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In vitro inhibition of adrenal catecholamine secretion by steroidal metabolites of ginseng saponins

Eiichi Tachikawa[a,*], Kenzo Kudo[a], Hideo Hasegawa[b], Takeshi Kashimoto[a], Kazuhiko Sasaki[c], Masao Miyazaki[d], Hideharu Taira[d], Jon M. Lindstrom[e]

[a]*Department of Pharmacology, School of Medicine, Iwate Medical University, Uchimaru 19-1, Morioka 020-8505, Japan*
[b]*Fermenta Herb Institute Inc.,1-30-15 Motohongo, Hachioji, Tokyo 192-0051, Japan*
[c]*Department of Physiology I, School of Medicine, Iwate Medical University, Uchimaru 19-1, Morioka 020-8505, Japan*
[d]*Faculty of Agriculture, Iwate University, 3-18-3 Ueda, Morioka 020-8550, Japan*
[e]*Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, PA 19104-6074, USA*

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Abstract

We reported previously that the protopanaxatriol saponins in *Panax ginseng* greatly reduce the secretion of catecholamines from bovine adrenal chromaffin cells stimulated by acetylcholine (ACh). However, protopanaxadiol saponins showed only slight inhibitory effects. Recent studies have demonstrated that oligosaccharides connected to the hydroxyl groups of the aglycone in ginseng saponins (ginsenosides) are in turn hydrolyzed in the digestive tract and absorbed into the circulation following oral administration of ginseng. Therefore, the present study was performed to investigate the effects of the major ginsenoside metabolites (M1, M2, M3, M4, M5, M11, and M12) on catecholamine secretion. All of these metabolites were shown to be potent inhibitors of ACh-evoked secretion, and M4 was the most effective. M4 blocked not only the ACh-induced Na^+ influx into the chromaffin cells but also the ACh-induced inward current into *Xenopus* oocytes expressing human α3β4 neuronal nicotinic ACh receptors. M4 reduced the secretion induced by high K^+, an activator of voltage-sensitive Ca^{2+} channels, to a much lesser extent than that evoked by ACh. M1, M2, M3, M5, and M12 are protopanaxadiol saponin-derived metabolites. Therefore, these results imply that the protopanaxadiol saponins are prodrugs, and they show more potent inhibitory activity following metabolism in the digestive tract. The results further suggest that the metabolites act on nicotinic ACh receptors, blocking Na^+ influx through the receptors, and consequently reduce the catecholamine secretion from bovine adrenal chromaffin cells. The inhibitory effect of ginsenoside metabolites is probably one of the mechanisms of action responsible for the pharmacological effects of ginseng.

Keywords: *Panax ginseng*; Saponin; Ginsenoside; Catecholamine secretion; Adrenal chromaffin cell; Nicotinic acetylcholine receptor

1. Introduction

The root of *Panax ginseng* C.A. Meyer has been widely used in Eastern Asia as a drug for nourishment, as a prophylactic tonic, and as a medicine to obtain perennial youth. Now, its utilization as a natural medicine has become widespread throughout the world. However, there is little pharmacological evidence for its efficacy. Ginseng is also one of the most important components in many traditional Chinese prescriptions called Kampo medicine in Japan. Therefore, it is speculated that ginseng influences the functions of various physiological systems and maintains homeostasis against disease. We have focused on the pharmacological effects of ginseng and its components on the nervous system, and have investigated the influence of ginseng on the function of adrenal chromaffin cells.

Because the adrenal medulla is derived from neural crest tissue, it is widely used as a nervous system model, in particular as a model of the sympathetic nervous system. The adrenal medulla secretes catecholamines in response to stimulation of the nicotinic ACh receptors, possibly composed of α3β4 subunits [1,2], by the neurotransmitter

* Corresponding author. Tel.: +81-19-651-5111; fax: +81-19-651-1677.
E-mail address: etachika@iwate-med.ac.jp (E. Tachikawa).
Abbreviations: ACh, acetylcholine; KRH, Krebs–Ringer–HEPES; $[Na^+]_i$, intracellular free sodium concentration; SBFI, sodium-binding benzofuran isophthalate.

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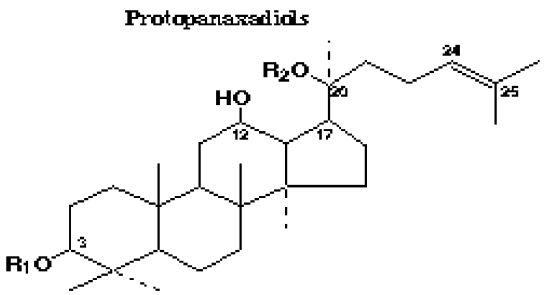
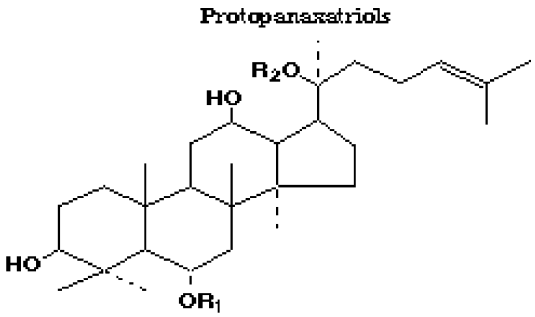
ACh, which is released from the splanchnic nerve terminals. Binding of ACh to the nicotinic receptors leads to depolarization of the cell membrane by an influx of Na^+ through the receptors, causing an influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels, and results in exocytotic catecholamine secretion [3–5].

We have reported previously that the crude saponin fraction extracted from the root of *P. ginseng*, but not the non-saponin fraction, reduces the ACh-evoked secretion of catecholamines from bovine adrenal chromaffin cells [6]. To date, over 30 kinds of ginseng saponins, known as "ginsenosides," have been identified. The ginsenosides are divided into two major groups, i.e. the oleanolic acid-type and dammarane-type, on the basis of the chemical structures of their aglycones (Fig. 1). The dammaranes have steroidal skeletons and are further classified into two groups, the protopanaxadiol and protopanaxatriol groups, according to differences in the number of alcohol hydroxyl groups attached to the aglycones. All of the protopanaxatriols tested in the study, ginsenoside-Rg_2, -Rf, -Re, etc., have been demonstrated to markedly inhibit ACh-evoked secretion from chromaffin cells due to the blockade of Na^+ influx into the cells through nicotinic ACh receptors [7]. However, almost all of the protopanaxadiols, ginsenoside-Rb_1, Rc, Rd, etc., have been shown to have only weak inhibitory effects on secretion, while the oleanolic acid saponin, ginsenoside-Ro, showed no such effect. The structure–inhibitory activity relationships have also been reported [8]. We concluded that this effect may be associated with some pharmacological effects of *P. ginseng* root.

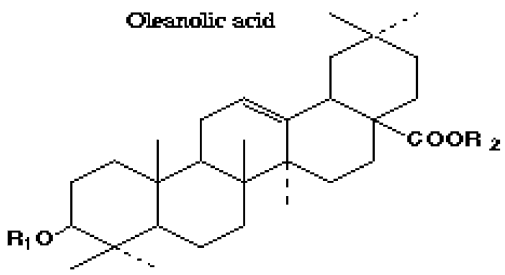
Recent studies have indicated that the sugar moieties of the ginsenosides are in turn hydrolyzed by gastric acid in the stomach and by the enzymes of bacteria in the large intestine, and absorbed into the circulation following oral administration of ginseng [9,10]. Moreover, incubation of ginsenosides with intestinal bacteria has been shown to result in the formation of several hydrolyzed ginsenosides



	R_1	R_2
ginsenoside-Rb_1:	-glc(2-1)glc	-glc(6-1)glc
-Rb_2:	-glc(2-1)glc	-glc(6-1)arap
-Rb_3:	-glc(2-1)glc	-glc(6-1)xyl
-Rc :	-glc(2-1)glc	-glc(6-1)araf
-Rd :	-glc(2-1)glc	-glc
-Rg_3:	-glc(2-1)glc	-H
-Rh_2:	-glc	-H
-Rs_1:	-glc(2-1)glc(6)Ac	-glc(6-1)arap
compound-M (M1):	-H	-glc
compound-Y (M2):	-H	-glc (6-1) arap
ginsenoside-Mc (M3):	-H	-glc (6-1) araf
ginsenoside-F_2 (M5):	-glc	-glc
20(s)-protopaxadiol (M12):	-H	-OH



	R_1	R_2
ginsenoside-Re :	-glc(2-1)rha	-glc
-Rf :	-glc(2-1)glc	-H
-Rg_1:	-glc	-glc
-Rg_2:	-glc(2-1)rha	-H
-Rh_1:	-glc	-H
20(s)-protopaxatriol (M4):	-H	-H
ginsenoside-F_1 (M11):	-H	-glc



	R_1	R_2
ginsenoside Ro:	-glcUA(2-1)	-glc

araf: α-L-arabinofuranosyl
arap: α-L-arabinopyranosyl
glc: β-D-glucopyranosyl
glcUA: β-D-glucuronic acid
rha: α-L-rhamnopyranosyl
xyl: β-D-xylopyranosyl
Ac: acetyl

Fig. 1. Structures of ginsenosides and their metabolites.

in vitro. These observations suggest that the metabolites of ginsenosides are the major active components of ginseng. The main hydrolytic metabolites of the protopanaxadiols are compound-M (M1), compound-Y (M2), ginsenoside-Mc (M3), ginsenoside-F_2 (M5), and 20(*S*)-protopanaxadiol (M12), and those of the protopanaxatriols are 20(*S*)-protopanaxatriol (M4) and ginsenoside-F_1 (M11) (Fig. 1). We investigated the effects of these metabolites on the secretion of catecholamines from bovine adrenal chromaffin cells. Our results indicated that all of these metabolites reduce the secretion evoked by ACh. Furthermore, we explored their mechanisms of action, and discussed their importance in the beneficial health effects of ginseng.

2. Materials and methods

2.1. Isolation and primary culture of bovine adrenal chromaffin cells

Bovine adrenal glands were kindly provided by the Iwate Chikusan Center. Adrenal chromaffin cells were prepared by collagenase digestion as described previously [11]. The isolated cells were suspended in Eagle's minimum essential medium containing 10% calf serum, 3 μM cytosine arabinoside, and antibiotics (100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.3 μg/mL of amphotericin B), and were maintained in monolayer culture in dishes 35 mm in diameter at a density of 2×10^6 cells or in 24-well plates at a density of 5×10^5 cells. The cells were cultured at 37° in a CO_2 incubator (95% air/5% CO_2). A total population of 2×10^6 cells contained 37.9 ± 1.8 μg catecholamines as epinephrine and norepinephrine.

2.2. Measurement of catecholamine secretion from chromaffin cells

After 4 days in culture, the cells were washed with prewarmed Krebs–Ringer–HEPES (KRH) buffer and then incubated with or without each ginsenoside metabolite in the same buffer for 15 min at 37°. They were then incubated with or without the metabolite used in the preincubation step in the presence or absence of ACh or high K^+ (56 mM K^+) for 7 min. The reaction was terminated by transferring the incubation medium to tubes in an ice-water bath. The catecholamines secreted into the medium were extracted with 0.4 M perchloric acid and adsorbed on aluminum hydroxide. Their levels were estimated by the ethylenediamine condensation method [12] using a fluorescence spectrophotometer (650-10s; Hitachi) at an excitation wavelength of 420 nm and an emission wavelength of 540 nm. At these wavelengths, epinephrine and norepinephrine show the same fluorescence intensity. Basal values (secretions) were subtracted from the data, and the stimulus-induced response was assigned a value of 100%.

2.3. Measurement of $[Na^+]_i$

Loading of the chromaffin cells with sodium-binding benzofuran isophthalate (SBFI) was performed by a modification of the method of Harootunian *et al*. [13]. The isolated cells were cultured for 4 days on coverslips cut to fit into the spectrofluorometer cuvette. The cultured cells on each coverslip were incubated with 10 μM SBFI tetraacetoxymethyl ester and 0.02% Pluronic F-127 in KRH buffer for 3 hr at 37° and washed three times with KRH buffer. The coverslips were then placed in the cuvette and preincubated with KRH buffer for 5 min at 37° in the fluorescence meter. Then, the test agents were added to the cuvette. Increases and decreases in the fluorescence induced by the SBFI–Na^+ complex were recorded simultaneously 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 (CAF-100, Japan Spectroscopic Co, Ltd.).

2.4. RNA preparation and two-electrode voltage clamp with Xenopus oocytes

Capped mRNAs for human nicotinic ACh receptor subunits, α3 and β4, were synthesized *in vitro* from the respective cDNA inserts in the pcDNAI vector (Invitrogen), using T7 RNA polymerase (mMESSAGE mMACHINE™, Ambion, Inc.). Oocytes were prepared for microinjection as described by Sakata *et al*. [14] and injected with 1 nL (10 ng/nL) of the subunit mRNAs. They were incubated with a modified Barth's solution (pH 7.6), consisting of 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.5 mM sodium pyruvate, and 7.5 mM Tris, supplemented with 40 units/mL penicillin and 1000 units/mL streptomycin, for 3 days at 18° after injection.

The oocytes expressing the α3β4 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's solution, consisting of 120 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES (pH 7.5) at 20–22°. Two recording microelectrodes (1–5 MΩ) filled with 3 M KCl were inserted into the animal pole under a binocular microscope. One electrode was connected to a preamplifier (Dagan 8500 clamp unit) and used to record the membrane potentials. The other was connected to the output of the same clamp unit to pass the current across the cell membrane. The ACh-induced inward current response was recorded under voltage clamp conditions at resting membrane potential (−40 mV).

2.5. [^{3}H]Nicotine binding to chromaffin cells

The binding of [^{3}H]nicotine to the receptors in the chromaffin cells was measured according to the method

described by Park *et al*. [15]. The cells in 24-well plates were rinsed twice with KRH buffer and preincubated with different concentrations of M4 or amitriptyline for 15 min at 25°. The cells were then incubated with 20 nM [^{3}H]nicotine (51.4 KBq) in the presence of different concentrations of M4 or amitriptyline used in the preincubation for 40 min at 25°. After incubation, the medium was immediately removed, and the cells were rinsed three times with ice-cold Ca^{2+}-free KRH buffer containing 200 μM EGTA. The cells were lysed with 10% Triton X-100 and scraped off the plates, and the radioactivity was measured by liquid scintillation counting (LSC-6100; Aloka). The nonspecific binding was determined by co-incubation with 1 mM unlabeled nicotine. The specific binding of [^{3}H]nicotine was obtained by subtracting the nonspecific binding from the total binding.

2.6. Statistics

Statistical evaluation of the data was performed by ANOVA. When a significant F value was found by ANOVA, Scheffe's test for multiple comparisons was performed to identify differences among groups. $P < 0.05$ was considered to indicate significance.

2.7. Materials

The metabolites of the ginsenosides were prepared by fermentation of the protopanaxadiol- or protopanaxatriol-type saponins by human intestinal bacteria as described elsewhere [10]. The metabolites were dissolved in DMSO. The final concentration of DMSO in the incubation medium was 0.1%, which had no effect upon the secretion of catecholamines from the bovine adrenal chromaffin cells or the inward ion current into the oocytes under the conditions used in this study. Oxygenated KRH buffer (pH 7.4) was used as the reaction medium unless otherwise noted, 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% BSA. In 56 mM KCl–KRH buffer, the amount of NaCl was reduced to maintain the isotonicity of the medium. SBFI tetraacetoxymethyl ester was obtained from Molecular Probes Inc. L-(−)-[*N-methyl*-^{3}H]Nicotine (257 TBq/mmol) was obtained from NEN Life Science Products, Inc.

3. Results

3.1. Effects of ginsenoside metabolites on catecholamine secretion from chromaffin cells

First, we evaluated the influence of ginsenoside metabolites on the secretion of catecholamines from bovine adrenal chromaffin cells stimulated by ACh. Chromaffin cells were preincubated with various concentrations of each metabolite (M1, M2, M3, M4, M5, M11, and M12) and then incubated with or without ACh (20 μM) in the presence of the same metabolite. All of the metabolites examined inhibited the ACh-evoked secretion of catecholamines in a concentration-dependent manner (Fig. 2). M4, an end product derived from the protopanaxatriol saponins, exhibited the most potent inhibitory effect: M4 showed inhibition at concentrations as low as 500 nM, and at the higher concentration of 10 μM, this metabolite inhibited secretion by 53%. The rank order of IC_{50} values (μM) was as follows: M4 (9) > M12 (15) > M2 (18) > M3 (19) > M11 (22) > M5 (36) > M1 (38). These metabolites had no effect on spontaneous (basal) secretion from non-stimulated cells.

3.2. Reversibility of M4-induced inhibition of catecholamine secretion

The inhibitory properties of the metabolites were examined using M4. Preincubation of chromaffin cells with M4

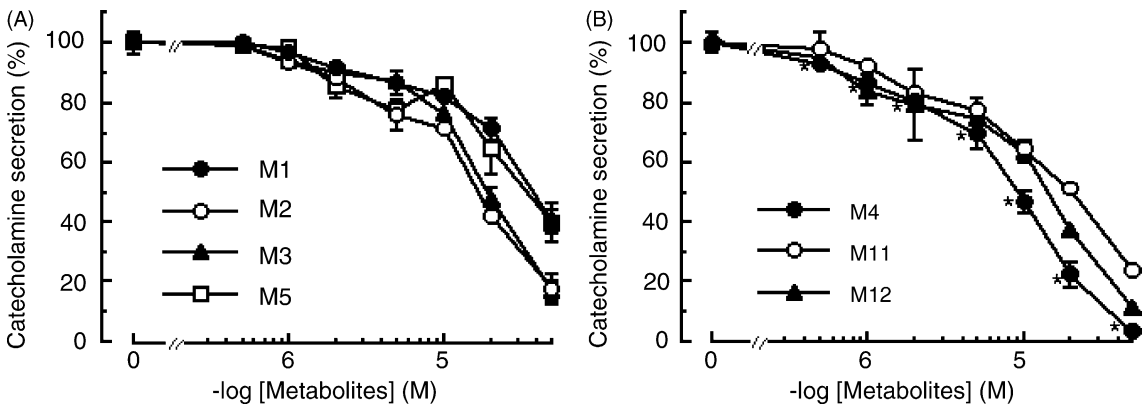


Fig. 2. Effects of ginsenoside metabolites on the secretion of catecholamines from bovine adrenal chromaffin cells. The cultured chromaffin cells were preincubated with different concentrations of M1, M2, M3, or M5 (A), and M4, M11, or M12 (B) (500 nM–50 μM) in KRH buffer at 37° for 15 min, and then incubated with the same metabolite in the presence or absence of ACh (20 μM) for 7 min. The amounts of catecholamines secreted into the medium were determined as described in Section 2. ACh-evoked secretion was assigned a value of 100%. ACh-evoked and basal catecholamine secretions were 6.2 ± 0.3 μg/2 × 10^6 cells and 0.3 ± 0.1 μg/2 × 10^6 cells, respectively. Values are means ± SD from four experiments. $^*P < 0.01$, compared with ACh-evoked secretion.

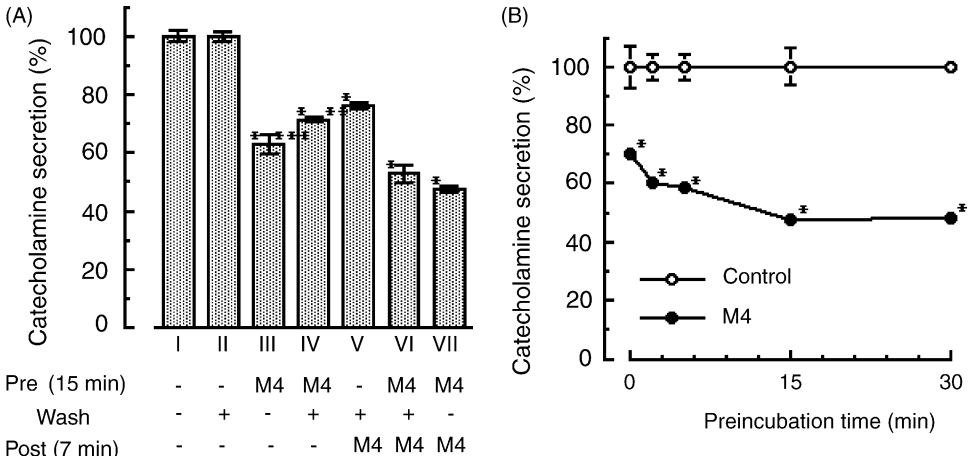


Fig. 3. Reversibility of the inhibitory effect of M4 on catecholamine secretion and effect of preincubation of chromaffin cells with M4 on catecholamine secretion. (A) After preincubation with (III, IV, VI, and VII) or without M4 (10 μM) (I, II, and V) for 15 min, the cells were washed (II, IV, V, and VI) and then incubated with (V–VII) or without M4 (10 μM) (I–IV) in the presence or absence of ACh (20 μM) for 7 min. (B) Chromaffin cells were preincubated with or without M4 (10 μM) for 0–30 min, and then incubated with or without M4 (10 μM) in the presence or absence of ACh (20 μM) for 7 min. The amounts of catecholamines secreted into the medium were determined as described in Section 2. ACh-evoked secretion was assigned a value of 100%. (A) ACh-evoked and basal catecholamine secretions were 6.1 ± 0.5 μg/2 × 10^6 cells and 0.2 ± 0.1 μg/2 × 10^6 cells, respectively. Values are means ± SD from four experiments. ǂ$P < 0.005$, compared with ACh-evoked secretion (I). ǂǂ$P < 0.01$, compared with VI. ǂǂǂ$P < 0.01$, compared with V. (B) ACh-evoked and basal catecholamine secretions were 6.0 ± 0.5 and 0.2 ± 0.1 μg/2 × 10^6 cells, respectively. Values are means ± SD from at least four experiments. ǂ$P < 0.005$, compared with ACh-evoked secretion.

(10 μM) for 15 min followed by incubation with M4 in the presence of ACh (20 μM) for 7 min, inhibited the secretion of catecholamines by about 50% (Fig. 3A, VII). On the other hand, secretion was still considerably inhibited (29% inhibition) after preincubation of the cells with M4, washing three times with KRH buffer, and incubation without M4 in the presence of ACh (Fig. 3A, IV). However, the reduction was much less than that observed when M4 was present throughout both the preincubation and incubation steps (Fig. 3A, VI and VII). Thus, the M4 effect is not completely reversible. In addition, the inhibitory effect of M4 on secretion from the cells that were preincubated with the metabolite for 15 min (Fig. 3A, III) was greater than that in cells incubated in its presence for only 7 min (Fig. 3A, V). The duration of exposure to M4 seemed to be a determinant of the inhibitory potency. Therefore, the effect of preincubation time on the inhibitory effect of M4 on catecholamine secretion was examined. Preincubation of the cells with M4 (10 μM) for 2 min led to 40% inhibition of secretion, and preincubation for 15 min produced almost maximal inhibition (approximately 50%), while no preincubation resulted in 30% inhibition (Fig. 3B). Thus, the inhibitory effect of M4 was dependent on the preincubation time.

3.3. Effects of M4 on ACh-induced Na^+ influx into chromaffin cells and on high K^+-induced catecholamine secretion from the cells

The Na^+ influx into the cells through nicotinic ACh receptors and the consequent Ca^{2+} influx through voltage-sensitive Ca^{2+} channels are indispensable for ACh-induced catecholamine secretion from chromaffin [3–5]. ACh (100 μM) augmented the fluorescence ratio (340/380 nm) in SBFI-loaded cells, indicating an increase in $[Na^+]_i$ due to the external Na^+ influx into the cells (Fig. 4A) [7]. M4 reduced the ACh-induced Na^+ influx in a concentration-dependent manner. At 500 nM, M4 showed an inhibitory effect, and at 10 μM, this metabolite showed approximately 50% inhibition of Na^+ influx (at the peak). Thus, the concentration–response curve for the inhibitory effect of M4 on Na^+ influx was similar to that on catecholamine secretion.

On the other hand, M4 also inhibited the secretion of catecholamines induced by high K^+, a stimulus that activates voltage-sensitive Ca^{2+} channels direct membrane depolarization (Fig. 4B). However, the inhibitory effect ($IC_{50} = 45$ μM) was much less than that on ACh-evoked secretion.

3.4. M4 inhibition of the function of human neuronal nicotinic ACh receptors expressed in oocytes

As M4 reduced ACh-induced Na^+ influx into chromaffin cells, we next examined whether M4 affects nicotinic ACh receptors. The subunit combination of neuronal nicotinic ACh receptors associated with the secretion of catecholamines from bovine adrenal chromaffin cells stimulated by nicotinic agonists is thought to be mainly α3β4 [1,2]. Therefore, we expressed human α3β4 neuronal nicotinic ACh receptors in *Xenopus* oocytes and examined the effects of M4 on the ACh-induced inward ion current into the cells. M4 decreased the ACh-induced current in a concentration-dependent manner (Fig. 5). The inhibitions of M4 at 5 and 10 μM were 58 and 67%, respectively.

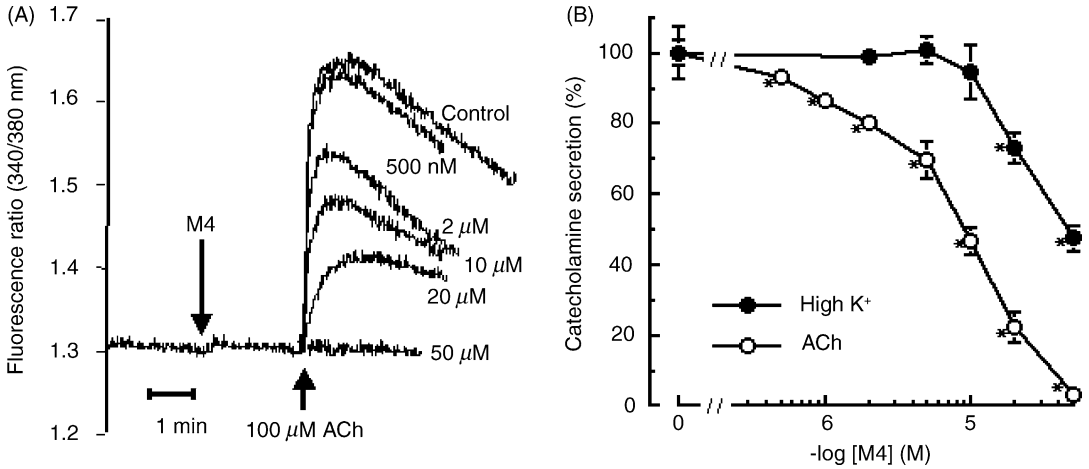


Fig. 4. Effects of M4 on $[Na^+]_i$ and high K^+-induced secretion of catecholamines in chromaffin cells. (A) SBFI-loaded chromaffin cells on the coverslip in the fluorescence meter were preincubated for 5 min at 37°, and then M4 or ACh (100 μM) was added. The fluorescence was recorded before and after the addition of the test agents. The change in $[Na^+]_i$ was expressed as the ratio of fluorescence at an excitation wavelength of 340 to that of 380 nm. Data are from a representative sample of at least four experiments. (B) After preincubation of the cells with different concentrations of M4 for 15 min, the cells were incubated with M4 at the same concentration as used for preincubation in the presence or absence of high K^+ or ACh (20 μM) for 7 min. The amounts of catecholamines secreted into the medium were determined as described in Section 2. The stimulus-induced secretion was assigned a value of 100%. High K^+-induced and ACh-evoked catecholamine secretions were 8.2 ± 0.7 μg/2 × 10^6 cells and 5.9 ± 0.4 μg/2 × 10^6 cells, respectively. Basal catecholamine secretion was 0.3 ± 0.1 μg/2 × 10^6 cells. Values are means ± SD from four experiments. $^*P < 0.01$, compared with stimulus-induced secretion.

3.5. Effect of external ACh concentration on M4 inhibition and effect of M4 on [^{3}H]nicotine binding

When chromaffin cells were incubated with different concentrations of ACh, secretion was shown to be augmented by increasing ACh concentration (10–200 μM). Maximal secretion was seen at 100 μM ACh. M4 at concentrations of 2–200 μM gave a parallel shift to the right of the concentration–response curve to ACh (Fig. 6A).

Next, the effects of M4 on the binding of [^{3}H]nicotine to the receptor were examined. The specific binding of [^{3}H]nicotine was 80,771 ± 4,505 DPM when the chromaffin cells (5×10^5 cells) were incubated with 20 nM [^{3}H]nicotine. However, M4 (1–50 μM) did not alter the specific binding of [^{3}H]nicotine to the chromaffin cells, while amitriptyline (1–50 μM), a tricyclic antidepressant known as a competitor for the nicotine binding to the chromaffin cells [16], decreased the specific binding (Fig. 6B).

4. Discussion

All of the ginsenoside metabolites examined in this study markedly reduced the secretion of catecholamines from bovine adrenal chromaffin cells stimulated by ACh (Fig. 2). Recent studies have shown that the ginsenosides in orally administrated ginseng are rapidly metabolized in the digestive tract and absorbed into the circulation [9,10]. The oligosaccharides connected to the hydroxyl groups of the aglycones are in turn hydrolyzed by gastric acid and by the enzymes produced by the intestinal bacteria. The major

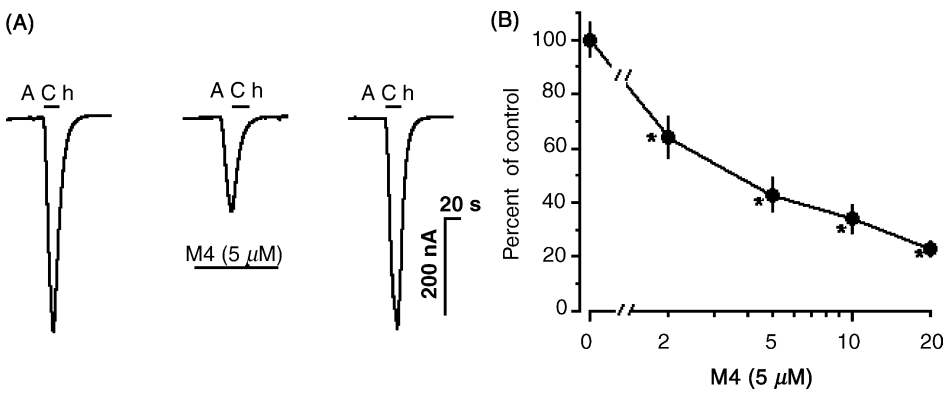


Fig. 5. Effects of M4 on ACh-induced inward currents in *Xenopus* oocytes expressing human nicotinic receptor α3β4 subunits. M4 (2–50 μM) was applied to the oocytes for 1 min before stimulation, and then the oocytes were perfused with ACh (100 μM) in the presence of M4 for 2 min. (A) A representative sample of tracings obtained from a single oocyte clamped at −40 mV. (B) Concentration–response curves for the inhibitory effect of M4 on the ACh-induced response. Values are means ± SD from at least four experiments. $^*P < 0.01$, compared with the ACh-induced inward current.

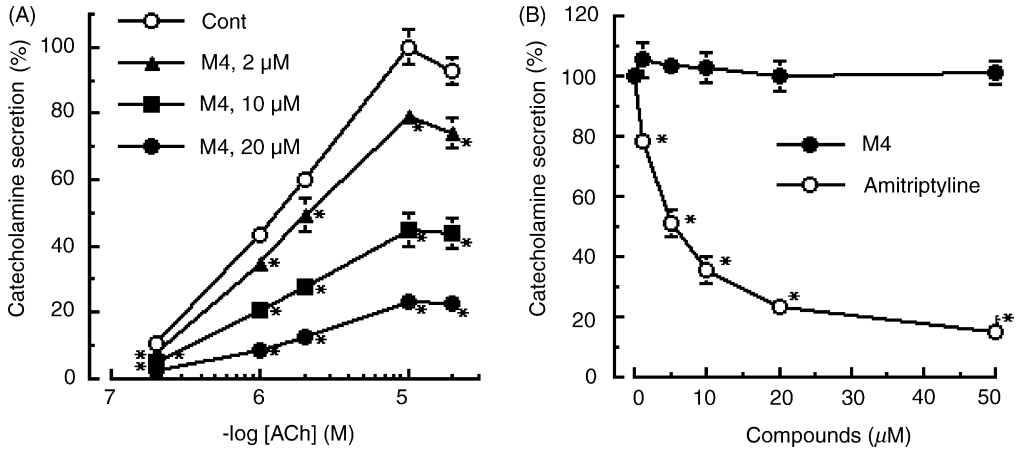


Fig. 6. Effects of M4 or amitriptyline on the secretion of catecholamines and on the specific binding of [^{3}H]nicotine in chromaffin cells. (A) After preincubation of the cells with different concentrations of M4 for 15 min, the cells were incubated with M4 at the same concentration as used for preincubation in the presence of various concentrations of ACh for 7 min. The amounts of catecholamines secreted into the medium were determined as described in Section 2. ACh (at 100 μM)-evoked secretion was assigned a value of 100%. ACh (at 100 μM)-evoked and basal catecholamine secretions were 10.5 ± 0.7 μg/2×10^6 cells and 0.3 ± 0.1 μg/2×10^6 cells, respectively. Values are means ± SD from four experiments. (B) After preincubation of the cells with different concentrations of M4 or amitriptyline for 15 min at 25°, the cells were incubated with M4 or amitriptyline at the same concentration as used for preincubation in the presence of 20 nM [^{3}H]nicotine (51.4 KBq) for 40 min at 25°. The specific binding of [^{3}H]nicotine was determined as described in Section 2. The specific binding was assigned a value of 100%. The total and the nonspecific binding of [^{3}H]nicotine were 115,469 ± 4,504 DPM and 34,698 ± 1,302 DPM, respectively. Values are means ± SD from four experiments. $^*P < 0.01$, compared with control.

metabolites of the protopanaxatriol saponins are M4 and M11, and those of the protopanaxadiol saponins are M1, M2, M3, and M5 (Fig. 1). M2, M3, M5, and M11 are intermediate metabolites, and M1 (20(*S*)-protopanaxadiol monoglucoside) and M4 (20(*S*)-protopanaxatriol) are the end metabolites derived from the protopanaxadiols and the protopanaxatriols, respectively. M12, a complete aglycone derivative of the protopanaxadiols, is currently considered an artifact. The glucose at the 20-position in M1 seems to be resistant to hydrolysis. M1, M2, M3, M4 and M12 have been shown to be detected in blood after the oral administration of ginseng or total saponin to rats [10]. We have demonstrated previously that the protopanaxatriol ginsenosides strongly inhibit the ACh-evoked secretion of catecholamines from chromaffin cells, whereas the protopanaxadiols show only a slight inhibitory effect. The IC_{50} values of the diol-type saponins, in particular, ginsenoside-Rb_1, -Rb_2, -Rb_3, -Rc, -Rd and -Rs, on catecholamine secretions were much higher than 100 μM [7,8]. In this study, the IC_{50} values of the protopanaxadiol metabolites were as follows: M12, 15 μM; M2, 18 μM; M3, 19 μM; M5, 36 μM; and M1, 38 μM. Hasegawa [17] has reported that M1 level in tissues (liver) reaches at least 12 μg/mL (≒20 μM), which inhibited the ACh-evoked catecholamine secretion by approximately 20% in this study (Fig. 2A), 2 hr after the oral administration of the metabolite (10 mg/kg) to rats. The M1 administration of 10 mg/kg orally (p.o.) to rats does not seem to be overdosage. Because content of the total saponin is about 7% in ginseng [18], it should contained 350 mg in 5 g of ginseng, a usual dose for human therapy, which is roughly estimated to be 6–7 mg saponin/kg. Furthermore, a diol-type saponin ginsenoside-Rb_1 has been shown to be completely hydrolyzed to M1 in mouse caecum and colorectum [19]. Therefore, it is highly probable that the protopanaxadiol-type ginsenosides are prodrugs, which are metabolized in the digestive tract and reveal their inhibitory activity *in vivo*. On the other hand, the protopanaxatriol-type ginsenosides were shown to retain their inhibitory potency even after metabolism.

M4 blocked the ACh-induced Na^+ influx into the cells in a concentration-dependent manner, similar to its effect on ACh-evoked catecholamine secretion (Figs. 2B and 4A). On the other hand, much higher concentrations of M4 were required for the inhibition of secretion induced by high K^+, a stimulus that activates voltage-sensitive Ca^{2+} channels regardless of the response of the nicotinic receptors (Fig. 4B). Therefore, the inhibitory effect of M4 on the ACh-evoked secretion of catecholamines is due mainly to the direct blockade of Na^+ influx into the cells through nicotinic ACh receptors, rather than Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. The functional subunit combination of nicotinic ACh receptors associated with catecholamine secretion in adrenal chromaffin cells is considered to be α3β4 [1,2]. M4 also reduced the ACh-induced inward ion current into oocytes expressing cloned nicotinic receptors comprised of α3β4 subunits (Fig. 5). This observation not only strongly supports the view described above, but also suggests that M4 directly modulates the function of nicotinic ACh receptors. However, the inhibitory effect of M4 on the ACh-induced inward ion current was more potent than those on the ACh-induced secretion of catecholamines and Na^+ influx in the chromaffin cells (Figs. 2B, 4A and 5). The discrepancy may be explained by the following reasons; there are species differences of the nicotinic ACh receptors (bovine and human ones); and the nicotinic receptors in the

chromaffin cells may contain α5 subunit, which has been reported to be expressed in the cells in addition to α3 and β4 subunits [1] and recently found to modulate the function of the reconstituted nicotinic ACh receptors [20,21].

On the other hand, the inhibitory effect of M4 on catecholamine secretion was not overcome by increasing the external ACh concentration (Fig. 6A), and the specific binding of [^{3}H]nicotine to the cells was not altered by M4 (Fig. 6B), suggesting that the inhibitory effect of M4 is not mediated by competition with the binding site of ACh to the receptors. Furthermore, as the inhibitory effect of M4 on secretion was not completely reversible (Fig. 3A) and was dependent on the preincubation time of the cells with M4 (Fig. 3B), it is highly likely that M4 enters the cells and acts on the nicotinic ACh receptors from the cytoplasmic side of the plasma membrane. A very recent report has shown that dansyl M1, a fluorescent analog of M1, is observed in the cytosol of B16-BL6 mouse melanoma cells 15-min after incubation of the cells with the fluorescent probe [22]. This strongly indicates that ginsenoside metabolites can penetrate the plasma membrane. However, we cannot exclude the possibility that M4 is incorporated into the lipid bilayer of the plasma membrane, which produces a change in the environment of the membrane adjacent to the nicotinic receptors. Léna and Changeux have proposed that the binding sites of lipophilic, non-competitive blockers of nicotinic ACh receptors lie at the interface between the receptor protein and membrane lipids [23]. Further studies, therefore, are required to determine the action site of ginsenoside metabolites.

Many reports have shown that ginseng and its major components, the saponins, ameliorate the symptoms and lesions evoked by stress (anti-stress action) [24–26]. For example, improvements have been reported in gastrointestinal symptoms (anorexia, dyspepsia, etc.), gastric ulcer, fatigue, boredom, anxiety, and essential hypertension in stressed animals and humans treated with ginseng or saponins. In peripheral systems, catecholamines act as hormones and neurotransmitters in the adrenal medulla and sympathetic nerve terminals, respectively, and they are secreted in large quantities under conditions of stress. Consequently, the organs that are responsive to those transmitters are stimulated to cope with stress. However, long-term or excessive stress results in the over-secretion of catecholamines, leading to over-reaction and exhaustion of the organs. Furthermore, in autonomic nervous systems, the stress leads to an imbalance between sympathetic and parasympathetic nervous systems. It is possible that ginseng suppresses these phenomena induced under stressful conditions *via* the reduction of catecholamine secretion, and leads to recovery of homeostasis *in vivo*. These effects may account, at least in part, for the anti-stress action of ginseng. Stress is considered to lead to the development of almost all types of disease. Therefore, the beneficial effects of ginseng on stress might be why it has become known as a panacea or prophylactic natural medicine.

In summary, all of the ginsenoside metabolites examined in this study inhibited the secretion of catecholamines from bovine adrenal chromaffin cells, a model of sympathetic nerves, stimulated by ACh. The inhibition was attributable to the blockade of Na^+ influx into the cells through nicotinic ACh receptors. This implies that ginsenosides show stronger inhibitory effects in concert after their metabolism in the digestive tract, and this may be associated with the pharmacological effects of ginseng.

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