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Characterization of ginseng saponin ginsenoside-Rg_3 inhibition of catecholamine secretion in bovine adrenal chromaffin cells

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Abstract

Since ginsenoside-Rg_3, one of the panaxadiol saponins isolated from the ginseng root, significantly inhibited the secretion of catecholamines from bovine adrenal chromaffin cells stimulated by acetylcholine (ACh), the properties of ginsenoside-Rg_3 inhibition were investigated. Although ginsenoside-Rg_3 inhibited the secretion evoked by ACh in a concentration-dependent manner, it affected the secretion stimulated by high K^+ or veratridine, an activator of the voltage-sensitive Ca^{2+} or Na^+ channels, only slightly. The ACh-induced Na^+ and Ca^{2+} influxes into the cells were also reduced by ginsenoside-Rg_3. The inhibitory effect of this saponin on the secretion of catecholamines was not altered by increasing the external concentration of ACh or Ca^{2+}. The ACh-evoked secretion of catecholamines was completely restored in cells that were preincubated with 10 μM ginsenoside-Rg_3 and then incubated without the saponin, whereas secretion was not completely restored in cells that were preincubated with 30 μM of this compound. Above 30 μM ginsenoside-Rg_3 increased the fluorescence anisotropy of diphenylhexatriene in the cells. Furthermore, the inhibitory effect of ginsenoside-Rg_3 at 30 μM on the ACh-evoked secretion of catecholamines was dependent upon the preincubation time, but this was not the case at 10 μM. These results strongly suggest that ginsenoside-Rg_3 blocks the nicotinic ACh receptor-operated cation channels, inhibits Na^+ influx through the channels, and consequently reduces both Ca^{2+} influx and catecholamine secretion in bovine adrenal chromaffin cells. In addition to this action, the ginsenoside at higher concentrations modulates the fluidity of the plasma membrane, which probably contributes to the observed reduction in the secretion of catecholamines.

Keywords: Ginseng saponin; Ginsenoside; Catecholamine secretion; Adrenal chromaffin cell; Nicotinic acetylcholine receptor-operated cation channel; Membrane fluidity

1. Introduction

The root of *Panax ginseng* C. A. Meyer is widely known as one of the most important components in many Chinese traditional prescriptions, called kampo medicine in Japan, and itself is also commonly used to treat various diseases and maintain health. The oldest Chinese traditional medical book, *Shengnong Ben-cao Jing*, mentions that the ginseng root has many effects (e.g. replenishment of vital energy, tranquilization, mood elevation, and prevention of aging). Among the many pharmacological effects of the ginseng root, we have focused on the tranquilizing action and have investigated the influence of the root on the nervous systems, especially the autonomic nervous systems, using bovine adrenal chromaffin cells.

The adrenal medulla secretes catecholamines mainly via stimulation of its nicotinic ACh receptors by ACh, which is released from the terminal of the splanchnic nerve. Binding of ACh to these nicotinic receptors leads to a depolarization of the cell membrane by an influx of Na^+ through receptor-operated cation channels, causing an influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels, which results in catecholamine secretion by exocytosis [1–3]. Therefore, adrenal chromaffin cells are widely used as a model to study catecholamine secretion in response to stimulation by the sympathetic nervous system.

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Abbreviations: ACh, acetylcholine; KRH, Krebs-Ringer-HEPES; $[Na^+]_i$, intracellular free sodium concentration; SBFI, sodium-binding benzofuran isophthalate; and DPH, 1,6-diphenyl-1,3,5-hexatriene.

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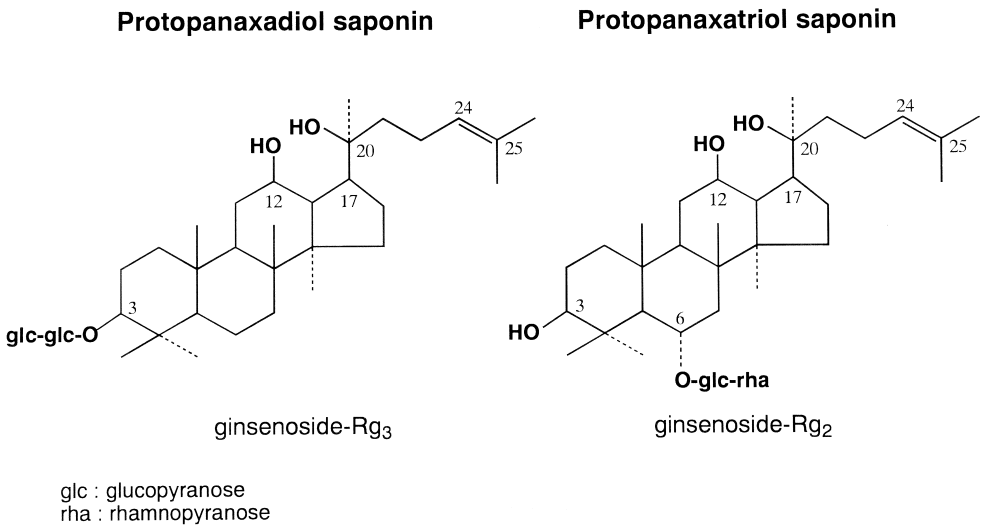

Fig. 1. Structures of ginsenoside-Rg_2 and -Rg_3.

We have found that an ingredient isolated from the ginseng root, i.e. ginseng saponins (ginsenosides), inhibited the secretion of catecholamines from bovine adrenal chromaffin cells stimulated by ACh [4,5]. The ginseng saponins are classified into three groups, the protopanaxadiol, protopanaxatriol and oleanolic acid saponins, on the basis of the chemical structures of their aglycones. The inhibitory effects of the protopanaxatriols on the secretion of catecholamines were very strong, whereas those of the protopanaxadiols and the oleanolic acid saponin (ginsenoside-Ro) were only slight. There was a structure–activity relationship between the inhibitory effects and the structures of the ginsenosides [6]. Furthermore, we have demonstrated that ginsenoside-Rg_2 (Fig. 1), a panaxatriol showing the greatest inhibition among the tested ginsenosides, regulates the nicotinic ACh receptor-operated cation channels, inhibiting Na^+ influx through the channels, and consequently reduces both Ca^{2+} influx and catecholamine secretion in the cells [5].

On the other hand, ginsenoside-Rg_3, a panaxadiol (Fig. 1), has been found to cause an exceptionally strong inhibition of the ACh-evoked secretion of catecholamines, comparable to that of ginsenoside-Rg_2 [5,6]. Moreover, it diminished not only ACh-induced but also histamine-, angiotensin II-, neurotensin-, and γ-aminobutyric acid-induced secretion of catecholamines from chromaffin cells as well as muscarine- and histamine-induced contractions of the ileum in the guinea pig. However, ginsenoside-Rg_2 selectively blocked only the responses mediated by ionotropic receptors [nicotinic ACh and γ-aminobutyric acid ($GABA_A$) receptors] [7].

Therefore, in this study, we investigated whether the mechanism by which ginsenoside-Rg_3 inhibits the ACh-evoked secretion of catecholamines from chromaffin cells is the same as that of ginsenoside-Rg_2.

2. Materials and methods

2.1. Materials

Ginsenoside-Rg_2 and -Rg_3 were supplied by the Korea Tobacco & Ginseng Corp. and the Japan Korea Red Ginseng Co., Ltd. The purities of the ginsenosides were checked by thin-layer chromatography and nuclear magnetic resonance according to the method of Kawashima and Samukawa [8] and were found to be >98% pure. The ginsenosides were dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the incubation medium was 1%, which had no effect upon the secretion of catecholamines from bovine adrenal chromaffin cells under the conditions used in this study. Oxygenated 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 0.5% BSA. In 56 mM KCl-KRH buffer, the amount of NaCl was reduced to maintain the isotonicity of the medium. Tissue culture instruments were obtained from the Falcon Plastics Co. Eagle's minimum essential medium was from Nissui Seiyaku. SBFI tetraacetoxymethyl ester and DPH were from Molecular Probes Inc. $^{45}CaCl_2$ (0.185 to 1.85 GBq/mg calcium) was from Amersham International, Ltd. 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 provided by the Center of Iwate Livestock Industry. Adrenal chromaffin cells were prepared by the method of collagenase digestion as described previously [9]. The isolated cells were suspended in Eagle's minimum essential medium containing 10% calf

serum, 3.0 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 as a monolayer culture in 35-mm diameter dishes at a density of 2×10^6 cells. The cells were cultured at 37° in a CO_2 incubator (95% air/5% CO_2). A total of 2×10^6 cells contained 38.9 ± 1.3 μg of catecholamines as epinephrine and norepinephrine, and their ratio was determined to be 72 and 28%, respectively, by HPLC.

2.3. Measurements of catecholamine secretion from, and $^{45}Ca^{2+}$ influx into, chromaffin cells

After 4 days of culturing, the cells were washed twice with prewarmed KRH buffer and then preincubated with or without ginsenoside-Rg_3 in KRH buffer for 10 min at 37°. They were then incubated with or without ginsenoside-Rg_3 in the presence or absence of ACh, high K^+ (56 mM K^+), or veratridine for 7 min. 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 adsorbed on aluminum hydroxide. Their amounts were estimated by the ethylenediamine condensation method [10] 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 showed the same fluorescence intensity.

After preincubation of the cells with or without ginsenoside-Rg_3 in KRH buffer for 10 min, the cells were incubated for 7 min with or without ginsenoside-Rg_3 in the presence of $^{45}Ca^{2+}$ (37 KBq) in the plain or the ACh-containing medium. The medium was removed, and the cells were immediately cooled on ice and washed three times with ice-cold Ca^{2+}-free KRH buffer. The cells were scraped and solubilized in 10% Triton X-100. Radioactivity was determined using a liquid scintillation counter (LSC-900; Aloka) [9].

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

Loading of the chromaffin cells with SBFI was performed by a modification of the method of Harootunian et al. [11]. 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 10 min at 37° in the fluorescence meter. Then the test agents were added to the cuvette. Increases and decreases in the fluorescence induced from the SBFI–Na^+ complex were recorded simultaneously at excitation wavelengths of 340 and 380 nm, respectively, and at an emission wavelength of 500 nm. The change in

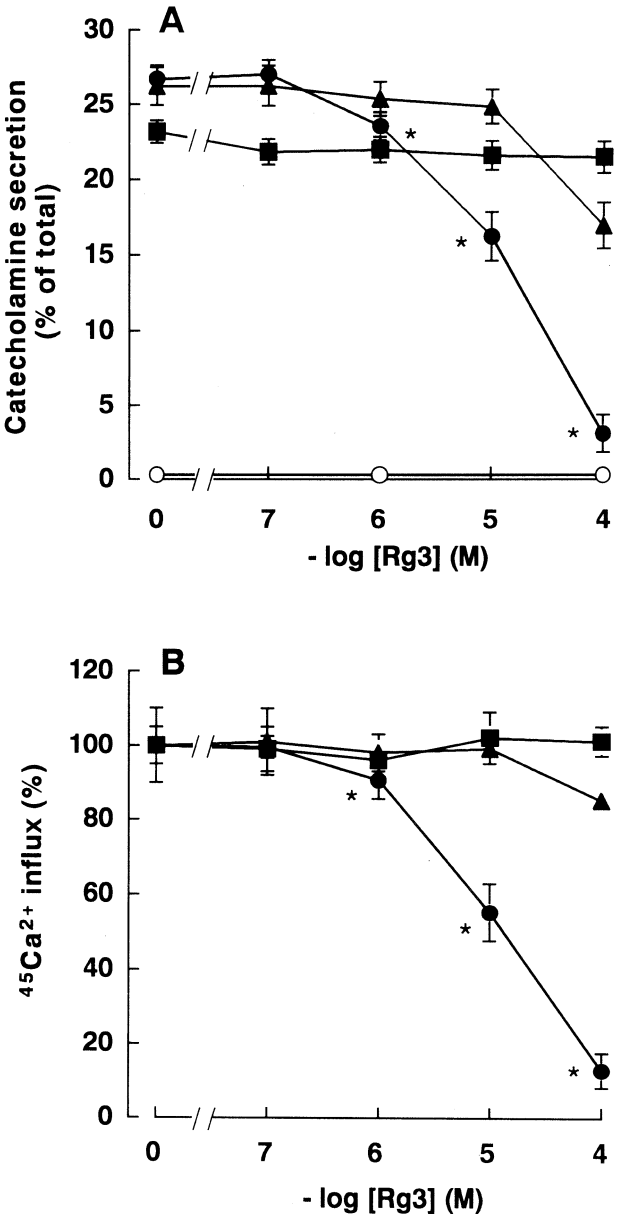

Fig. 2. Effects of different concentrations of ginsenoside-Rg_3 on catecholamine secretion (A) and Ca^{2+} influx (B) in bovine adrenal chromaffin cells. Cultured chromaffin cells were washed twice with prewarmed KRH buffer and preincubated with different concentrations of ginsenoside-Rg_3 (Rg_3) in the KRH buffer for 10 min at 37°. (A) Then the cells were incubated with different concentrations of the saponin used above in the absence (○) or presence of 50 μM ACh (●), 56 mM K^+ (■), or 50 μM veratridine (▲) for 7 min. The amount of catecholamines secreted from the cells into the medium was determined as described in "Materials and methods" and was expressed as a percentage of the total cellular catecholamines. (B) The cells were then incubated with different concentrations of the saponin in the KRH buffer containing 37 KBq $^{45}Ca^{2+}$ in the absence or presence of 50 μM ACh (●), 56 mM K^+ (■), or 50 μM veratridine (▲) for 7 min. The amount of Ca^{2+} influx into the cells was measured as described in "Materials and methods." The values of the basal Ca^{2+} influx were subtracted from the data, and the secretagogue-induced Ca^{2+} influxes were assigned the value of 100%. The ACh-, the K^+-, and the veratridine-induced Ca^{2+} influxes were 9.01 ± 0.52, 11.08 ± 0.73, and 10.34 ± 0.69 nmol/2×10^6 cells, respectively, and the basal influx was 1.43 ± 0.27 nmol/2×10^6 cells. Values are means ± SD from at least four experiments. Key: (*) $P <$ 0.05, compared with the ACh-induced responses.

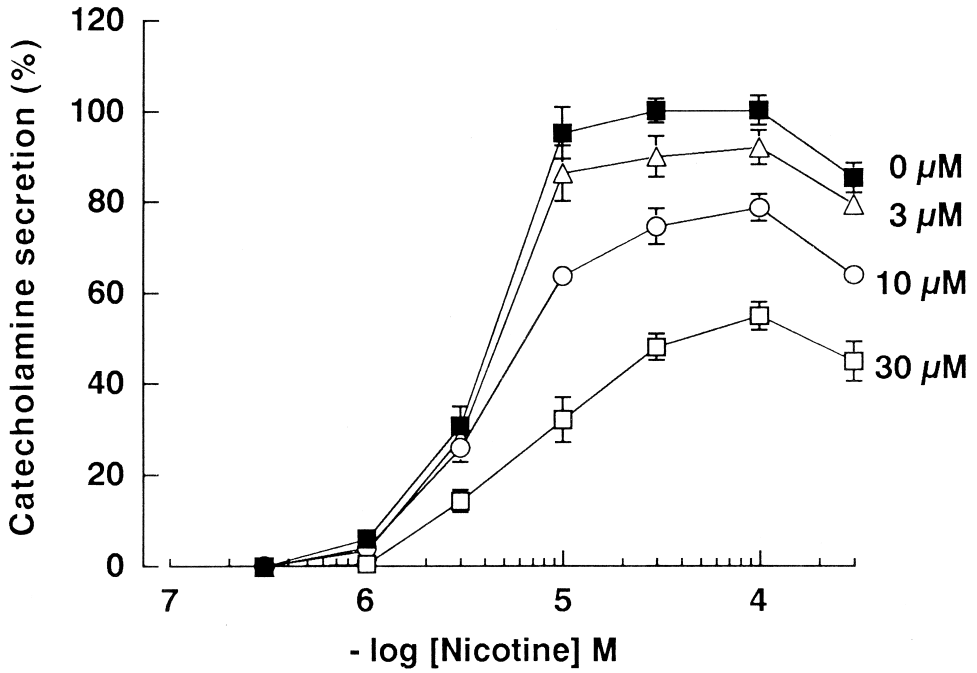

Fig. 3. Effects of ginsenoside-Rg_3 on catecholamine secretion from the chromaffin cells induced by l-nicotine. The cells were preincubated without (■) or with ginsenoside-Rg_3 (3 μM, Δ; 10 μM, ❍; and 30 μM, ❑) for 10 min at 37° and then incubated with or without different concentrations of l-nicotine (300 pM-300 μM) for 7 min in the presence or absence of ginsenoside-Rg_3. Catecholamines secreted from the cells into the medium were determined as described in "Materials and methods." The values of the basal secretion were subtracted from the data, and the nicotine-induced maximal response was assigned the value of 100%. The basal and the nicotine-induced maximal secretions were 0.3 ± 0.1 and 28.2 ± 0.9% of the total cellular catecholamines, respectively. Values are means ± SD from at least four experiments.

$[Na^+]_i$ was expressed as the ratio of the fluorescence at excitation wavelengths of 340 and 380 nm [5].

2.5. Measurement of fluorescence anisotropy

The fluorescence anisotropy of DPH in the chromaffin cells was measured as described elsewhere [12]. The suspension of adrenal chromaffin cells with KRH buffer was incubated with a final concentration of 0.5 μM DPH for 2 min at 37°. The fluorescence intensity of the probe was measured in a spectrofluorometer equipped with excitation and emission polarizers. The excitation and emission wavelengths used for DPH were 363 and 428 nm, respectively. Steady-state fluorescence anisotropy, γ, was calculated according to the equation

$$\gamma = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

where I_{VV} and I_{VH} are the fluorescence intensities measured with a vertical polarizer and analyzer mounted vertically and horizontally, respectively. $G = I_{HV}/I_{HH}$ is the correction factor [13].

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 the groups. $P < 0.05$ was considered to be indicative of significance.

3. Results

3.1. Effects of ginsenoside-Rg_3 on catecholamine secretion from, and Ca^{2+} influx into, bovine adrenal chromaffin cells

We examined the effects of ginsenoside-Rg_3 (Fig. 1) on the secretion of catecholamines from bovine adrenal chromaffin cells stimulated by ACh (50 μM), high K^+ (56 mM), or veratridine (50 μM) (Fig. 2A). Ginsenoside-Rg_3 at 1 μM significantly reduced the ACh-evoked secretion of catecholamines concentration-dependently (1–100 μM), whereas it had no effect on the secretion induced by high K^+, which directly depolarizes the cell membranes and results in Ca^{2+} influx into the cells through voltage-sensitive Ca^{2+} channels and, consequently, catecholamine secretion from the chromaffin cells [14]. On the other hand, the secretion induced by veratridine, an activator of voltage-sensitive Na^+ channels [15], was not affected by 100 nM-10 μM ginsenoside-Rg_3, but at a higher concentration (100 μM), it was diminished slightly.

To further confirm the action of ginsenoside-Rg_3 on the nicotinic ACh receptors, we examined the effect of this

saponin on the secretion of catecholamines induced by l-nicotine. Ginsenoside-Rg_3 also inhibited the nicotine-induced secretion (Fig. 3). Ginsenoside-Rg_3 at concentrations of 3–30 μM shifted the concentration–response curve of nicotine (300 pM-300 μM) to the right. Schild plot analysis showed that the slope of ginsenoside-Rg_3 was 1.99, suggesting that the mode of antagonism of this saponin is unsurmountable.

Ca^{2+} influx into the bovine chromaffin cells is essential for triggering catecholamine secretion [1]. Ginsenoside-Rg_3 diminished the ACh-induced Ca^{2+} influx into the cells in a concentration-dependent manner (1–100 μM), whereas it had little or only a slight effect on the high K^+- or the veratridine-induced Ca^{2+} influx (Fig. 2B).

3.2. Effects of ginsenoside-Rg_3 on the ACh-induced Na^+ influx into the cells

Na^+ influx into adrenal chromaffin cells is a crucial first step in the process of ACh-evoked catecholamine secretion [15]. Stimulation of SBFI-loaded cells with ACh (50 μM) led to a rapid and marked increase in the fluorescence ratio (Fig. 4A), indicating that ACh augmented Na^+ influx into the chromaffin cells through the nicotinic ACh receptor-operated cation channels. The ACh-induced Na^+ influx was little affected by 100 nM ginsenoside-Rg_3 and it was decreased slightly at 1 μM (Fig. 4, B and C). Ginsenoside-Rg_3 at 10 μM greatly reduced the ACh-induced Na^+ influx, and at 100 μM it almost abolished the influx (Fig. 4, D and E). Thus, the concentration–response curves for the ginsenoside inhibition of the ACh-induced Na^+ and Ca^{2+} influxes and secretion were quite similar. The 50% inhibitory concentration (IC_{50}) values of ginsenoside-Rg_3 on catecholamine secretion, Ca^{2+} influx, and Na^+ influx were 14, 16, and 8 μM, respectively.

3.3. Effects of external ACh and Ca^{2+} concentrations on the ginsenoside-Rg_3 inhibition of catecholamine secretion

The character of the ginsenoside-Rg_3 inhibition of the ACh-induced secretion was investigated. An increment of the external ACh (20–200 μM) or Ca^{2+} concentration (2.6–7.8 mM) produced an increase in secretion. However, inhibition by ginsenoside-Rg_3 was affected only slightly by increasing the ACh (50–57% inhibition) (Fig. 5A) or Ca^{2+} concentration (46–50% inhibition) (Fig. 5B).

3.4. Reversibility of the ginsenoside-Rg3 inhibition of catecholamine secretion

We have reported that the inhibitory effect of ginsenoside-Rg_2 (Fig. 1) on ACh-evoked secretion is reversible [5]. Therefore, to compare the inhibitory properties of ginsenoside-Rg_3 with those of -Rg_2, we examined the reversibility of ginsenoside-Rg_3 inhibition. The cells were preincubated with or without 10 or 30 μM ginsenoside-Rg_3, washed three times with the prewarmed KRH buffer, and then incubated with or without 10 or 30 μM ginsenoside-Rg_3 in the presence or absence of 50 μM ACh. As shown in Fig. 6A (column V), in the cells that were preincubated with 10 μM ginsenoside-Rg_3, washed with the buffer and incubated with ACh, catecholamine secretion was restored completely. On the other hand, the effect of 30 μM ginsenoside-Rg_3 was still maintained but only partly diminished (Fig.

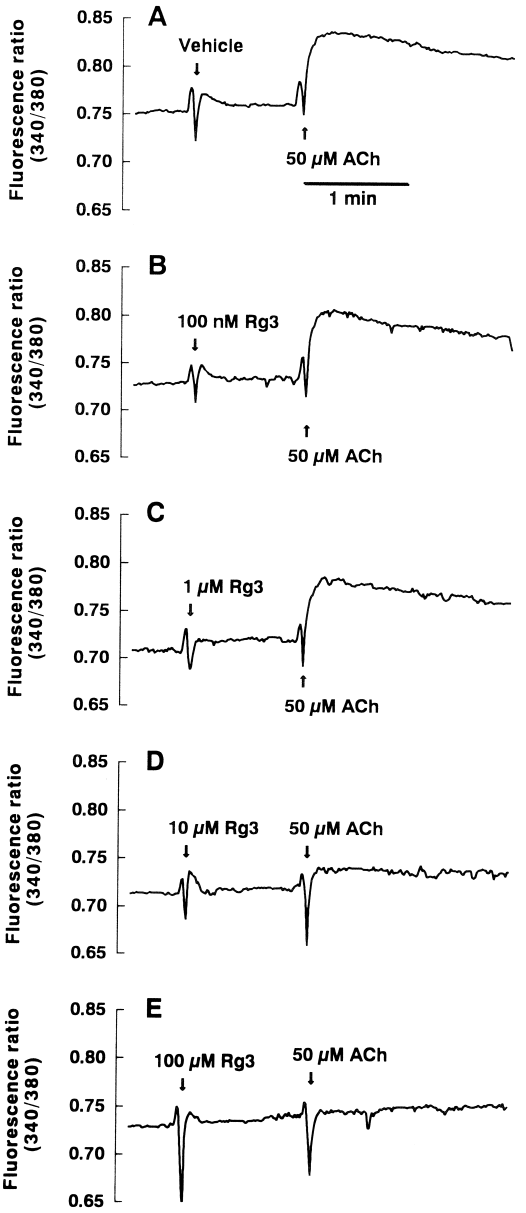

Fig. 4. Effects of ginsenoside-Rg_3 on the ACh-induced increase in $[Na^+]_i$. The SBFI-loaded cells were preincubated with KRH buffer in a fluorescence meter cuvette for 10 min at 37° and then incubated with different concentrations of ginsenoside-Rg_3 (Rg_3) (0–100 μM) for 1 min. ACh (50 μM) was added to the cuvette in the fluorescence meter. The change in fluorescence was recorded before and after the addition of the test agents. The $[Na^+]_i$ was expressed as a ratio of the fluorescence at an excitation wavelength of 340 nm to that of 380 nm. Data are from a representative sample of four experiments.

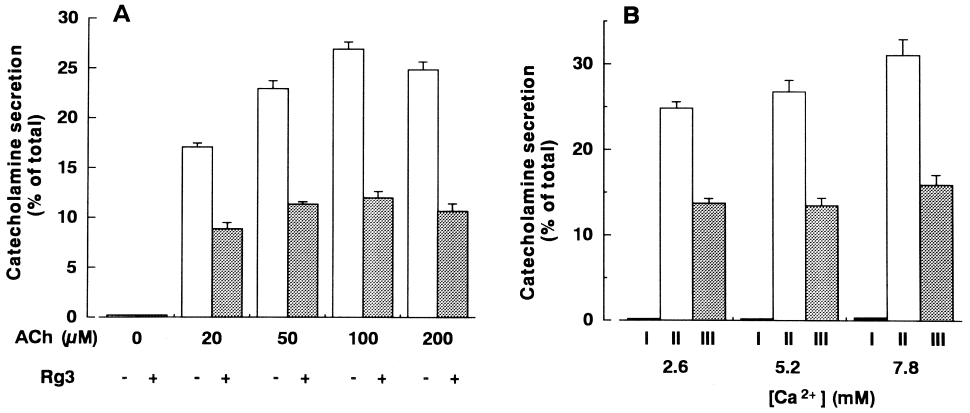

Fig. 5. Effects of external ACh and Ca^{2+} concentrations on the ginsenoside-Rg_3 inhibition of catecholamine secretion. The cultured cells were washed twice with prewarmed KRH buffer and preincubated with or without 30 μM ginsenoside-Rg_3 for 10 min at 37°. (A) The cells were then incubated with different concentrations of ACh (0–200 μM) in the presence or absence of 30 μM ginsenoside-Rg_3 (Rg_3) for 7 min. (B) The cells were then incubated with (II and III) or without 50 μM ACh (I) in the presence (III) or absence of 30 μM ginsenoside-Rg_3 (I and II) in the KRH buffer containing different concentrations of Ca^{2+} (2.6 to 7.8 mM) for 7 min. Catecholamines secreted from the cells into the medium were determined as described in "Materials and methods." The secretion was expressed as a percentage of total cellular catecholamines. Values are means ± SD from at least four experiments.

6B, column V). Although further cell washing (another four times) was added after the preincubation, it did not alter the effect of the inhibition. These results indicate that the inhibitory effect of ginsenoside-Rg_3 at the lower concentration is reversible.

3.5. Effects of ginsenoside-Rg_2 and -Rg_3 on fluorescence anisotropy

DPH, a fluorescent dye, penetrates through the plasma membrane and localizes to the hydrophobic core of the membrane. The probe is virtually nonfluorescent in water. Therefore, the fluorescence anisotropy perhaps mainly reflects the fluidity of the middle of the lipid bilayer in the membrane [16]. Ginsenoside-Rg_3 at 10 μM did not alter the fluorescence anisotropy, whereas at 30 μM it greatly augmented the anisotropy (Table 1). On the other hand, neither 10 nor 30 μM ginsenoside-Rg_2 changed it (Table 1). The effect of ginsenoside-Rg_3 concentrations over 30 μM on membrane fluidity could not be observed because these concentrations affected the fluorescence intensity.

3.6. Effects of preincubation on the ginsenoside-Rg_2 and -Rg_3 inhibition of catecholamine secretion

The preincubation time of the cells (0–10 min) with ginsenoside-Rg_2 or -Rg_3 was examined. As shown in Fig. 7, when 30 μM ginsenoside-Rg_3 was added together with 50 μM ACh to the cells (0-min preincubation), catecholamine secretion was inhibited by 21%. Preincubation with ginsenoside-Rg_3 for an additional 2 min enhanced the inhibition (40%) and with a 5-min preincubation, inhibition reached a plateau (45%). Thus, the effect of ginsenoside-Rg_3 was dependent upon preincubation time. On the other hand, the inhibitory effects of 10 μM ginsenoside-Rg_3 and -Rg_2 were little affected by the preincubation times (0–10 min).

4. Discussion

4.1. Inhibition of catecholamine secretion by ginsenoside-Rg_3

Ginsenoside-Rg_3, a protopanaxadiol saponin (Fig. 1), produced an exceptionally strong reduction in catecholamine secretion from bovine adrenal chromaffin cells stimulated by ACh [6]. Its inhibitory effect was comparable to that of ginsenoside-Rg_2, a protopanaxatriol saponin (Fig. 1), which showed the strongest inhibition of secretion of the ginseng saponins [5]. In this study, ginsenoside-Rg_3 inhibited both ACh-induced Ca^{2+} and Na^+ influxes in a concentration-dependent manner similar to that observed with the ACh-evoked secretion of catecholamines (Figs. 2 and 4). However, it had no or only a slight effect on the catecholamine secretion and Ca^{2+} influx induced by high K^+ concentration or veratridine (Fig. 2), an activator of the voltage-sensitive Ca^{2+} or Na^+ channels [14,15]. These results strongly suggest that ginsenoside-Rg_3 acts on the nicotinic ACh receptor-operated cation channels but not on the voltage-sensitive Ca^{2+} or Na^+ channels. Furthermore, the ginsenoside-Rg_3 inhibition was not overcome by increasing the external ACh and Ca^{2+} concentrations (Fig. 5, A and B, respectively), indicating that the inhibitory effect of ginsenoside-Rg_3 is distinct from that of the competitive antagonists of the nicotinic ACh receptors, such as trimethaphan [17,18], and that of blockers of the L-type voltage-sensitive Ca^{2+} channels, which are competitive with external Ca^{2+} concentrations, such as diltiazem [19]. In fact, the mode of the ginsenoside-Rg_3 antagonism was non-competitive with nicotine (Fig. 3). Taken together, therefore, it is highly probable that ginsenoside-Rg_3 as well as ginsenoside-Rg_2 reduces the ACh-evoked secretion of catecholamines by blocking the Na^+ influx into the cells through the nicotinic ACh receptor-operated cation chan-

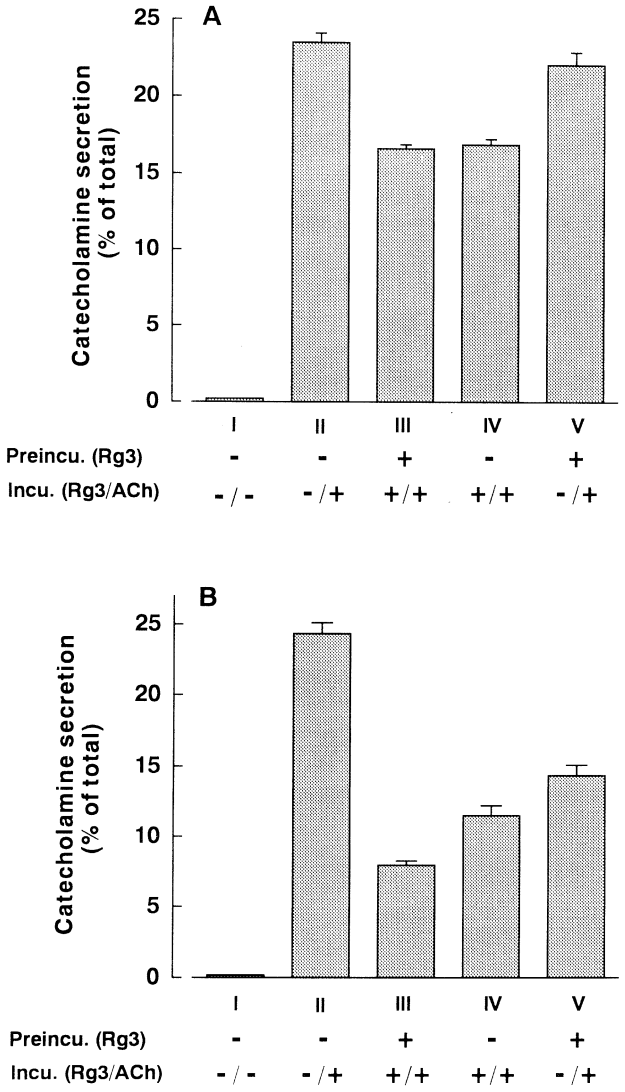

Fig. 6. Reversibility of the ginsenoside-Rg_3 inhibition of catecholamine secretion. The cultured cells were preincubated with (III and V) or without 10 μM (A) or 30 μM (B) ginsenoside-Rg_3 (I, II, and IV) for 10 min at 37° (Preincu.). The cells were washed three times with KRH buffer and then incubated with (III and IV) or without 10 μM (A) or 30 μM (B) ginsenoside-Rg_3 (I, II, and V) in the presence (II-V) or absence of ACh (I) for 7 min (Incu.). The amount of catecholamines secreted from the cells into the medium was determined as described in "Materials and methods." Values are means ± SD from four experiments.

nels. Further study of the action of ginsenoside-Rg_3 on the receptor-operated cation channels in the chromaffin cells is now in progress, using oocytes expressing nicotinic ACh receptors.

4.2. Actions of ginsenoside-Rg_3 and -Rg_2 on chromaffin cell membranes

To compare the properties of ginsenoside-Rg_3 and -Rg_2 inhibition, we examined the reversibility of the inhibitory effect of ginsenoside-Rg_3 on the ACh-evoked secretion of catecholamines (Fig. 6). Our previous report demonstrated

Table 1
Effects of ginsenoside-Rg_3 and -Rg_2 on fluorescence anisotropy of DPH in adrenal chromaffin cells

Concn (μM)	Fluorescence anisotropy	
	G-Rg_3	G-Rg_2
0	0.153 ± 0.002	0.153 ± 0.007
10	0.151 ± 0.001	0.150 ± 0.001
30	0.170 ± 0.010*	0.149 ± 0.002

Adrenal chromaffin cells were incubated with 0.5 μM DPH in a spectrofluorometer cuvette at 37° for 2 min, and then different concentrations of ginsenoside (G)-Rg_3 or -Rg_2 were added. After further incubation for 3 min, fluorescence anisotropy was measured as described in "Materials and methods." Values are the means ± SD from four experiments.

* $P < 0.01$, compared with the control.

that the inhibitory effect of ginsenoside-Rg_2 (100 μM) on ACh-evoked secretion of catecholamines from chromaffin cells, which were preincubated with this saponin and then incubated without it, was no longer observed, indicating that the ginsenoside-Rg_2 inhibition is reversible [5]. On the other hand, although the inhibitory effect of ginsenoside-Rg_3 at a low concentration (10 μM) also was completely reversed (Fig. 6A), at a higher concentration (30 μM) inhibition was partially maintained in the incubation medium, even in the absence of the saponin (Fig. 6B). Furthermore, the effect of ginsenoside-Rg_3 at 30 μM on the ACh-evoked secretion of catecholamines was dependent upon the preincubation time but that of ginsenoside-Rg_3 or -Rg_2 at 10 μM was independent of it (Fig. 7). Thus, ginsenoside-Rg_3 has another action besides directly acting on nicotinic ACh receptors. Ginsenoside-Rg_3 at 30 μM significantly augmented the fluorescence anisotropy of DPH in the chromaffin cells (Table 1), suggesting that this saponin increases membrane microviscosity. However, ginsenoside-Rg_3 at 10 μM and ginsenoside-Rg_2 at 10 and 30 μM had no such effect (Table 1). It is not clear why only ginsenoside-Rg_3 has the membrane-modifying action. The fact that ginsenoside-Rg_3 is eluted into a more highly lipophilic fraction than ginsenoside-Rg_2, as shown by silica gel chromatography [20], may be the reason for ginsenoside-Rg_3 showing such an effect on the membrane and perhaps also accounts for the finding that the inhibitory effect of ginsenoside-Rg_3 at 30 μM on the secretion was not fully reversible and was dependent upon the preincubation time. Therefore, ginsenoside-Rg_3 at the higher concentration probably inhibits Na^+ influx not only directly by blocking the nicotinic ACh receptor-operated cation channels but also indirectly by reducing plasma membrane fluidity.

We have observed that ginsenoside-Rg_3 suppresses the responses induced by various receptor stimuli as well as by ACh [7]. This saponin significantly inhibited histamine-, angiotensin II-, bradykinin-, neurotensin-, and γ-aminobutyric acid-induced secretions of catecholamines from bovine adrenal chromaffin cells and muscarine- and histamine-induced contractions of the ileum in the guinea pig [7]. Accordingly, the membrane-modifying action of ginsen-

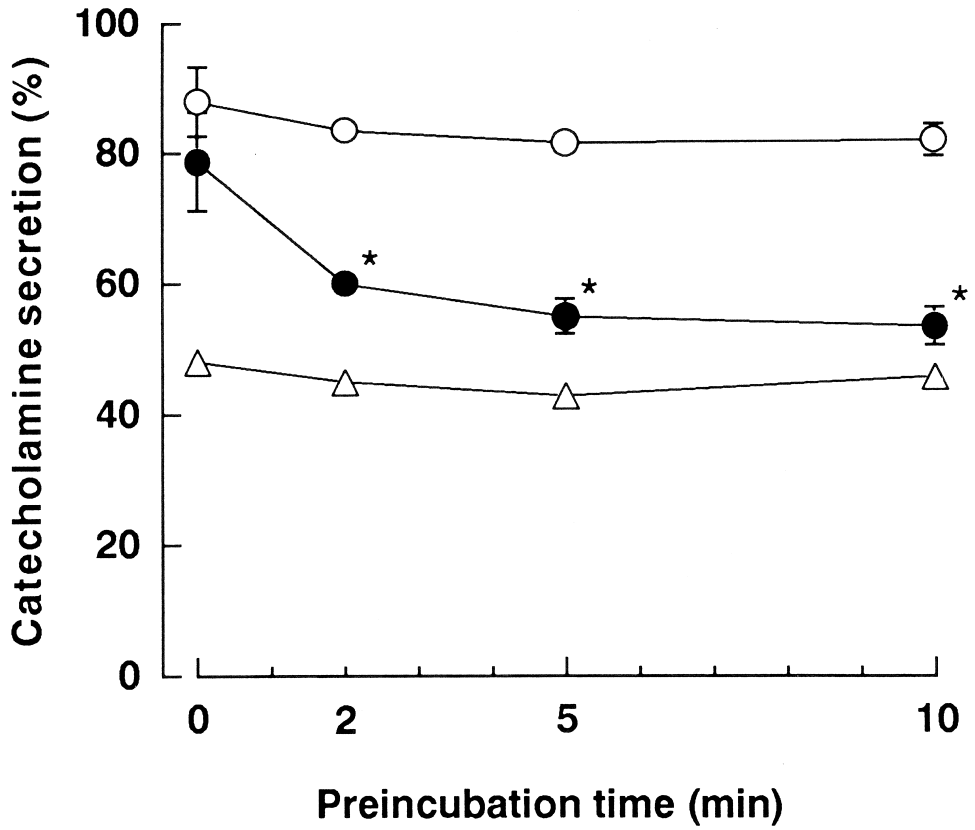

Fig. 7. Effects of preincubation time on the ginsenoside-Rg_2 and -Rg_3 inhibition of catecholamine secretion. The cultured cells were preincubated with or without 10 μM ginsenoside-Rg_2 (Δ), 10 μM ginsenoside-Rg_3 (❍), or 30 μM (●) ginsenoside-Rg_3 for 0–10 min at 37°. The cells were then incubated with or without 50 μM ACh in the presence or absence of 10 μM ginsenoside-Rg_2, or 10 or 30 μM ginsenoside-Rg_3 for 7 min. Catecholamines secreted from the cells into the medium were determined as described in "Materials and methods." The values of the basal secretion were subtracted from the data, and the ACh-evoked secretion of catecholamines was assigned the value of 100%. The ACh-evoked and the basal secretions were 23.4 ± 0.9 and 0.4 ± 0.1% of total cellular catecholamines, respectively. Values are means ± SD from at least four experiments. Key: (*) $P < 0.05$, compared with the 0-min preincubation.

oside-Rg_3 probably also contributes, at least in part, to their inhibition. In fact, there are several reports showing that alterations in membrane microviscosity may modulate the receptor–ligand interaction. It has been presumed that the increase in the membrane viscosity of rat cerebral cortex or pig pulmonary endothelial cells is related to the loss of an α_1-adrenergic or insulin receptor [21,22]. In addition, increased membrane viscosity in the rat frontal cortex has been reported to produce decreases in the affinity and binding sites of the muscarinic ACh receptor [23]. Thus, the reduction in fluidity of the plasma membranes leads to the negative alterations in receptor kinetics.

In conclusion, the protopanaxadiol saponin ginsenoside-Rg_3 inhibited the ACh-evoked secretion of catecholamines in bovine adrenal chromaffin cells due to suppression of Na^+ influx into the cells through nicotinic ACh receptor-operated cation channels. It is probable that the inhibition by this saponin of Na^+ influx is attributable to both direct modulation of the receptor-operated cation channels and indirect action on the channels via the alteration in the membrane fluidity.

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