Inhibiyory Effects of Ginseng Saponins Metabolized in Degestive Tract on Adrenal Secretion of Catecholamines *In vitro*

Eiichi Tachikawa[1], Hideo Hasegawa[2], Kudo Kenzo[1], Takeshi Kashimoto[1], Yoshikazu Miyate[1,3], Atsushi Kakizaki[1,3], Katsuo Takahashi[3] and Eiji Takahashi[1]

[1]*Department of Pharmacology, School of Medicine, Iwate Medical University, Uchimaru 19-1, Morioka 020-8505, Japan*

[2]*Fermenta Herb Institute Inc., 1-30-15 Motohongo, Hachioji, Tokyo 192-0051, Japan*

[3]*Department of Pharmacy, Iwate Medical University Hospital, Uchimaru 19-1, Morioka 020-8505, Japan*

Abstract

We have previously found that the saponins but not other components in the ginseng reduce the secretion of catecholamines (CAs) from bovine adrenal chromaffin cells, a model of sympathetic nerves, evoked by acetylcholine (ACh) due to the blockade of Na^+ influx through nicotinic ACh receptor-operated cation channels, and it has been concluded that the inhibitory effect may be associated with the anti-stress action of ginseng. However, the saponins, which showed the great reduction of the CA secretion, were mainly the protopanaxiatriols. The protopanaxadiol and oleanolic acid saponins had a little or little such effect. Recent studies demonstrated that the oligosaccharides connected to the hydroxyl groups of the aglycones of the saponins are in turn hydrolyzed by gastric acid and enzymes in the intestinal bacteria when the ginseng is orally administrated. In this study, the effects of their major 6 kinds of metabolites on the secretion of CAs were investigated. All metabolites (M1, 2, 3 and 5 derived from the protopanaxadiols, and M4 and 11 from the protopanaxiatriols) reduced the ACh-evoked secretion from the cells. In the metabolites, the M4 inhibition was the most potent (IC_{50} (μM): M4 (9) < M2 (18) < M3 (19) < M11 (22) < M5 (36) < M1 (38)). Although M4 also reduced the CA secretion induced by high K^+, a stimulation activating voltage-sensitive Ca^{2+} channels, the inhibitory effect was much less than that on the ACh-evoked secretion. M4 inhibited the ACh-induced Na^+ influx into the cells in a concentration-dependent manner similar to that of the inhibition of the ACh-evoked secretion. When the cells were washed by the incubation buffer after the preincubation of the cells with M4 and then incubated without M4 in the presence of ACh, the M4 inhibition was not completely abolished. On the other hand, its inhibition was maintained even by increasing the external ACh

concentration. These results indicate that the saponins are metabolized to the more active substances in the digestive tract and the metabolites attenuate the secretion of CAs from bovine adrenal chromaffin cells stimulated by ACh due to the noncompetitive blockade of the ACh-induced Na^+ influx into the cells. These findings may further explain the anti-stress action of ginseng.

Introduction

We have investigated the effects of the ginseng components on the secretion of CAs from bovine adrenal chromaffin cells and found that the saponins (ginsenosides) but not other components in ginseng reduce the secretion from the chromaffin cells evoked by ACh[1,2].

The adrenal chromaffin cells are know as a useful model of the sympathetic nerves, because they are embryologically derived from neuronal crest tissue. The chromaffin cells secrete CAs via a stimulation of the nicotinic ACh receptors by ACh, which is released from the terminal of the splanchnic nerve. The process of CA secretion is as follows: 1) ACh binds to the nicotinic receptors; 2) the influx of Na^+ occurs through the receptor-operated cation channels; 3) the cell membrane is depolarized and Ca^{2+} influx causes through the voltage-sensitive Ca^{2+} channels; and 4) the exocytic CA secretion occurs[3].

In the ginsenosides, the protopanaxatriol saponins greatly inhibited the ACh-evoked secretion of CAs, but the protopanaxadiol and the oleanolic acid saponins had slight effect or no effect. The ginsenoside inhibition of the secretion was attributable to the blockade of Na^+ influx through nicotinic ACh receptor operated-cation channels[1].

Recent works have demonstrated that the oligosaccarides connected to the hydroxyl group in the aglycone of ginsenosides are hydrolyzed by digestive tract and adsorbed into circulation when the ginseng is orally administered[4]. Therefore, the ginsenoside metabolites may be genuine substances which reveal pharmacological activities *in vivo*. In this study, we explored the effects of the representative ginsenoside metabolites (M1, M2, M3, M4, M5 and M11) (Fig. 1) on the secretion of CAs from bovine adrenal chromaffin cells.

Materials and Methods

Materials

Ginseng saponins were supplied by Korea Tobacco & Ginseng Corporation and Japan Korea

Red Ginseng Co., Ltd. (Kobe, Japan). Purities of the ginseng saponins were checked by TLC and NMR according to the method of Kawashima and Samukawa[5], and they were more than 98% pure. The metabolites of the ginseng saponins were prepared from the fermentation of the protopanaxadiol- or protopanaxatriol-type saponins by human intestinal bacteria. Oxygenated Krebs-Ringer-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (KRH buffer) (pH 7.4) was used as the incubation medium and was composed of 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, 5.6 mM glucose, and 0.1% bovine serum albumin. In 56 mM KCl-KRH buffer, the amount of NaCl was reduced to maintain the isotonictiy of the medium. All other chemicals were of the highest grade available from commercial sources.

Isolation and primary culture of bovine adrenal chromaffin cells

Bovine adrenal glands were kindly provided by the Center of Iwate Livestock Industry. Adrenal chromaffin cells were prepared by the method of collagenase digestion as previously described[6]. To remove the nonchromaffin cells including fibroblasts or epithelial cells, the differential plating procedure was used[7]. The chromaffin dells were maintained as a monolayer culture in 35-mm diameter dishes at a density of 2×10^6 cells. The cells were cultured at 37°C in a CO_2 incubator (95% air/5% CO_2) for four days. The purity of the cultured cells was confirmed by the Grimelius method[8] and the final cell preparation contained at least 80-90% chromaffin cells.

Measurements of CA secretion

After four days of culturing, the chromaffin cells were washed twice with KRH buffer and then preincubated with or without the ginsenoside metabolites in KRH buffer for 15 min at 37°C. The cells were incubated with or without secretagogues for 7 min in the presence or absence of the above metabolites except as otherwise described below. The reaction was terminated by transferring the incubation medium to tubes in an ice-cold bath. The CAs secreted into the medium were extracted with 0.4 M perchloric acid and adsorbed on aluminum hydroxide. Their amounts were estimated by the ethylenediamine condensation method, using a fluorescence spectrophotometer (650-10S; Hitachi, Tokyo, Japan) at an excitation wavelength of 420 nm and an emission wavelength of 540 nm.

Measurement of intracellular free Na^+ concentration ($[Na^+]_i$)

The isolated cells were incubated with 10 μM sodium-binding benzoforan isophthalate (SBFI) tetraacetoxy methyl ester and 0.02% Pluronic F-127 in KRH buffer for 3 hrs at 37°C and washed

three times with KRH buffer. The cells were preincubated with KRH buffer for 10 min at 37°C in the fluorescence meter, and then the test agents were added. Increases and decreases in the fluorescence induced from the SBFI-Na^+ complex were simultaneously recorded at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 500 nm. The change in $[Na^+]_i$ was expressed as the ratio of the fluorescence at excitation wavelengths of 340 and 380 nm.

Two-electrode voltage clamp with Xenopus oocytes

The oocytes expressing human α3β4 nicotinic ACh receptors were placed in a small recording chamber (120 μl volume), and superfused by gravity feed (3-4 ml/min) with a modified frog Ringer solution, consisting of 120 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ and 5 mM HEPES (pH 7.5) at 20-22°C. Two recording microelectrodes (1-5 MΩ) filled with 3 M KCl were inserted into the animal pole under binocular microscopic control. One electrode was connected to the preamplifier (Dagan 8500 clamp unit) and used for recording the membrane potentials. The other was connected to the output of the same clamp unit to pass the current across the cell membrane. ACh-induced inward current response was recorded under the voltage clamp at the resting membrane potential (-40 mV).

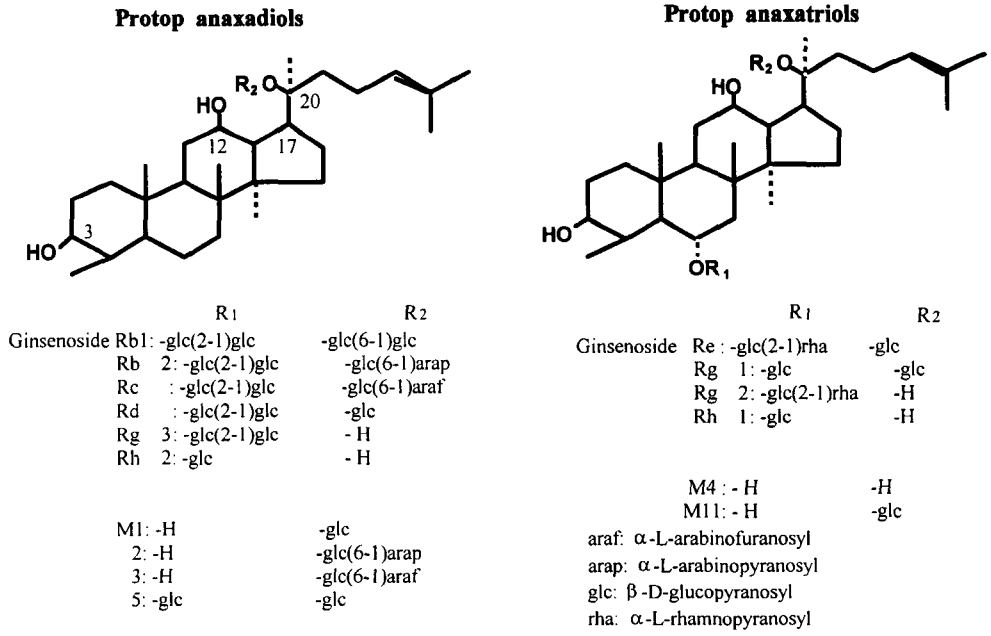

Fig. 1. Structures of ginsenosides and their metabolites.

Table 1. IC_{50} Values of ginsenoside metabolites on ACh-induced CA secretion

Ginsenoside metabolites	IC_{50} (μM)
M1	38
M2	18
M3	19
M4	9
M5	36
M11	22

Results and Discussion

Effects of ginsenoside metabolites on CA secretion

We checked 6 kinds of representative ginsenoside metabolites, M1, M2, M3, M4, M5 and M11 (Fig. 1), on the secretion of CAs from bovine adrenal chromaffin cells stimulated by ACh. All metabolites greatly reduced the ACh-evoked secretion in a concentration-dependent manner. Although M4 was most effective, there were not great differences of the inhibitory potencies among these metabolites. The rank order of IC_{50} values (μM) was as follows; M4 (9) > M2 (18) > M3 (19) > M11 (22) > M5 (36) > M1 (38) (Table 1).

Properties of M4 inhibition of CA secretion

We investigated the inhibitory mechanism of the ginsenoside metabolites using M4. M4 inhibited the ACh-induced Na^+ influx into the cells in a concentration-dependent manner (Fig. 2) similar to that of the inhibition of the ACh-evoked CA secretion. On the other hand, it also reduced the secretion induced by high K^+, which is a stimulator activating voltage-sensitive Ca^{2+} channels (Fig. 3). However, the inhibitory effect of M4 was much less than that evoked by ACh.

When the chromaffin cells were preincubated with 10 μM M4 for 15 min and then incubated with M4 in the presence of ACh, the secretion of CAs was inhibited by about 50%. On the other hand, after preincubation of the cells with M4, when the cells were washed with KRH buffer and then incubated without M4 in the presence of ACh, the ACh-evoked secretion was still considerably inhibited (data not shown). This suggests that the inhibitory effect of M4 is not completely reversible.

The preincubation of the cells with 10 μM M4 for 2 min led to 40% inhibition of the ACh-evoked secretion, while the no-preincubation resulted in 30% inhibition. For 15 min of the pre-

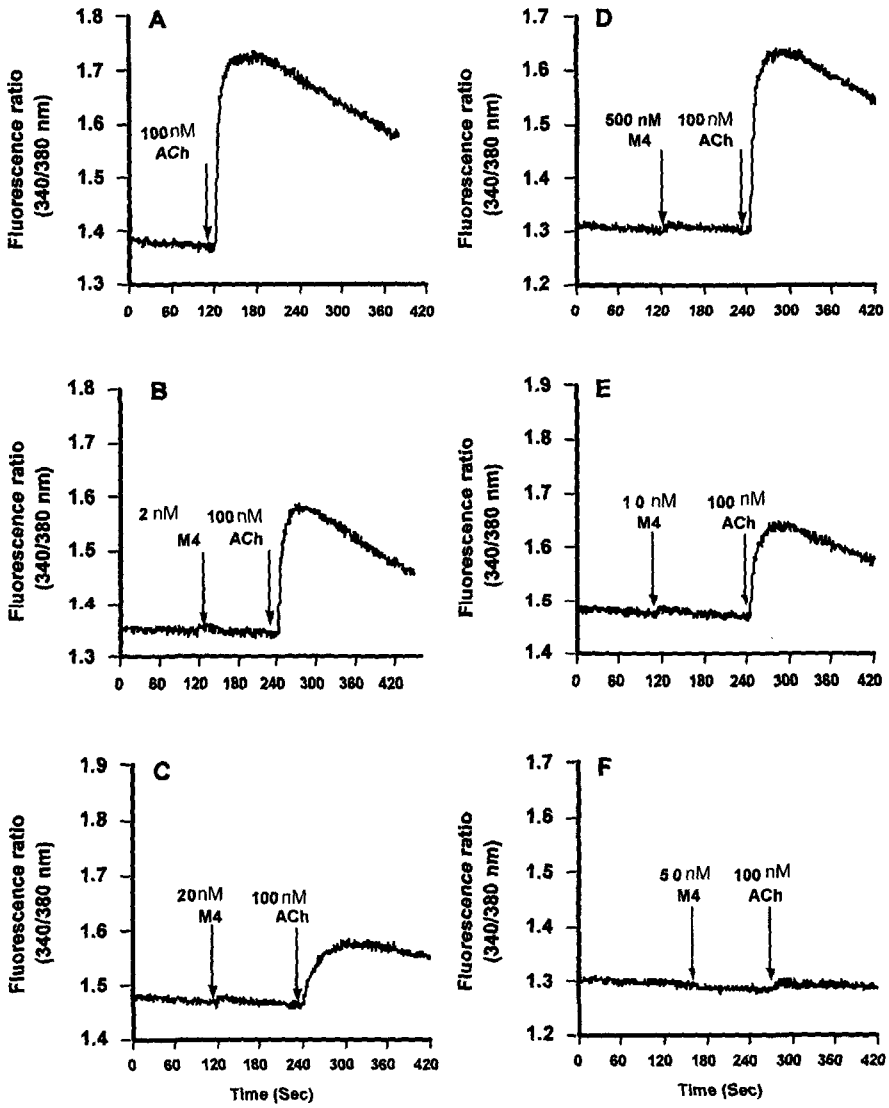

Fig. 2. Effect of M4 on $[Na^+]_i$ in bovine adrenal chromaffin cells.

incubation, the inhibition was almost maximal (50% inhibition) (data not shown). Thus, the M4 inhibition of the CA secretion is dependent on the preincubation time.

M4 inhibition of inward current into oocytes expressing human neuronal nicotinic ACh receptors

The subunit combination of nicotinic ACh receptors associated with the CA secretion from

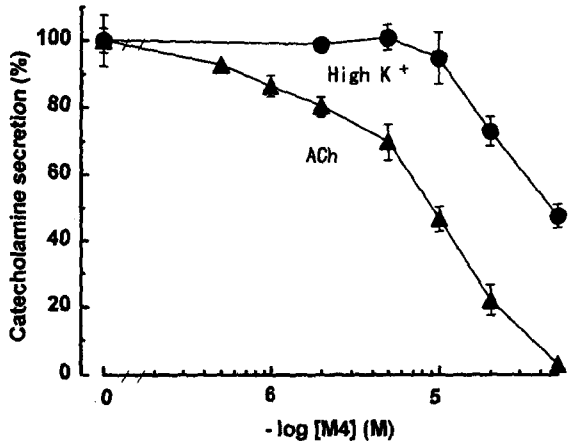

Fig. 3. Effects of M4 on high K^+- and ACh- induced secretion of catecholamines. The high K^+- and the ACh-induced secretions were assigned a value of 100%.

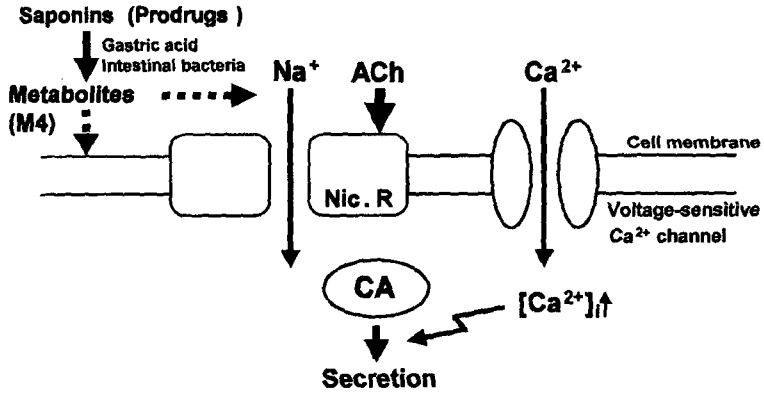

Fig. 4. Mechanism of M4 inhibition of catecholamine secretion.

bovine adrenal chromaffin cells is suggested to be at least $\alpha3\beta4$[9]. Therefore, the effect of M4 on the inward current into oocytes expressing human $\alpha3\beta4$ nicotinic ACh receptors. M4 dose-dependently inhibited the ACh-induced current (data not shown).

All ginsenoside metabolites inhibited the secretion of CAs from bovine adrenal chromaffin cells stimulated by ACh. M1, M2, M3 and M5 are the metabolites derived from the protopanaxadiol saponins, which were weak inhibitors of the CA secretion[1]. Accordingly, on the basis of our results, ginsenosides, especially the protopanaxadiols, are prodrugs and reveal the inhibitory abilities when they are metabolized in digestive tract. On the other hand, the protopanaxatriol saponins, which are strong inhibitors of the secretion[1], maintain their ability even if they are

metabolized.

M4 blocked both the ACh-induced Na^+ influx into the chromaffin cells and inward current into the oocyte expressing human $\alpha 3\beta 4$ nicotinic ACh receptors. On the other hand, the much higher concentrations of M4 were required for the inhibition of the secretion induced by high K^+. These evidences suggest that the ginsenoside metabolites reduce the ACh-evoked secretion of CAs mainly due to the block of Na^+ influx into the cells through nicotinic ACh receptor-operated cation channels. However, the inhibitory effect of M4 was dependent on the preincubation time and was not completely reversible. Therefore, the ginsenoside metabolites may act on not only the nicotinic receptor protein but also on the lipid bilayer of the plasma membrane.

The ginseng and its major component, the saponins, have been reported to improve the symptoms and the lesions evoked by stress[10]. The adrenal medulla and the autonomic nervous systems are extremely important organs that respond to stress. Under stressful circumstance, they secret CAs and cope with stress. However, long-term or excessive stress leads to the over-secretion and consequently causes the exhaustion of various organs. Therefore, it is highly possible that the ginsenosides and their metabolites suppress the secretion of CAs and exert the anti-stress action.

References

1. Tachikawa E, Kudo K, Kashimoto T and Takahashi E. (1995) Ginseng saponins reduce acetylcholine-evoked Na^+ influx and catecholamine secretion in bovine adrenal chromaffin cells. *J. Pharmacol. Exp. Ther.* **273**: 629-636
2. Kudo K, Tachikawa E, Kashimoto T and Takahashi E. (1998) Properties of ginseng saponin inhibition of catecholamine secretion in bovine adrenal chromaffin cells. *Eur. J. Pharmacol* **341**: 139-144
3. Tachikawa E, Takahashi S, Kashimoto T. (1989) *p*-Chloromercuribenzoate causes Ca^{2+}-dependent exocytotic catecholamine secretion from cultured bovine adrenal medullary cells. *J. Neurochem* **53**: 19-26
4. Hasegawa H, Sung J-H, Matsumiya S, Uchiyama M. (1996) Main ginseng saponin metabolites formed by intestinal bacteria. *Planta Med* **62**: 453-457
5. Kawashima Y, and Samukawa K. (1986) Studies of ginseng. *J Med Pharm Soc WAKAN-YAKU* **3**: 236-237

6. Tachikawa E, Takahashi S, Furumachi K, Kashimoto T, Iida A, Nagaoka Y, Fujita T and Takaishi Y. (1991) Trichosporin-B-III, an α-aminoisobutyric acid-containing peptide, causes Ca^{2+}-dependent catecholamine secretion from adrenal medullary cells. *Mol Pharmacol* **40**: 790-797
7. Waymire JC, Bennett WF, Boehme L, Hankins L, Glimer-Waymire K and Haycock JW. (1983) Bovine adrenal chromaffin cells: high yield purification and viability in suspension culture. *J. Neurosci Methods* **7**: 329-351
8. Pearse AG (1985) Histochemistry: Theoretical and Applied. Churchill, New York p966
9. Tachikawa E, Mizuma K, Kudo K, Kashimoto T, Yamato S and Ohta S (2001) Characterization of the functional subunit combination of nicotinic acetylcholine receptors in bovine adrenal chromaffin cells. *Neurosci Lett* **312**: 161-164
10. Attele AS, Wu JA, Yuan C-S (1999) Ginseng pharmacology: multiple constituents and multiple actions. *Biochem Pharmacol* **58**: 1685-1693