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Effect of Ginseng Saponin on Gap Junction Channel Reconstituted with Connexin32

Eun Jung Hong, Keun Huh[1] and Seung Keun Rhee*

Department of Biochemistry, [1]Department of Pharmacology, Yeungnam University, Kyongsan 712-749, Korea

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Panax-ginseng saponin has been known to exert various pharmacological effects on cellular metabolism. This study was performed to determine the effect of ginseng saponin on gap junction channel-mediated intercellular communication, using an established *in vitro* system of reconstituted gap junction channels. Gap junction channels are a specialized plasma membrane fraction, which are permeable to relatively large water-soluble molecules. The sucrose permeable property of reconstituted gap junction channels was completely inhibited with 0.1% (w/v) of ginseng saponin. We also compared the effect of ginseng saponin with that of Triton X-100, a nonionic detergent, on the same system. Triton X-100 showed significantly different effect on sucrose-permeability of gap junction channel from that was affected by ginseng saponin. The structures of liposomes containing gap junction channels was significantly destroyed by Triton X-100.

Key words : Ginseng, Gap junction channel, Connexin32, Sucrose-permeability, Triton X-100, Reconstitution

INTRODUCTION

Gap junction channels provide a unique pathway through which direct intercellular communications occur in most mammalian tissues (Loewenstein 1981; Spray and Bennett, 1985). A number of studies showed that gap junction channel-mediated intercellular communication controlled cell growth, differentiation, and development (Ginzberg and Gilula, 1979; Hertzberg and Jonson 1988).

The root of Panax ginseng has been widely used as restorative drug, sedative, psychic energizer, and an agent to counter senile changes and to prolong vital force in oriental countries including China (Tang and Eisenbrand, 1992). Recently, detailed studies on ginseng have shown that it has various physiological activities in many cell types, including neuron and smooth muscle cell (Murase and other groups reported that ginseng has been shown have some components similar to nerve growth factor (NGF) and epidermal growth factor (Zhang *et al.*, 1991; Murase *et al.*, 1994). In addition, other experimental evidences indicated that ginseng saponin modulates cell division, tumor metastasis, and smooth muscle contraction (Masunaga *et al.*, 1994; Yokozaya *et al.*, 1994; Sato *et al.*, 1994). These studies led us to assume that the components of ginseng could affect the intercellular communication induced by gap junction channel because the gating of gap junction channels has been known to closely related with regulation of cell cycle, transformation, electric and metabolic couplings among neighboring cells.

In the present study, we described direct effects of ginseng saponin on reconstituted gap junction channels, which allow neighboring cells to exchange their cellular metabolites including secondary messengers such as cyclic nucleotides, inositol 1,4,5 tris-phosphate, Ca^{2+}. As mentioned above, since ginseng saponin has shown to modulate various cellular activities, it would be very meaningful to observe how ginseng saponin affect the gating of gap junction channels.

Correspondence to: Seung Keun Rhee, Department of Biochemistry, Yeungnam University, Kyongsan 712-749, Korea

MATERIALS AND METHODS

Materials

Phospholipids (PC, PS, Rhodamine-labeled PE) were purchased from Avanti Polar Lipids, Inc. (Birmingham, USA). Tween 20 (non-ionic detergent), nitro blue tetrazolium (NBT), diisopropyl-fluorophosphate (DIFP), and (3-[(3-Chloamidopropyl)-dimethylammonio]-1-propansulfonate) were obtained from Sigma Chemical Co. (St. Louis, USA). Bio-Gel (A-0.5 m, exclusion limit: 500,000 Da) and silver-staining kit were obtained from Bio-Rad Laboratories (R*ichmon* d, USA). Im-

mobilon PVDF membrane was purchased from Enprotech (Boston, USA). Alkaline phosphatase-conjugated goat anti-mouse IgG and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Boehringer Mannheim Biochemicals (Indiana Police, USA). Sprague-Dowley rats (CD strain, 35-days old, female) were obtained from Life Science Inc. (Taegu, Korea). Ginseng saponin was kindly provided by Korea Tobacco and Ginseng Corporation.

Purification of connexin32 (gap junction channel-forming protein in rat liver) by immunoaffinity chromatography.

Connexin32 was affinity-purified from a CHAP-solubilized plasma membrane fraction of rat liver using a monoclonal antibody (M12.13), as previously described (Rhee *et. al.*, 1989). A plasma membrane fraction of liver tissue was prepared by Percoll-gradient centrifugation (Prpic *et al.*, 1984), and then solubilized with CHAPS (final conc.: 1%) in phosphate buffer (50 mM Na-phosphate, 50 mM NaCl, 5 mM EDTA, 3 mM NaN_3, pH 7.0). The criterion used for solubilization of connexin32 was to take the supernatant following centrifugation at 100,000×g for 1 hr. The solubilized fraction was applied to an affinity column containing Sepharose beads to which a monoclonal antibody (M12.13, Goodenough *et al.*, 1988) was attached. Connexin32 bound to the immunobeads was eluted by brief exposure to urea buffer (10 mM HEPES, 10 KCl, 0.1 EDTA, 3 mM NaN_3 and 459 Urea, pH 4.0), and eluent was rapidly neutralized by adding one-tenth volume of 1M HEPES (pH 7.5). Samples were subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1972). Proteins on the gel were electro-transferred to Immobilon PVDF membrane with a semi-dry blotter in standard transfer buffer (25 mM Tris-HCl, 192 mM glycine, 15% Me-OH, pH 8.3) at current density of 0.8 mA/cm^2 for 36 minutes. The membrane was incubated with primary antibody (M12.13) at 5 μg/ml for 1 hour at room temperature. After washing with PBS-Tween 20 (0.5%) for 30 minutes with changes every 10 minutes, the membrane was incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG) at 5 μg/ml for 1 hour, and then developed in 0.1 mg/ml NBT and 0.05 mg/ml BCIP in alkaline phosphate buffer (10 mM Tris, 10 mM $MgCl_2$, 100 mM NaCl, pH 9.5). For the visualization of protein bands, the gel was silver-stained following the manufacturer's protocol.

Reconstitution of gap junction channel into liposomes

Protein in the immunoaffinity column eluates were mixed with phospholipid solution. Phosphatidyl choline (PC), phosphatidyl serine (PS) and Rhodamine-labeled phosphatidyl ethanolamine (PE) dissolved in chloroform were mixed at a concentration ratio of 2 : 1 : 0.3. The lipid mixture was dried to a thin film under a stream of nitrogen gas, and then desiccated to remove any residual traces of organic solvent for 20 minutes at room temperature. The lipid film was suspended with urea buffer (459 mM urea, 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, pH 7.4) containing 1% CHAPS and immunoaffinity-purified connexin32 at a concentration of 2 μg/ml. The protein/lipid/detergent mixture (usually 2 ml volume) was incubated on ice for at least 20 minutes with occasional gentle swirling, and then dialysis was performed at 4°C, as described in Nakade *et al.*, (1994). Urea buffer (1,000-fold excess volume) used as dialysis buffer was changed every 8 hours for 72 hours.

Transport-specific density shift technique

The procedure used to assay functional properties of reconstituted gap junction channel based on its sucrose permeability was described and characterized by Harris *et al.*, (1989). Linear iso-osmolar density gradients were formed from the urea and sucrose buffer (450 mM sucrose, 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, pH 7.4) in 3 ml ultracentrifuge tubes (Hitachi, Japan) using gradient maker. An aliquot of proteoliposme mixture (100 μl) was layered on the top of each gradient. Gradients were typically spun at 300,000×g for 2 hours in swinging bucket rotor (SW 60, Hitachi, Japan) at 4°C. The specific intensity of Rhodamine fluorescence of gradient fractions (200 μl) was measured with a Perkin-Elmer 650-10S spectrophotometer (560 nm excitation; 590 nm emission).

RESULTS AND DISCUSSION

Purification and reconstitution of gap junction channels into liposomes

When the immunoaffinity purified gap junction channel-forming protein from rat liver (connexin32) was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), two major bands were identified at positions of 27 and 47 kDa. Western blotting analysis revealed that these two bands were monomeric and dimeric form of connexin32, gap junction channel-forming protein in liver tissue (Fig. 1) (Goodenough *et al.*, 1988). In order to incorporate the immunoaffinity-purified connexin32 into phospholipid vesicles, we used dialysis method rather than gel-filtration, avoiding protein dilution during reconstitution. In our previous studies the dialysis method led 10-fold dilution of connexin32, although it prepared homogeneous size of proteoliposomes.

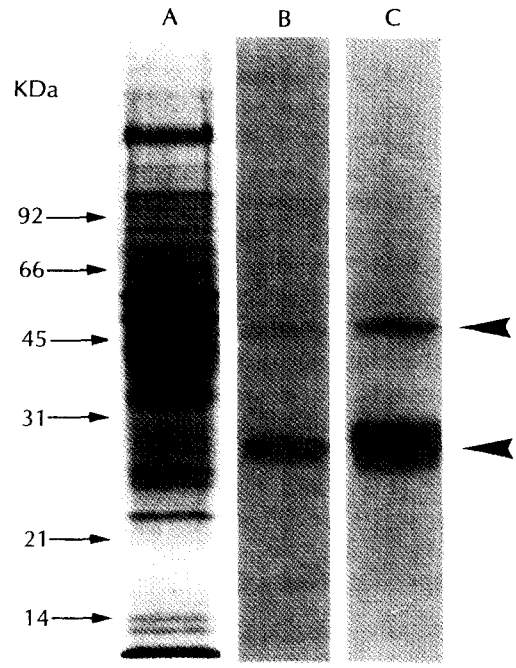

Fig. 1. SDS polyacrylamide gel electrophoresis and Western blotting of immunoaffinity-purified connexin32 (gap junction-forming protein in rat liver). Lane A: silver staining of solubilized plasma membrane fraction of rat liver homogenate, Lane B: silver staining of immunoaffinity-purified connexin32, Lane C: Western blotting of immunoaffinity-purified connexin32 using a monoclonal antibody (M12.13)

For the functional analysis of the reconstituted channels, sucrose-permeable property was assayed in an iso-osmalar urea-sucrose density gradient. Phospholipid vesicles that contain gap junction channels should be permeable to sucrose, and get higher specific density than those vesicles without gap junction channels (Fig 2A). Following centrifugation of the reconstituted phospholipid vesicles on urea-sucrose density gradient at 200,000×g for 2 hours, phospholipid vesicles were separated into two layers: sucrose-impermeable upper one and sucrose-permeable lower one (Fig 2B). A precise distribution of phospholipid vesicles on gradient were analyzed by measuring the distribution of Rhodamine-labeld phosphatidyl ethanolamine (PE) (Fig. 3A). In order to confirm that the density increase in phospholipid vesicles is due to gap junction channel, vesicles at both upper and lower position in density gradient were subjected to immuno-dot blot assay (Fig 3B). Phospholipid vesicles at higher density contained connexin32, indicating that the sucrose-permeability was induced by gap junction channels. Occasionally, the phospholipid vesicles at low density position also contained small fraction of connexin32. This phenomenon is probably due to incomplete formation of gap junction channel or irreversible protein denaturation during purification.

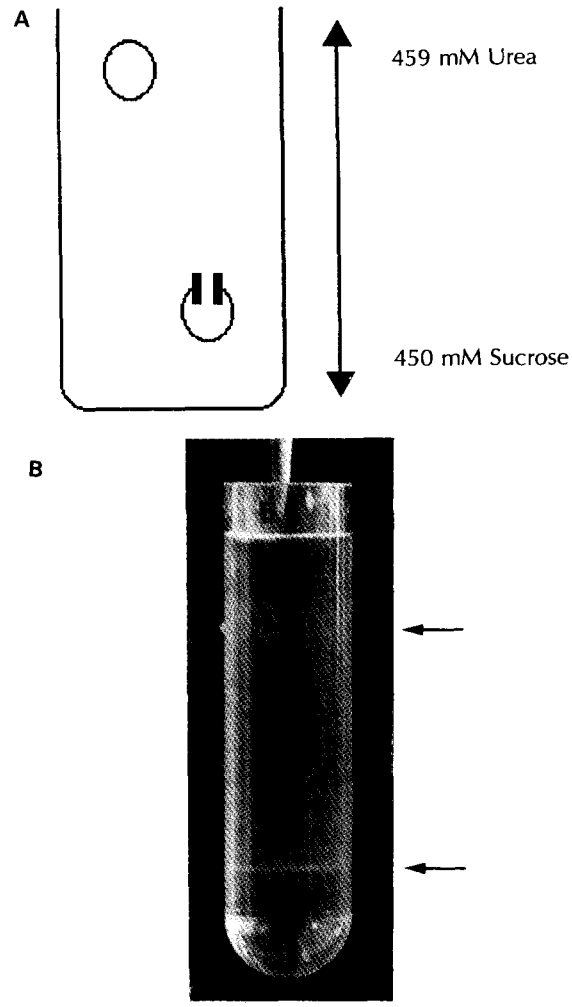

Fig. 2. A: Mechanism of transport-specific density shift method by which sucrose-permeable vesicles containing gap junction channels are separated from sucrose-impermeable vesicles. B: Phospholipid vesicles were divided into two layers, differing their densities after centrifugation for 2 hours at 300,000×g. The vesicles were visualized by using Rhodamine-labeled PE. Upper and lower arrows represent sucrose-impermeable and sucrose-permeable vesicles, respectively.

Effect of ginseng saponin on sucrose-permeability of reconstituted gap junction channel

Ginseng has been known to contain a number of substances that have various physiological effects. In this study, the effect of ginseng saponin was observed on the reconstituted gap junction channel. Various concentrations of ginseng saponin (ranging from 1×10^{-4} to 0.1%) were applied to an urea-sucrose density gradient, on which the reconstituted phospholipid vesicles were loaded to assess their sucrose-permeable property (Fig. 2B). As shown in Fig. 4, the sucrose-permeable fraction of phospholipid vesicles exclusively disappeared on the urea-sucrose density

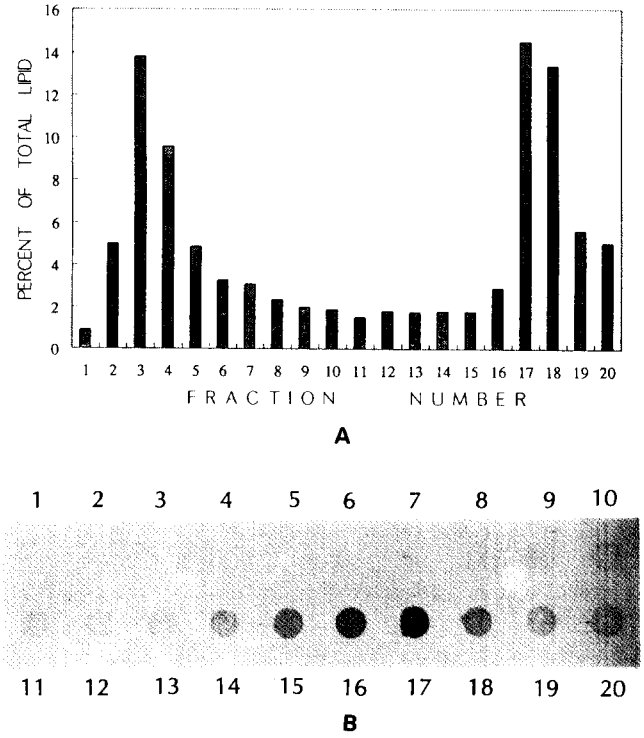

Fig. 3. A: Distribution of phospholipid vesicles after centrifugation was determined by measuring the fluorescent intensities of each fraction from top to bottom of urea-sucrose gradient (1 to 20). B: Distribution of connexin32 on urea-sucrose gradient after centrifugation was determined by immuno-dot blotting using a monoclonal antibody.

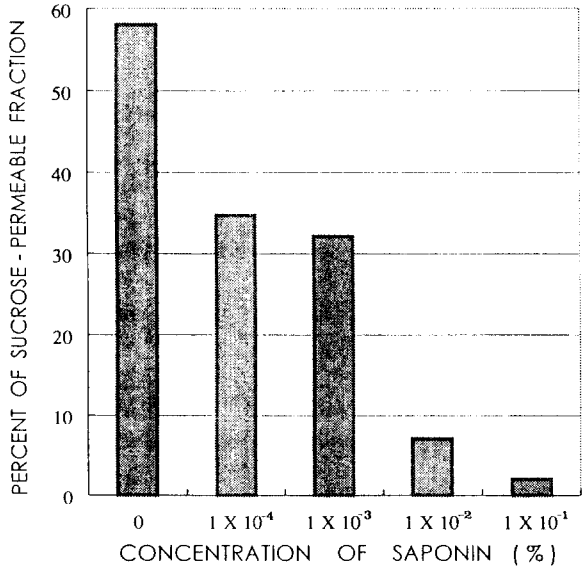

Fig. 4. The effect of ginseng-extract on the sucrose-permeability of reconstituted gap junction channels. The graph was prepared from data obtained by five experiments.

gradient containing 0.1% of ginseng saponin. In comparison to control experiment (in the absence of ginseng saponin), as the higher concentration of ginseng was exposed to the reconstituted channels, the less

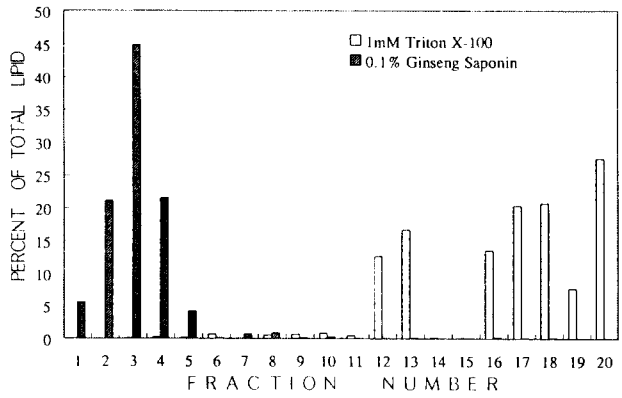

Fig. 5. The effects of ginseng-extract (0.1%) and Triton X-100 (1 mM) on the sucrose-permeability of reconstituted gap junction channels. The distribution of phospholipid vesicles on urea-sucrose gradients containing either ginseng-extract or Triton X-100 was determined by measuring fluorescent intensities of Rhodamine-PE.

fraction of phospholipid vesicles were increased in their densities.

Ginseng saponin has been known to act as a surfactant, which decreases surface tension and dissolves lipids, like conventional detergents (Joo, 1993). Accordingly, we suspected that the inhibitory effect of ginseng saponin on the sucrose-permeability of gap junction channel might come from the detergent-like characteristics of ginseng. In order to compare the effect of ginseng saponin on the sucrose-permeability of gap junction channel with the effect exerted by detergent, we observed the effect of Triton X-100, a non-ionic detergent, on the permeability of gap junction channel. Sucrose-permeability of gap junction channel was observed in urea-sucrose density gradients containing either 1% ginseng saponin or 1% Triton X-100 (Fig. 5). Phospholipid vesicles spun on the gradient containing Triton X-100 were exclusively localized at the bottom of gradient, indicating that the structures of liposomes were broken due to the amphipathic property of Triton X-100.

These results represent that the ginseng saponin inhibits the sucrose-permeability of gap junction channels. A number of studies have reported that gap junction channel-mediated intercellular communication is involved in the complex processes of cell growth and differentiation (Sheridan, 1976; Loewenstein, 1979). Furthermore, it has been also shown that intercellular communication through gap junction channels was significantly disrupted in the process of carcinogenesis and also in the maintenance of transformed phenotypes (Trosko *et al.*, 1983; Yamasaki and Katoh, 1988).

Interestingly, recent studies reported that ginseng saponin exerts a growth factor-like activity on various cell types (Zhang *et al.*, 1990; Murase *et al.*, 1994). If the ginseng saponin promotes cell growth as well as

proliferation, this mechanism might involve a modulation of the intercellular communications between neighboring cells via gap junction channels. The inhibitory effect of ginseng saponin on the sucrose-permeability of reconstituted gap junction channel observed in this study would contribute to better understanding of the biological actions of ginseng saponin.

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