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Properties of ginseng saponin inhibition of catecholamine secretion in bovine adrenal chromaffin cells

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Abstract

To investigate the relationship between the inhibitory effects of ginseng saponins (ginsenosides) on acetylcholine-evoked secretion of catecholamines and the structures of ginsenosides, we examined the effects of ginsenoside-Rg_3 and -Rh_2, which are panaxadiol saponins, 20(*R*)- and 20(*S*)-ginsenoside-Rg_2, which are epimers involving the hydroxyl group at C-20 of sapogenin, and other plant saponins on the acetylcholine-evoked secretion of catecholamines from cultured bovine adrenal chromaffin cells. The ginsenoside-Rg_3 (1–100 μM) and -Rh_2 (10–100 μM) greatly reduced the acetylcholine-evoked secretion in a concentration-dependent manner comparable to that of ginsenoside-Rg_2, a panaxatriol saponin, which was the most potent inhibitor in our previous study. 20(*R*)- and 20(*S*)-ginsenoside-Rg_2 (1–100 μM) similarly reduced the acetylcholine-evoked secretion. In contrast, saikosaponin-a, glycyrrhizin and the cardiac glycosides (100 nM–100 μM), digitoxin and digoxin, had no significant inhibitory effect on catecholamine secretion. Saikosaponin-c (10–100 μM), however, had an inhibitory effect, which was less than that of ginsenoside-Rg_2 and -Rg_3. These results strongly suggest that the inhibitory effects of ginsenosides on the acetylcholine-evoked secretion of catecholamines from bovine adrenal chromaffin cells are a unique property of ginseng. Further, the relationship between the inhibitory effects and the structures of ginsenosides is discussed. © 1998 Elsevier Science B.V.

Keywords: Ginsenoside; Ginseng saponin; Catecholamine; Chromaffin cell; Acetylcholine

1. Introduction

The root of *Panax ginseng* C.A. Meyer has been highly valued as a medicine from ancient times and has been widely used as an important component of Chinese prescriptions called 'Kan-pou medicine'. The oldest Chinese medical book, *Sheng-nong Ben-cao Jing*, stated that the ginseng root has various effects (e.g., replenishment of vital energy, tranquilization, elevation of mood and prevention of aging). These pharmacological effects led us to consider that the ginseng root may affect nervous systems. In fact, there are several reports showing that the ginseng saponins, which are isolated from the ginseng root, have effects on the nervous system (Saito et al., 1977; Yoshimura et al., 1988). However, the mechanisms underlying the pharmacological effects are not well understood.

The adrenal medulla can secret catecholamines via stimulation of nicotinic acetylcholine receptors by a physiological secretagogue, acetylcholine, which is released from the terminal of the splanchnic nerve. Binding of acetylcholine to nicotinic receptors leads to depolarization of the cell membrane by an influx of Na^+ through receptor-operated cation channels, causes an influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels and results in catecholamine secretion by exocytosis (Douglas and Poisner, 1961; Wilson and Kirshner, 1977; Holz et al., 1982). Therefore, adrenal chromaffin cells are widely used in studies of catecholamine secretion as a useful model of sympathetic neurons.

To investigate the effect of the root of the *Panax ginseng* on the nervous system, we examined the effects of two major parts, nonsaponin and crude saponin fractions extracted from the root, on the secretion of catecholamines

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from cultured bovine adrenal chromaffin cells stimulated by acetylcholine (Kudo et al., 1992). The result showed that the crude saponin, but not the nonsaponin, reduced the acetylcholine-evoked secretion. The ginseng saponins, which are called ginsenosides, are classified into three major groups, the panaxadiol, the panaxatriol and the oleananesaponin groups, on the basis of the chemical structures of their sapogenins (aglycones). We have demonstrated that all ginseng saponins (1–100 μM) (panaxadiol saponins: ginsenoside-Rb_1, -Rb_2, -Rb_3, -Rc, -Rd, -Rs_1; panaxatriol saponins: ginsenoside-Re, -Rf, -Rg_1, -Rg_2, -Rh_1), except for ginsenoside-Ro (oleananesaponin), have a tendency to reduce the acetylcholine-evoked catecholamine secretion from chromaffin cells. The inhibitory effects of the panaxatriol saponins were much greater than those of the panaxadiols. We investigated the mechanism of the ginsenoside-induced inhibition of catecholamine secretion using ginsenoside-Rg_2, which caused the greatest inhibition of the ginsenosides tested. It has been suggested that ginsenoside-Rg_2 acts on nicotinic acetylcholine receptor-operated cation channels, inhibiting Na^+ influx through the channels and consequently reducing both Ca^{2+} influx and catecholamine secretion in chromaffin cells (Tachikawa et al., 1995).

In the present study, we further examined the effects of ginsenoside-Rg_3 and -Rh_2, which are panaxadiol saponins, 20(*R*)- and 20(*S*)-ginsenoside-Rg_2, which are epimers of the ginsenoside, and five kinds of other saponins, which are saikosaponins, glycyrrhizin and cardiac glycosides, on the secretion of catecholamines from cultured bovine adrenal chromaffin cells stimulated by acetylcholine. The goal of our present study is to examine the relationship between the inhibitory effects and the structures of ginsenosides.

2. Materials and methods

2.1. Materials

Ginsenosides were kindly supplied by Korea Tobacco and Ginseng Corporation and Japan Korea Red Ginseng Co. (Kobe). The purity of the ginsenosides used in this study was checked by thin-layer chromatography and nuclear magnetic resonance according to the method of Kawashima and Samukawa (1986); they were found to be more than 98% pure. The ginsenosides were dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the incubation medium was 1%, which had no effect on the secretion of catecholamines from bovine adrenal chromaffin cells under the conditions used in this study. Oxygenated Krebs-Ringer-HEPES buffer (KRH buffer) (pH 7.4) was used as an incubation medium and contained (mM) 125 NaCl, 4.8 KCl, 2.6 $CaCl_2$, 1.2 $MgSO_4$, 25 HEPES, 5.6 glucose, and 0.5% bovine serum albumin. Tissue culture equipment was obtained from Falcon Plastics Co. (Cockeysville, MD). Eagle's minimum essential medium was obtained from Nissui Seiyaku (Tokyo). Calf serum, acetylcholine, digitoxin, digoxin and saikosaponins (saikosaponin-a and -c) were obtained from Nacarai Tesque (Kyoto). Glycyrrhizin was obtained from Wako Pure Chemical Industries (Osaka). All other chemicals were of the highest grade available from commercial sources.

2.2. 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 elsewhere (Tachikawa et al., 1989). The isolated cells were suspended in Eagle's minimum essential medium containing 10% calf serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.3 μg/ml amphotericin B) and were plated on 35 mm dishes at a density of 2×10^6 cells. The cells were cultured at 37°C in a CO_2 incubator (95% air and 5% CO_2) for 4 days. A total of 2×10^6 cells contained 35.4 ± 2.9 μg of catecholamines as epinephrine and norepinephrine.

2.3. Measurements of catecholamine secretion

After 4 days in culture, the chromaffin cells were washed twice with KRH buffer and then preincubated with or without ginsenosides or other saponins in KRH buffer for 10 min at 37°C. The cells were incubated with or without acetylcholine (50 μM) for 7 min in the presence or absence of ginsenosides or other saponins. The reaction was terminated by transferring the incubation medium to tubes in an ice-cold bath. The catecholamines secreted into the medium were extracted with 0.4 M perchloric acid and absorbed onto aluminum hydroxide (Tachikawa et al., 1995). Their amounts were estimated by the ethylenediamine condensation method (Weil-Malherbe and Bone, 1952), using a fluorescence spectrophotometer (650-10S; Hitachi, Tokyo). At the emission wavelengths used, epinephrine and norepinephrine had the same fluorescence intensity.

3. Results

3.1. Effects of ginsenoside-Rg_3 and -Rh_2 on catecholamine secretion from bovine adrenal chromaffin cells

Fig. 1 shows the structures of two panaxadiol saponins, ginsenoside-Rg_3 and -Rh_2, in addition to those of six kinds of panaxadiol saponins (ginsenoside-Rb_1, -Rb_2, -Rb_3, -Rc, -Rd, and -Rs_1) and five kinds of panaxatriol saponins (ginsenoside-Re, -Rf, -Rg_1, -Rg_2 and -Rh_1), which were previously used in our study (Tachikawa et al., 1995). We

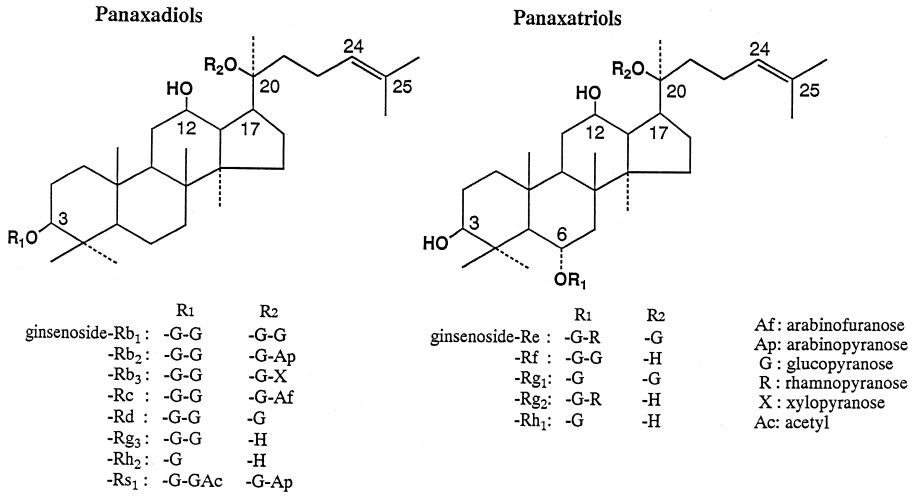

Fig. 1. Structures of ginseng saponins (ginsenosides).

examined the effects of ginsenoside-Rg_3 and -Rh_2 (100 nM–100 μM) on the acetylcholine-evoked secretion of catecholamines from bovine adrenal chromaffin cells (Fig. 2). The effect of ginsenoside-Rb_1, a representative panaxadiol saponin, on catecholamine secretion is also shown in Fig. 2. Both ginsenoside-Rg_3 and -Rh_2 reduced the acetylcholine-evoked secretion in a concentration-dependent manner. Ginsenoside-Rh_2 at 1 μM did not affect catecholamine secretion but at 10 μM, it inhibited the secretion by 16%. At 100 μM, the ginsenoside-Rh_2-induced inhibition was 55%. Ginsenoside-Rg_3 at 1 μM inhibited catecholamine secretion by 11%, and at 10 and 100 μM, the ginsenoside-Rg_3-induced inhibition was 50 and 95%, respectively. Neither ginsenoside-Rg_3 nor -Rh_2 had an effect on spontaneous (basal) catecholamine secretion from nonstimulated cells.

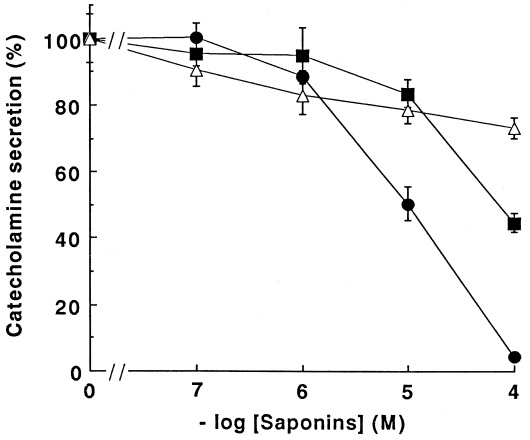

Fig. 2. Effects of ginsenosides on acetylcholine-evoked catecholamine secretion from bovine adrenal chromaffin cells. The cultured chromaffin cells were washed twice with KRH buffer and preincubated with KRH buffer in the presence or absence of different concentrations of ginsenoside-Rb_1 (△), ginsenoside-Rg_3 (●) or ginsenoside-Rh_2 (■) for 10 min at 37°C. The cells were then incubated for 7 min with or without acetylcholine (50 μM) in the presence or absence of different concentrations of the ginsenosides described above. Catecholamines secreted from the cells were determined as described in Section 2. The values of basal secretion were subtracted, and acetylcholine-evoked responses were assigned values of 100%. The acetylcholine-evoked secretion was 24.5 ± 1.3 μg catecholamines/2×10^6 cells, and the basal secretion was 0.4 ± 0.1 μg catecholamines/2×10^6 cells. Each value expresses the mean ± S.D. from four determinations.

The 50% inhibitory concentration (IC_{50}) values of the various ginsenosides on catecholamine secretion are given in Table 1. The order of IC_{50} values of the ginsenosides was as follows (μM): Rg_2 (4) > Rf (10), Rg_3 (10) > Re (16) > Rh_1 (30) > Rg_1 (40) > Rh_2 (80) > Rb_2 (> 100), Rb_1 (> 100), Rd (> 100), Rc (> 100), Rb_3 (> 100), Rs_1 (> 100).

Table 1
IC_{50} values of various ginsenosides on acetylcholine-evoked catecholamine secretion from bovine adrenal chromaffin cells

Ginsenoside	Sapogenin[a]	Sugar moiety[b] at			IC_{50} (μM)
		C-3	C-6	C-20	
Rg_2	triol		–G–R	—	4
Rf	triol		–G–G	—	10
Rg_3	diol	–G–G		—	10
Re	triol		–G–R	–G	16
Rh_1	triol		–G	—	30
Rg_1	triol		–G	–G	40
Rh_2	diol	–G		—	80
Rb_2	diol	–G–G		–G–Ap	> 100
Rb_1	diol	–G–G		–G–G	> 100
Rd	diol	–G–G		–G	> 100
Rc	diol	–G–G		–G–Af	> 100
Rb_3	diol	–G–G		–G–X	> 100
Rs_1	diol	–G–GAc		–G–Ap	> 100

The IC_{50} values were evaluated from the log dose-response curve of various ginsenosides on the acetylcholine-evoked secretion of catecholamines.

[a] triol: panaxatriol; diol: panaxadiol.

[b] Af: arabinofuranose; Ap: arabinopyranose; G: glucopyranose; R: rhamnopyranose; X: xylopyranose; Ac: acetyl.

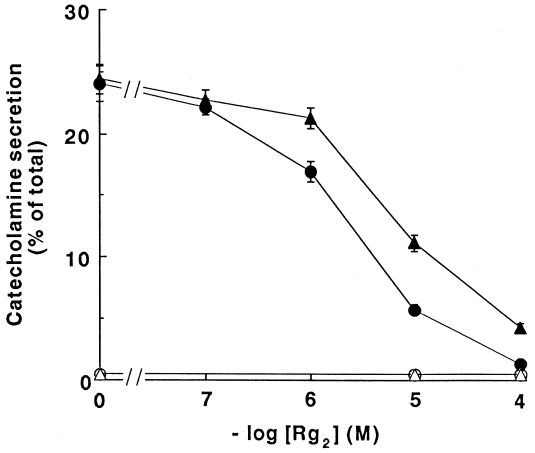

Fig. 3. Effects of 20(*R*)-ginsenoside-Rg_2 and 20(*S*)-ginsenoside-Rg_2 on catecholamine secretion from bovine adrenal chromaffin cells. The cultured chromaffin cells were washed twice with KRH buffer and preincubated with KRH buffer in the presence or absence of different concentrations of 20(*R*)-ginsenoside-Rg_2 (△, ▲) or 20(*S*)-ginsenoside-Rg_2 (○, ●) for 10 min at 37°C. The cells were then incubated for 7 min with (▲, ●) or without acetylcholine (50 μM) (△, ○) in the presence or absence of different concentrations of the ginsenosides described above. Catecholamines secreted from the cells were determined as described in Section 2. The amount of catecholamines was expressed as a percentage of the total cell content of catecholamines. Each value expresses the mean ± S.D. from four determinations.

3.2. Effects of 20(R)- and 20(S)-ginsenoside-Rg_2 on catecholamine secretion

Ginsenoside-Rg_2, the most potent inhibitor of catecholamine secretion, has epimers involving the hydroxyl group at the C-20 position. Therefore, we examined the effects of the epimers, 20(*R*)- and 20(*S*)-ginsenoside-Rg_2, on the acetylcholine-evoked catecholamine secretion from the cells. These saponins reduced catecholamine secretion from the cells in a concentration-dependent manner (1–100 μM) (Fig. 3). At 100 nM, 20(*R*)- and 20(*S*)-ginsenoside-Rg_2 had a slight effect on catecholamine secretion but at 1 μM they inhibited the secretion by 13 and 32%, respectively. The inhibition induced by 20(*R*)- and 20(*S*)-ginsenoside-Rg_2 at 10 μM was 54 and 76%, and at 100 μM, was 82 and 95%, respectively. IC_{50} values for 20(*R*)- and 20(*S*)-ginsenoside-Rg_2 were 8 and 3 μM, respectively. Thus, the inhibitory effects of 20(*S*)-ginsenoside-Rg_2 on catecholamine secretion were slightly greater than those of 20(*R*)-ginsenoside-Rg_2.

3.3. Effects of saikosaponins, glycyrrhizin and cardiac glycosides on catecholamine secretion

To examine whether saponins isolated from other plants can inhibit the acetylcholine-evoked secretion of catecholamines from chromaffin cells, we tested the effects of saikosaponin-a and -c, which are isolated from *Bupleurum falcatum* L., glycyrrhizin, which is from *Glycyrrhiza glabra* L., and cardiac glycosides, digoxin and digitoxin, which are from *Digitalis purpurea* L., on catecholamine secretion (Fig. 4A and B). Fig. 5 shows the structures of saikosaponins (saikosaponin-a and -c), glycyrrhizin and cardiac glycosides (digoxin and digitoxin).

Both saikosaponin-a and -c at 1 μM had no effect on the acetylcholine-evoked secretion (Fig. 4A). Saikosaponin-a at 10 μM inhibited catecholamine secretion by 47%, whereas at 100 μM, it greatly enhanced catecholamine secretion. Saikosaponin-a also increased the basal secretion at 100 μM but not at 1–10 μM. Saikosaponin-c at 10 and 100 μM inhibited the acetylcholine-evoked secretion of catecholamines by 19 and 66%, respectively, without affecting the basal secretion. The IC_{50} value was 55 μM. To assess plasma membrane integrity,

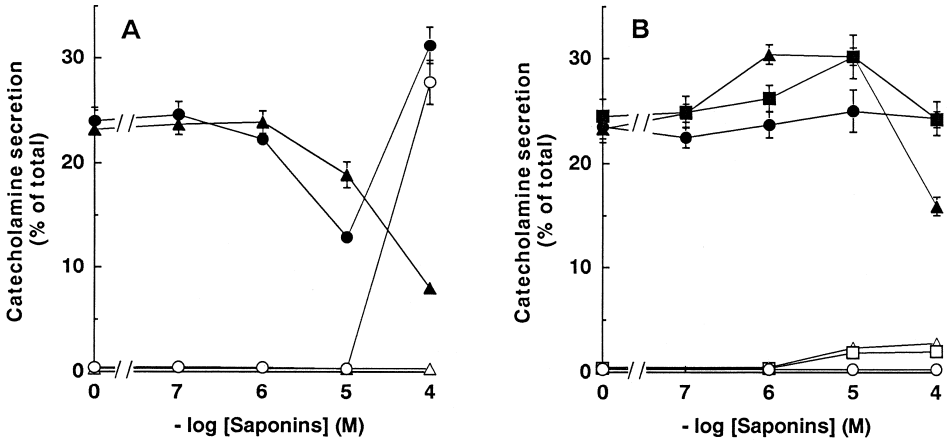

Fig. 4. Effects of other saponins on catecholamine secretion from bovine adrenal chromaffin cells. The cultured chromaffin cells were washed twice with KRH buffer and preincubated with KRH buffer in the presence or absence of different concentrations of (A) saikosaponin-a (○, ●) and saikosaponin-c (△, ▲), and (B) glycyrrhizin (○, ●), digoxin (□, ■) and digitoxin (△, ▲), for 10 min at 37°C. The cells were then incubated for 7 min with (●, ■, ▲) or without acetylcholine (50 μM) (○, □, △) in the presence or absence of different concentrations of the saponins described above. Catecholamines secreted from the cells were determined as described in Section 2. The amount of catecholamines was expressed as a percentage of the total cell content of catecholamines. Each value expresses the mean ± S.D. from four determinations.

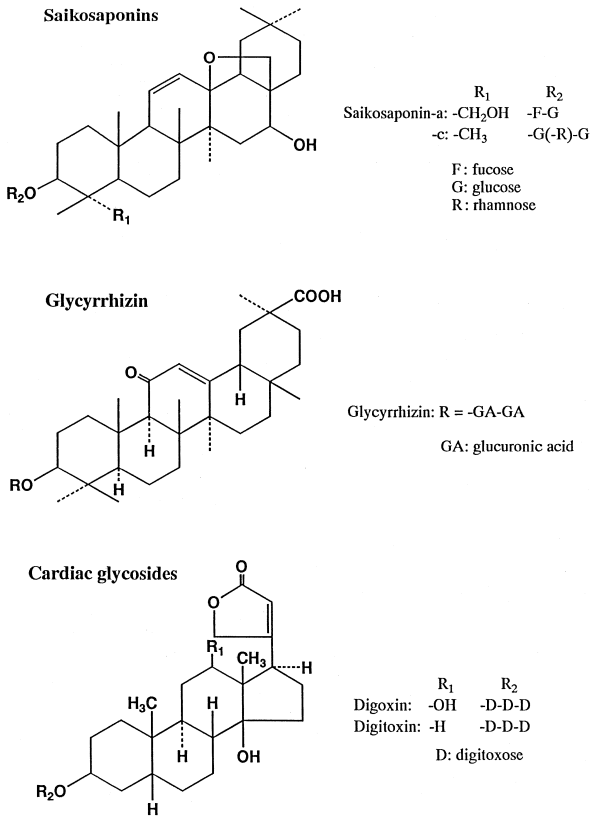

Fig. 5. Structures of saikosaponins (saikosaponin-a and -c), glycyrrhizin and cardiac glycosides (digoxin and digitoxin).

the ability of chromaffin cells to exclude trypan blue was examined. Chromaffin cells which were treated with saikosaponin-a or -c at 100 μM for 10 min were incubated with 2 mg/ml trypan blue in KRH buffer. More than 90% of the cells treated with saikosaponin-a were stained with trypan blue, while most cells treated with saikosaponin-c were able to exclude trypan blue (data not shown).

Glycyrrhizin (100 nM–100 μM) altered neither the acetylcholine-evoked nor the basal secretion of catecholamines from the cells (Fig. 4B).

Digoxin slightly enhanced the acetylcholine-evoked secretion only at 10 μM and at 1 and 100 μM it did not affect the secretion. Digitoxin at 1 and 10 μM enhanced catecholamine secretion but at 100 μM it inhibited the secretion by 43%. Digoxin and digitoxin at 10–100 μM slightly increased the basal secretion (Fig. 4B).

4. Discussion

We have already reported the effects of 12 kinds of ginsenosides (oleananesaponin: ginsenoside-Ro; panaxadiols: ginsenoside-Rb_1, -Rb_2, -Rb_3, -Rc, -Rd, -Rs_1; panaxatriols: ginsenoside-Re, -Rf, -Rg_1, -Rg_2, -Rh_1) on the acetylcholine-evoked catecholamine secretion from bovine adrenal chromaffin cells (Tachikawa et al., 1995). The results showed that, except for the oleananesaponin, the ginsenosides generally inhibited catecholamine secretion from chromaffin cells. The panaxatriol saponins produced the greatest inhibition of secretion, whereas the panaxadiol saponins were much weaker. Further, the results suggested that the inhibitory potencies of the ginsenosides might be related to the chemical structures of sapogenins (aglycones) and to the number or the type of sugar moieties (Tachikawa et al., 1995).

In this study, to clarify the relationship between the inhibitory effects and the structures of ginsenosides in detail, we further examined the effects of the two panaxadiol saponins, ginsenoside-Rg_3 and -Rh_2, on the acetylcholine-evoked catecholamine secretion from chromaffin cells. Ginsenoside-Rg_3 and -Rh_2 greatly reduced the secretion in a concentration-dependent manner (Fig. 2). The order of IC_{50} values of the two ginsenosides and the saponins previously used (Tachikawa et al., 1995) was as follows (μM): Rg_2 (4) > Rf (10), Rg_3 (10) > Re (16) > Rh_1 (30) > Rg_1 (40) > Rh_2 (80) > Rb_2 (> 100), Rb_1 (> 100), Rd (> 100), Rc (> 100), Rb_3 (> 100), Rs_1 (> 100) (Table 1). Thus, ginsenoside-Rg_3 was a potent inhibitor, comparable to ginsenoside-Rg_2, which was the most potent inhibitor in the previous study (Tachikawa et al., 1995), although ginsenoside-Rg_3 belongs to the panaxadiol saponins. All the panaxadiols, tested in the previous study, have sugar moieties at the C-20 position in the sapogenin, whereas ginsenoside-Rg_3 and -Rh_2 have no sugar at this position (Fig. 1). Ginsenoside-Rg_2 and -Rf, which were the most potent inhibitors of the panaxatriols, do not have the C-20 sugar. Furthermore, the inhibitory effects of ginsenoside-Rg_2 and -Rh_1, which are saponins without a glucose at the C-20 position in ginsenoside-Re and -Rg_1, respectively, were greater than those of ginsenoside-Re and -Rg_1 (Fig. 1 and Table 1). These results indicate that the inhibitory effects of ginsenosides are at least, in part, related to the C-20 sugar moieties. Of the panaxatriols, the inhibitory effect of ginsenoside-Rh_1 and -Rg_1, which have a monosugar at C-6, was weaker than that of ginsenoside-Rf and -Re, which have a disugar at C-6, respectively. This suggests that the inhibitory effects of ginsenosides are also affected by the number of sugars at the C-6 position.

20(R)- and 20(S)-ginsenoside-Rg_2 are epimers of ginsenoside-Rg_2 with regard to the hydroxyl group at the C-20 position. The inhibitory effects of 20(S)-ginsenoside-Rg_2 were slightly greater than those of 20(R)-ginsenoside-Rg_2 (Fig. 3). Therefore, the stereospecificity of the hydroxyl group at the C-20 in the ginsenosides seems to contribute to the inhibitory effects.

Thus, the number and the position of sugar moieties and their stereospecificity are associated with the inhibitory potency of the ginsenosides. The polarity of the ginsenosides is also related to their inhibitory effects on catecholamine secretion. The ginseng saponins have been isolated and purified according to differences in polarity by using chromatography (Kitagawa et al., 1983; Samukawa

et al., 1995). The order of polarity of the ginsenosides is as follows: $Rh_2 < Rh_1 < Rg_3 < Rg_2 < Rg_1 < Rf < Re < Rd < Rc < Rb_3 < Rb_2 < Rb_1 < Ro$. Thus, there is roughly a reverse relationship between the inhibitory potency and the polarity of the ginsenosides. The polarity and structure of the ginsenosides, therefore, may affect their affinity for nicotinic acetylcholine receptor cation channels.

Other saponins derived from three kinds of plants showed a variety of effects on the secretion of catecholamines from chromaffin cells evoked by acetylcholine (Fig. 4). Glycyrrhizin (100 nM–100 μM), cardiac glycosides, digoxin (100 nM–100 μM) and digitoxin (100 nM–10 μM) had no inhibitory effect on the acetylcholine-evoked secretion, although digitoxin at 100 μM, the highest concentration tested in the study, inhibited catecholamine secretion. Both digoxin (10 μM) and digitoxin (1–10 μM) slightly potentiated the acetylcholine-evoked secretion (Fig. 4B). This is probably due to the inhibition of Na^+–K^+ ATPase by the cardiac glycosides, because many data document that cardiac glycosides inhibit Na^+–K^+ ATPase and increase the spontaneous or secretagogue-induced secretion of catecholamines from bovine adrenal chromaffin cells (Sorimachi et al., 1981; Pocock, 1983a,b). Saikosaponin-a at 10 μM inhibited the acetylcholine-evoked secretion, but at 100 μM it increased not only the acetylcholine-evoked secretion but also spontaneous secretion. Abe et al. (1978) have reported that saikosaponin-a (13–39 μM) has strong hemolytic activity. Furthermore, in this study, more than 90% of the chromaffin cells which were treated with saikosaponin-a at 100 μM for 10 min were stained with trypan blue. Therefore, the stimulatory effects of saikosaponin-a at 100 μM on catecholamine secretion may be attributed to the passive diffusion of catecholamines through the cells damaged by the saponin. Saikosaponin-c (10–100 μM) reduced the acetylcholine-evoked secretion, but the inhibitory potency (IC_{50}: 55 μM) was not greater than that of the potent inhibitors among the ginseng saponins, ginsenoside-Rg_2 and -Rf (IC_{50}: 4 and 10 μM) (Tachikawa et al., 1995). Hence, the inhibitory effect on the acetylcholine-evoked secretion of catecholamines seems to be relatively specific for the ginseng saponins (ginseng sapogenins) and not for saponins from other plants.

It is known that the sugar moieties attached to C-20 in the sapogenins of ginsenoside-Rb_1, -Rb_2 and -Rg_1 are easily hydrolyzed by rat gastric juice, enteric enzymes (Karikura et al., 1991), or mild acidic conditions (Han et al., 1982). Accordingly, the panaxadiol saponins, ginsenoside-Rb_1, -Rb_2, -Rb_3, -Rc and -Rd, which were less potent inhibitors of catecholamine secretion, are decomposed and converted to ginsenoside-Rg_3, a potent inhibitor of catecholamine secretion. This indicates the possibility that the panaxadiols may be changed in the body to ginsenoside-Rg_3, which, together with the panaxatriols, may depress the activity of the sympathetic nervous system.

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