I).

In the previous paper [2], we reported that *p*-nitrophenyl α-L-arabinofuranoside inhibited the agglutination but *p*-nitrophenyl β-D-gluco-

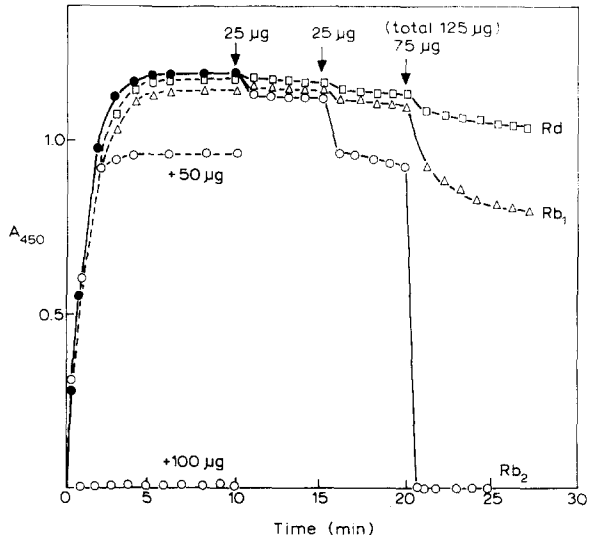

Fig. 6. Inhibitory effects on agglutination by ginsenoside-Rc toward sonicated egg PC vesicles assayed by the turbidity change. Each inhibitor – ginsenoside-Rb_2 (50 μg and 100 μg, O------O), Rb_1 (50 μg, Δ------Δ) or Rd (50 μg, □-----□) was simultaneously mixed with ginsenoside-Rc (150 μg) and sonicated egg PC vesicles in 0.5 ml of phosphate-buffered saline (final concn. 0.5 mM PC, 0.28 mM Rc). The turbidity was measured as to the absorbance at 450 nm. The control (●———●) was assayed using the same condition without an inhibitor. After the agglutination by ginsenoside-Rc was caused, various amounts (25 μg, 25 μg, 75 μg) of ginsenoside-Rb_2 (O——O), Rb_1 (Δ——Δ) or Rd (□——□) were added to the control mixture step by step as shown with arrows.

TABLE I

INHIBITION BY VARIOUS GINSENOSIDES AND SUGAR DERIVATIVES OF LIPOSOMAL AGGLUTINATION BY GINSENOSIDE-Rc

Inhibitors	Concn.	Maximal agglutination (%)
Control (without inhibitor)		100
Ginsenoside-Rc	150 μg (0.28 mM)	
egg PC	0.50 mM	
p-Nitrophenyl α-L-arabinofuranoside	5 mM	88
	10 mM	6
p-Nitrophenyl β-D-glucopyranoside	10 mM	97
	20 mM	71
Ginsenoside-Rb_1	50 μg	95
	125 μg (0.23 mM)	66
Ginsenoside-Rb_2	50 μg	81
	125 μg (0.23 mM)	0
Ginsenoside-Rd	50 μg	99
	125 μg (0.26 mM)	89

pyranoside did not inhibit at the final concentration of 10 mM (Table I). From the results of inhibition tests described above, it was elucidated that ginsenoside-Rb_2 with an arabinose residue and *p*-nitrophenyl α-L-arabinofuranoside strongly inhibited the agglutination by ginsenoside-Rc of egg phosphatidylcholine vesicles compared with the inhibitions by ginsenosides with no arabinose residue and *p*-nitrophenyl β-D-glucopyranoside. These inhibtions seemed to be caused by the interaction of arabinose residue in inhibitors with the polar head groups of phosphatidylcholine vesicles (Figs. 4 A and B). Ginsenoside-Rb_2 was also seemed to inhibit the agglutination by insertion into the lipid bilayer (Fig. 5A).

Discussion

Ginsenoside-Rc which has an α-L-arabinofuranosyl residue at its non-reducing terminus exhibited remarkable agglutinability toward egg phosphatidylcholine vesicles. We studied the interaction of saponin with phosphatidylcholine vesicles by means of ESR spectrometry. As spin-probes, we used SL-PA labeled at the polar head group of phosphatidic acid to elucidate the interaction of the specific sugar residue in saponin with the polar head groups of phospholipids. The anisotropy index, τ_B/τ_C, and the rotational correlation time, τ_R, were calculated from the ESR spectra for SL-PA. When agglutination occurred on the addition of ginsenoside-Rc, a great decrease in τ_B/τ_C and an increase in τ_R were observed. It seemed that ginsenoside-Rc could interact with the polar head groups of phospholipids, which resulted in the restriction of the movement of SL-PA. In other ginsenosides which have not agglutinability, only ginsenoside-Rb_2 having arabinopyranosyl residue strongly interacted with the poalr head groups of phospholipid vesicles. *p*-Nitrophenyl α-L-arabinofuranoside could also interact with the polar head groups. It seemed that the specific sugar residue, arabinose, in ginsenosides and sugar derivatives could interact with the polar head groups of vesicles. This finding is an example of a phospholipid recognizing a specific sugar residue. In recent years, Chapman et al. have reported the interaction of a nonreducing disaccharide of glucose, trehalose, with phos-

pholipids [14–16]. Interactions of neutral polysaccharides, Dextran, with phospholipids have been reported by Schachter [17] and Aducci and coworkers [18].

We also studied the interaction of saponin with the hydrophobic part of the phospholipids by using 7SL-PC spin-labeled at the fatty acyl groups of phosphatidylcholine. The order parameter of 7SL-PC in the lipid bilayer of vesicles greatly increased when agglutination occurred on the addition of ginsenoside-Rc. Therefore, it was suggested that ginsenoside-Rc could be inserted into the lipid bilayer of phosphatidylcholine vesicles and then interacted with the fatty acyl part in the lipid bilayer.

On the basis of the results of these studies, a possible process of agglutination by ginsenoside-Rc of phosphatidylcholine vesicles was proposed that can be summarized as follows: insertion of ginsenoside-Rc into the lipid bilayer in the membranes and interaction with the fatty acyl groups of phospholipids (step 1) → binding to the polar head groups of other phosphatidylcholine vesicles (step 2) → expression of agglutination by the cross-linking with other vesicles (step 3).

Ginsenoside-Rb_2 having less agglutinability toward egg phosphatidylcholine could also interact with both the polar head groups and the fatty acyl groups of phospholipids, the interactions of which were the same as those of ginsenoside-Rc. Why does ginsenoside-Rb_2 not show agglutinability compared with ginsenoside-Rc, nevertheless the results of interactions of ginsenoside-Rb_2 using spin-labels-7SL-PC and SL-PA were the same as those of ginsenoside-Rc? To dissolve the question, we are studying the reaction mixtures of ginsenosides and sonicated egg phosphatidylcholine vesicles by ^{13}C-NMR spectrometry [19]. The preliminary results of NMR spectrometry have suggested that the strength of cross-linking of ginsenoside-Rc with other phosphatidylcholine vesicles may be stronger than that of ginsenoside-Rb_2. Further studies are required to elucidate the mechanism of the agglutination by ginsenoside-Rc of egg phosphatidylcholine vesicles.

The agglutinability of ginsenoside-Rc toward native cell membranes which contain cholesterol or glycoconjugates such as glycophorin was suppressed, although data were not shown in this paper [20]. In fact, only slight agglutination was observed toward erythrocytes in comparison with egg yolk phosphatidylcholine vesicles [1]. It has been demonstrated that ginsenoside-Rc has an antitumor activity [21]. It remains to be elucidated whether or not the interaction of ginsenoside-Rc with phosphatidylcholine vesicles is related to that with membranes of tumor cells.

Acknowledgements

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