Phytoestrogen Cimicifugoside-Mediated Inhibition of Catecholamine Secretion by Blocking Nicotinic Acetylcholine Receptor in Bovine Adrenal Chromaffin Cells

Kyung-Chul Woo, Yong-Soo Park, Dong-Jae Jun, Jeong-Ok Lim, Woon-Yi Baek, Byung-Sun Suh, and Kyong-Tai Kim

Department of Life Science (K.C.W., Y.S.P., D.J.J., K.T.K.), Division of Molecular and Life Science, Pohang University of Science and Technology, Pohang, South Korea; Department of Life Science and Food Engineering (K.C.W., B.S.S.), Handong Global University, Pohang, South Korea; Medical Research Institute (J.O.L.), School of Medicine, Kyungpook National University, Daegu, South Korea; Department of Anesthesiology (W.Y.B.), School of Medicine, Kyungpook National University, Daegu, South Korea.
Inhibition of catecholamine secretion by cimicifugoside

Corresponding author:

Dr. Kyong-Tai Kim,

Department of Life Science, POSTECH, San 31, Hyoja Dong, Pohang, 790-784, Korea.

Tel: 82-54-279-2297

Fax: 82-54-279-2199

mail to: ktk@postech.ac.kr

This manuscript is including 40 printed text pages, 10 figures, and 40 references.

The number of words

Abstract ................................................................. 158 words

Introduction .............................................................. 451 words

Discussion .............................................................. 1082 words

Abbreviations

nAChR: Nicotinic acetylcholine receptor; VSSC: Voltage sensitive sodium channels;
VSCC: Voltage-sensitive calcium channels; CF: Cimicifugoside; MMA: Mecamylamine; BR: Bradykinin receptors

Neuropharmacology
Abstract

We investigated the effect of phytoestrogen cimicifugoside (CF), one of the pharmacologically active ingredients of the medicinal plant *Cimicifuga Racemosa* (Black cohosh) which has been used to treat many kinds of neuronal and menopausal symptoms, such as arthritis, menopausal depression, and nerve pain. Cimicifugoside inhibited calcium increase induced by 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), a nAChR agonist in bovine adrenal chromaffin cells with a half maximal inhibitory concentration (IC$_{50}$) of 18 ± 2µM. In contrast, cimicifugoside did not affect the calcium increases evoked by high K$^+$, veratridine, and bradykinin. The DMPP-induced sodium increase was also inhibited by cimicifugoside with IC$_{50}$ of 2 ± 0.3µM, suggesting that the activity of nAChRs is inhibited by cimicifugoside. Cimicifugoside did not affect the KCl-induced secretion but markedly inhibited the DMPP-induced catecholamine secretion which was monitored by carbon-fiber amperometry in real time, and by high performance liquid chromatography (HPLC) through electrochemical detection. The results suggest that cimicifugoside selectively inhibits nAChR-mediated response in bovine chromaffin cells.
Estrogen plays an important role in differentiation, proliferation, homeostasis, and female reproductive functions. The long term genomic effects of estrogen are known to be mediated by the binding of estrogen to intracellular receptors. The activated estrogen-receptor complex then acts as a transcription factor that induces the transcription of target gene and protein synthesis. In addition to the known genomic activities, the action of steroids on neuronal functions has been reported to consist of two distinct genomic and nongenomic mechanisms (McEwen, 1991). In several reports, nongenomic effects of steroid hormone were investigated, for example, on L-type voltage-sensitive calcium channels (VSCCs) (Nakajima et al., 1995; Ruehlmann et al., 1998; Yamamoto, 1995; Kim et al., 2000b), purinergic receptors (P2X7) (Cario et al., 1998), nicotinic acetylcholine receptors (nAChRs) (Uki et al., 1999), and Maxi-K+ channels (Valverde et al., 1999). We also have shown that 17β-estradiol inhibited L-and N-type voltage sensitive calcium channels and P2X2 receptors in PC12 cells (Kim et al., 2000a). The nongenomic effects are characterized by a rapid time course (<10min) and a high insensitivity of signals to inhibitors of gene expression (Dar and Zinder, 1997; Wehling, 1997; Nemere and Farach-Carson, 1998). Although the effects of estrogen on various cell types have been extensively studied, relatively little attention has been paid to effects of phytoestrogen in neuroendocrine cells.

Bovine adrenal chromaffin cells are neuroendocrine cells that have been widely
used as a model system for the study of catecholamine secretion (Kilpatrik et al., 1982; 1980). When acetylcholine secreted from splanchnic nerve terminal binds to acetylcholine receptors on adrenal chromaffin cell, influx of extracellular cation such as sodium and calcium through AChRs occurred and cell membrane is depolarized. Then, the depolarization activates voltage-sensitive calcium channels that increase intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]). Finally, increased intracellular Ca$^{2+}$ triggers exocytotic machineries to evoke catecholamine secretion. Catecholamines such as dopamine, norepinephrine, and epinephrine are synthesized in the brain, chromaffin cells, sympathetic nerves, and sympathetic ganglia and play important roles in stress and emotional behavior (Cooper et al., 1991). Many psychotropic drugs are known to act in catecholamine-containing neurons (Seeman and Van Tol, 1994). Therefore, compounds that modulate catecholamine secretion may be used as potential therapeutic drugs for the treatment of affective disorders.

Since cimicifugoside, a triterpene glycoside whose biological function has not been studied well, is the main ingredient of *Cimicifuga Racemosa* (common name: Black Cohosh), we investigated the effect of cimicifugoside in the catecholamine secretion from bovine adrenal chromaffin cells. Due to its steroid backbone structure, cimicifugoside has been called phytoestrogen on some occasions (Fig. 1).

We found that cimicifugoside, a member of phytoestrogen, specifically and
nongenomically inhibits nAChR-mediated effects evoked by DMPP, thereby leading to the inhibition of nAChR-mediated sodium, calcium increase and catecholamine secretion.
Materials and methods

Materials

Cimicifugoside was purchased from Chromadex Inc. (Laguna Hills, CA, USA). Fura-2/AM, SBFI/AM, and Pluronic F-127 were purchased from Molecular Probe Inc. (Eugene, OR, USA) \[^{3}H\] nicotine was purchased from NEN Life Science Products (Boston, MA, USA). UB 165 and 5-lodo-A-85380 were purchased from Tocris Inc. (Ellisville, MO, USA) DMPP, bradykinin, veratridine, mecamylamine and other reagents were purchased from Sigma (St. Louis, MO, USA) and Merck (Gibbstown, NJ, USA).

Preparation of chromaffin cells

Chromaffin cells were isolated from bovine adrenal medulla by two-step collagenase digestion as previously described (Kilpatrick et al., 1980). For the measurement of catecholamine secretion and the \[^{3}H\] nicotine binding assay, cells were plated in 24-well plates at a density of $5 \times 10^5$ cells/well. Chromaffin cells transferred to 100mm culture dishes ($1 \times 10^7$ cells per dish) were used to measure cytosolic free calcium and sodium concentrations. The cells were maintained in Dulbecco’s modified Eagles Medium/F-12 (Life Technologies Inc., Grand Island, NY,
USA) containing 10% bovine calf serum (Hyclone, Logan, UT, USA) and 1% antibiotics (Life Technologies Inc.). Chromaffin cells were incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C for 3-5 days before use.

**Measurement of catecholamine secretion by HPLC**

Catecholamine secretion from chromaffin cells was measured in 24-well plates following the method reported previously (Eaton et al., 2000; Cheng et al., 1992; 1993). In brief, cells were rinsed two times with Ca²⁺-containing Locke’s solution containing: 157.4mM NaCl, 5.6mM KCl, 2.2mM CaCl₂, 1.2mM MgCl₂, 5.6mM D-glucose, 5mM HEPES, 3.6mM NaHCO₃, pH 7.4 titrated by NaOH and were incubated at 37°C for 5min in each case. The cells were subsequently stimulated with the drugs under test. After the incubation, the medium was removed from each well and transferred to a test tube containing (10% v/v) 0.1N HCl. A 20µl aliquot of each 500µl sample was injected onto the HPLC (BAS-480, BioAnalytical System Inc. IN, USA) C18 column (150 × 1mm) with electrochemical detection. The potential used was +770 mV versus Ag/AgCl, with a classic 3mm glassy carbon electrode. The ranges of sensitivity for the electrode were 100nA and 50nA with a flow rate of 1ml/min. The 2L of mobile phase included: 0.55g heptanesulfonic acid, 0.2g EDTA, 80ml acetonitrile, 12ml 85% phosphoric acid, 16ml triethylamine with the pH adjusted to 2.5 with H₃PO₄.
and filtered with 0.45micron membrane filter. Stock catecholamine (NE, EP, DA) solutions were used as standards.

**Amperometric measurement of exocytosis**

Recordings were performed at room temperature as described previously (Kim et al., 2000a). Chromaffin cells were buffered with amine-free solution containing: 137.5mM NaCl, 2.5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 10mM D-glucose, 10mM HEPES, pH 7.3 titrated by NaOH. Carbon-fiber electrodes were fabricated from 5 to 11µm carbon fibers (PAN T650 or P25: Amoco Performance Products) and polypropylene 10µl micropipettor tips. A carbon-fiber electrode, back-filled with 3M KCl to connect to the headstage, was attached to a single cell. Measurements were begun after this electrode current fell below 10 pA. The amperometric current, generated by oxidation of catecholamines, was measured using an axopatch 200B amplifier (Axon Instruments Inc., Foster City, CA, USA) and operated in the voltage-clamp mode at a holding potential of +650 mV. Amperometric signals were low-pass filtered at 1 kHz, then sampled 0.5 kHz. For data acquisition and analysis, pCLAMP 8 software (Axon Instruments Inc., Foster City, CA, USA) was used as well as IGOR software (WaveMetrics) especially for visualizing large amounts of numeric data. Solutions were exchanged by a local perfusion system that allows complete
exchange of medium bathing the cells within 2 s.

\([\text{Ca}^{2+}]_i\) measurement and calcium imaging

Cytosolic free calcium concentration \([\text{Ca}^{2+}]_i\) was determined with the help of the fluorescent \(\text{Ca}^{2+}\) indicator fura-2 as reported previously (Park et al., 1998). Briefly, the chromaffin cell suspension was incubated with fresh serum-free DMEM/F12 medium containing fura-2/AM (3\(\mu\)M) for 40 min at 37°C with continuous stirring. The cells were then washed with Locke’s solution containing: 157.4 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 5.6 mM D-glucose, 5 mM HEPES, 3.6 mM NaHCO\(_3\), pH 7.4 titrated by NaOH and left at room temperature until use. Sulfinpyrazone (250\(\mu\)M) was added to all solutions to prevent dye leakage. Fluorescence ratios were measured by an alternative wavelength time scanning method (dual excitation at 340 nm and 380 nm: emission at 500 nm). Calibration of the fluorescent signal in terms of \([\text{Ca}^{2+}]_i\) was performed as described previously (Grynkiewicz et al., 1985), according to the formula

\[
[\text{Ca}^{2+}]_i = \left[\frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)}\right] \times \left(\frac{S_f}{S_b}\right) \times K_D.
\]

R\(_{\text{max}}\) and R\(_{\text{min}}\) are the ratios obtained when fura-2/AM is saturated with \(\text{Ca}^{2+}\) and when EGTA is used to remove \(\text{Ca}^{2+}\), respectively. S\(_f\) and S\(_b\) are the proportionality coefficients of \(\text{Ca}^{2+}\)-free and \(\text{Ca}^{2+}\)-saturated fura-2/AM, respectively. For multiphoton confocal microscopic calcium imaging, chromaffin cells plated on poly-D-lysine-coated cover
slips were pre-loaded with 5 µM Fluo-4/AM dye. After incubation for 30 min at 37 °C, the cells were washed two times with Locke’s solution to remove excess dye and examined under the confocal microscope. Groups of chromaffin cells were selected under the microscope. Measurements of intracellular calcium were performed with the Bio-Rad Radiance 2100 confocal microscope (Bio-Rad, Inc., Maylands, UK) equipped with a 40× objective (0.75 numerical apertures). The calcium-sensitive Fluo-4 dye was excited by the 488nm from an argon laser and the emission fluorescence monitored at 515 / 30nm was selected by a band-pass filter. During fluorescence data collection, each scan of a 512 × 512 pixel image took 0.35 s, and the interval between each image scan was ~2 s. Images were stored and processed with laser pix software (Bio-Rad Inc., Maylands, UK). The regions of interest (ROIs) distributed across the image provided an intensity versus time graphic output.

\([Na^+]_i\) measurement and whole-cell patch clamp

Cytosolic free Na\(^+\) concentration ([Na\(^+\)]\(_i\)) was measured using the fluorescent Na\(^+\) indicator SBFI as previously described in the report (Park et al., 1999a). In brief, the chromaffin cell suspension was incubated in fresh DMEM/F-12 medium containing 15µM SBFI/AM, 10% bovine calf serum, and 0.2% Pluronic F-127 for 2h at 37°C with continuous stirring. The cells were then washed twice with fresh DMEM/F-12 medium.
and left at room temperature until use. Sulfinpyrazone (250µM) was added to all solutions to prevent dye leakage. Fluorescence ratios were measured by an alternative dual excitation at 340nm and 380nm, and emission at 530nm. Changes in [Na⁺]ᵢ are presented as fluorescence ratios. Whole-cell patch-clamp recordings were performed to measure inward sodium current through nAChRs with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and Digidata 1200 interface. Isolated chromaffin cells were plated on poly-D-lysine-coated glass chip in 35mm culture dish and cultured for 2-3 days at 37°C under a 5% CO₂-containing atmosphere. The pipettes were fire-polished and had a typical resistance of 5~6 MΩ. The bath solution contained 137.5mM NaCl, 2.5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 10mM glucose, and 10mM HEPES titrated to pH 7.3 with NaOH. The intracellular solution contained 140mM CsCl, 3mM EGTA, 1mM MgCl₂, and 10mM HEPES titrated to pH 7.3 with CsOH. Currents were filtered at 1 kHz, and then sampled at 5 kHz. Step pulses were applied from 0 mV to -120 mV for 360 ms with an interpulse interval of 1 s and voltage ramp was performed from -120 mV to +50 mV for 250 ms to show voltage-current relationship of nAChRs. For data acquisition and analysis, pCLAMP 8 software (Axon instruments, Foster City, CA) was used. Solutions were exchanged by a local perfusion system that allows complete exchange of medium bathing the cells within 2 s.
[^3]H nicotine binding analysis

Binding of[^3]H nicotine to intact cells was measured as previously described (Kilpatrik et al., 1982). Intact chromaffin cells in 24-well plate (5 \times 10^5 cells/well) were washed twice with Locke’s solution. They were incubated with 40nM[^3]H nicotine and various concentrations of cimicifugoside for 40 min at 25°C. Then the cells were washed once with 2ml Ca\(^{2+}\)-free Locke’s solution containing 100\(\mu\)M EGTA. Finally, the cells were lysed and scraped in 0.5ml of ice-cold 5% trichloroacetic acid and radioactivity was measured by liquid scintillation counter. Nonspecific binding, determined by coincubation with 1mM nicotine, amounted to less than 20% of total binding, and was routinely subtracted from the total binding. The binding data were analyzed and expressed as percentage of total binding.

Statistical analysis

All quantitative data were expressed as means ± SEM. Half maximal inhibitory concentration (IC\(_{50}\)) values were calculated with the MicroCal Origin for Window program.
Results

Inhibitory effect of cimicifugoside on nAChR-mediated exocytosis

To study the effect of cimicifugoside on DMPP-evoked catecholamine secretion, we treated chromaffin cells with cimicifugoside (from 1 to 100 µM) and the quantification was performed by HPLC (Fig. 2). Cimicifugoside by itself did not induce catecholamine secretion (data not shown). Values of basal catecholamine (norepinephrine) release in the absence of 10 µM DMPP were 3.8 ± 0.4 µM per 5 × 10^5 cells. 10 µM DMPP evoked norepinephrine secretion up to 27.9 ± 0.1 µM. In case of preincubation with cimicifugoside for 10 min, the induction values were decreased with concentration dependent manner (Fig 2A), but not in case of 60 mM KCl-induced secretion (Fig 2B). In addition, we could observe the same inhibition phenomenon in 5 min preincubation (data not shown). In order to better understand how cimicifugoside inhibits secretory response evoked by nicotinic stimulation, catecholamines secreted from single bovine chromaffin cell were measured using the amperometric method (Venton et al., 2002; Michael and Wightman, 1999). When a brief pulse (20 s) of 10 µM DMPP was applied to a single chromaffin cell, a fast and transient increase in current occurred (Fig. 3A). Addition of 60 µM cimicifugoside for 5 min before and during the DMPP pulse reduced the catecholamine secretion to 32 ±
5% (Fig. 3A and C). The cimicifugoside-induced inhibitory effect was partially reversed after washout of cimicifugoside (Fig. 3A). In contrast, cimicifugoside did not inhibit 60mM KCl-induced catecholamine secretion (Fig. 3B and D).

Inhibitory effect of cimicifugoside on nAChR-mediated calcium increase

Since the increase in $[\text{Ca}^{2+}]_i$ is an essential step in the catecholamine secretion process, we investigated the effect of cimicifugoside on the $[\text{Ca}^{2+}]_i$ increase. Cimicifugoside (up to 300μM) by itself had no effect on $[\text{Ca}^{2+}]_i$ (data not shown), whereas 10μM DMPP induced a prominent rise in $[\text{Ca}^{2+}]_i$ (trace a in Fig. 4A) and the calcium increase was inhibited by cimicifugoside (trace b and c in Fig. 4A) in a concentration-dependent manner with a half-maximal inhibitory concentration (IC$_{50}$) of 18 ± 2μM (Fig. 4B). We have observed the inhibitory effect of cimicifugoside on calcium increase evoked by nicotine with similar potency (Fig. 10B). Incubation of cells with 300μM cimicifugoside resulted in the complete inhibition of DMPP-induced calcium increase. In order to assess the mechanism of cimicifugoside action we investigated the effects of different concentration of DMPP in the presence of 20μM cimicifugoside (Fig. 4C). We could observe half maximal inhibitory effect of cimicifugoside in each concentration of DMPP. We then examined the time course of the cimicifugoside effect on the 10μM DMPP-induced $[\text{Ca}^{2+}]_i$ elevation (Fig. 4D).
When the cells were treated with 60µM cimicifugoside and 10µM DMPP simultaneously, there was no inhibition on [Ca^{2+}]_i rise. In addition, the result shows that the inhibition effect of cimicifugoside was influenced by the preincubation time and that at least 3 min of incubation appears to be necessary to exert the maximal cimicifugoside effect. We also monitored calcium increase in single chromaffin cell using multiphoton confocal microscopic calcium imaging (Fig. 5). In the confocal image of when the cells were treated with 60µM cimicifugoside for 5min, in the presence of 10µM DMPP the fluorescence intensity of the confocal image was remarkably reduced when 10µM DMPP was applied (Fig. 5A). In contrast, cimicifugoside did not affect KCl-evoked calcium entry (Fig. 5B).

**Receptor specificity of cimicifugoside**

The specificity of cimicifugoside-induced inhibition was examined by testing the effects of cimicifugoside on voltage-sensitive calcium channels (VSCCs), voltage-sensitive sodium channels (VSSCs), and phospholipase C (PLC)-linked bradykinin receptor (B2R) signaling. As shown in Fig. 6, calcium increase induced by 50mM KCl was not inhibited by pretreatment with 20µM cimicifugoside, suggesting that voltage-sensitive calcium channels were not affected by cimicifugoside. Veratridine is a plant alkaloid that opens voltage-sensitive sodium channels by binding to the
pharmacological site 2 on the sodium channels and slowing its inactivation (Catterall, 1980). In bovine chromaffin cells, veratridine-induced activation of sodium channels was known to cause membrane depolarization (Friedman et al., 1985; Kitayama et al., 1990) thereby leading to slow and weak calcium increase through voltage-sensitive calcium channels (Heldman et al., 1996). The calcium increase by veratridine was not affected by cimicifugoside, either. Bradykinin is known to activate PLC-linked B2 bradykinin receptors in bovine chromaffin cells (McMillian et al., 1992; Park et al., 1999b). A half maximal inhibitory concentration of cimicifugoside in the DMPP-induced \([\text{Ca}^{2+}]_i\) elevation had no effect on the bradykinin-evoked calcium increase. The data together suggested that cimicifugoside have no significant inhibitory effect on calcium channels, sodium channels, and PLC-linked receptors. Therefore, it seems that the effect of cimicifugoside on nAChR is highly specific.

**Inhibitory effect of cimicifugoside on the sodium influx through the nAChR**

Since both calcium channels and nAChRs were activated by nicotinic stimulation, inhibition of DMPP-induced calcium increase can result from the inhibition of nAChRs or calcium channels. In order to verify whether nAChRs are inhibited by cimicifugoside, we investigated the effect of cimicifugoside on DMPP-induced sodium increase that occurs only through nAChRs. As shown in Fig. 7, DMPP induced an
increase in cytosolic sodium. Cimicifugoside inhibited the DMPP-induced sodium increase in a concentration-dependent manner with IC$_{50}$ of $2 \pm 0.3$ µM (Fig. 7B), and 60µM cimicifugoside completely inhibited the DMPP effect (trace ‘b’ in Fig. 7A). The inhibitory effect of cimicifugoside on the sodium current through nAChRs was also measured by whole-cell patch clamp recording (Fig. 7C,D,and E). The sodium currents induced by 10µM DMPP were remarkably reduced when the cells were costimulated with 3µM or 60µM cimicifugoside. The inhibition concentration of sodium influx by cimicifugoside was similar to those by 17β-estradiol, but less effective than by prototype antagonist mecamylamine (data not shown). The results suggested that the inhibition of DMPP-induced sodium increase by cimicifugoside result from the direct inhibition of nAChRs.

Nicotine binding

Since nicotine works on nAChR as a ligand we tested whether the binding of [$^3$H]nicotine to nAChRs was inhibited by cimicifugoside. We previously demonstrated clozapine showed concentration dependent competition with [$^3$H]nicotine in the same condition (Park et al., 2001). As shown in Fig. 8, cimicifugoside did not significantly compete for binding with [$^3$H]nicotine, suggesting that its binding site is distinct from that of agonist including nicotine and acetylcholine.
Comparison of inhibitory effects among cimicifugoside and other antagonists

It has been reported that local anesthetics such as lidocaine, procaine, QX-222 inhibit the function of nAChRs in a noncompetitive manner (Gentry and Lukas, 2001). Therefore, we compared the inhibitory potency of cimicifugoside with that of lidocaine and prototype antagonist mecamylamine under experimental conditions. As shown in Fig. 9, all of them inhibited DMPP-induced calcium increase in which the most potent agent was mecamylamine and lidocaine was the less potent. We determined whether cimicifugoside had subtype specificity. Intracellular calcium was evoked by 5-iodo-A-85380 and UB165 which are selective agonists to α4β2 and α3β2, respectively (Fig. 10A). Due to UB165 had more potent effect on calcium increase (EC50: 40nM) than A-85380 (EC50: 90nM), presumably α3β2 type nAChRs may be more expressed than α4β2 types in bovine adrenal chromaffin cell. Maximum calcium induction by selective agonists we tested, as well as DMPP and nicotine, were inhibited by cimicifugoside with similar potency (Fig. 10B). The results suggested that CF is broad spectrum nAChR inhibitor.
Discussion

*Cimicifuga racemosa* was historically used by the American indians to assist and ease the pain of childbirth, and also as an antidote against snake bites. It is an antispasmodic herb that can relieve cramps in the pelvic area while increasing blood supply to this area. It is used to treat for painful periods with cramps and any inflammatory condition in the pelvic area associated with spasm, tension or uterine discharge. It is also used in the treatment of nerve pain, headaches, arthritic pain and inflammation (British Herbal Pharmacopoeia, 1983). Recently the extract of *C. Racemosa* containing phytoestrogen has been used to treat menopausal women. Phytoestrogens such as isoflavone extracted from soybean has been mostly highlighted as an agent for hormone replacement therapy (HRT) of estrogen deficiency in menopausal women. Most of women are experiencing menopausal symptoms caused by estrogen deficiency which results in hyperactivity of neuronal conduction such as hot flushes, the most prominent neurovegetative symptom (Ginsburg et al., 1981). In recent study, it was reported that ethanol extract CR BNO 1055 of *C. Racemosa* was more effective in release of menopausal symptom than another phytoestrogen-containing candidates (Wuttke et al., 2003; Jarry et al., 2003). Although cimicifugoside is an important phytoestrogen ingredient of *C. Racemosa,*
little study has been conducted so far to elucidate its action mechanism.

Our data clearly suggest that catecholamine secretion induced by nicotinic acetylcholine receptor-mediated stimulation is specifically inhibited by cimicifugoside and that these inhibitory effects are caused by the inhibition of sodium influx through ionotropic receptor, nAChRs. The reason why the IC$_{50}$ of cimicifugoside on sodium increase (2µM) was considerably lower than that on calcium rise (18µM) may be that electrophysiologically higher concentration of antagonist is needed to suppress the activation of voltage-sensitive calcium channels compare to that needed to suppress sodium current. In other words, the amount of cimicifugoside sufficient to inhibit intracellular sodium increase can be insufficient to inhibit calcium rise. In addition, we observed that cimicifugoside inhibits cytosolic calcium increase a downstream phenomenon of membrane depolarization caused by the activation of nAChRs, and those other direct pathways which could activate voltage-sensitive calcium channels are not significantly influenced by cimicifugoside. Although calcium channels are activated in the activation process of nAChRs, both the inhibition of DMPP-induced sodium increase and the absence of inhibitory effect on high K$^+$-induced calcium increase clearly indicated that nAChRs, but not calcium channels, are the specific target of cimicifugoside. In bovine chromaffin cells, 5-lodo-A-85380 and UB165, which are selective agonists of α4β2 and α3β2, respectively, evoked intracellular
calcium increase. Both 5-lodo-A-85380- and UB165-induced calcium rise was inhibited by cimicifugoside to similar extents, suggesting that cimicifugoside did not have specificity for a certain type of nAChR. Even we used quite high concentration of cimicifugoside (＞50µM) to visualize its inhibitory effect, considering the amount (2.5% triterpene glycosides of total extract) among ingredients of C. racemosa, the concentration may be reached in clinical reality. In addition, the applicable dose for local anesthetics, such as lidocaine can be converted to micromolar range for its effective working.

In contrast to rapid onset inhibitors such as borneol (Park et al., 2003), cimicifugoside exhibits a rather slow onset, requiring at least 3 min of preincubation, suggesting that cimicifugoside does not interact directly with extracellular domain of nAChRs. According to the inhibition of calcium induction by selective agonist, it seems to lack receptor subtype selectivity, but functions as a broad spectrum inhibitory modulator. Furthermore, lack of inhibition by cimicifugoside in [³H]nicotine binding to nAChRs indicate that cimicifugoside acts as a noncompetitive inhibitor of nAChR. A variety of pharmacological agents are known to modulate the transitions between conformations by directly binding to several sites on nAChRs that are topographically distinct from the acetylcholine binding sites (Lena and Changeux, 1993). Many physiological or pharmacological effectors modify the properties of
nAChRs even though they do not significantly affect the binding of agonist such as acetylcholine and nicotine. These molecules, known as noncompetitive blockers, inhibit the ion channel gating activity of the nAChRs through mechanisms that differ from those of the competitive blockers (Lowenick et al., 2001). Our data suggest that cimicifugoside is likely to act as a noncompetitive blocker of nAChRs. In chromaffin cell it has been reported previously that a synthetic glucocorticoid, dexamethasone, binds to the specific site located on the outer cell membrane, and suppresses the $I_{Ach}$ level in a noncompetitive manner, and that steroids may change the lipid environment of nAChRs, thus leading to the suppression of $I_{Ach}$. In other words, it is possible that the structure of steroids, which is changed by the cleavage of cholesterol side chain, thus contributes to the direct inhibition of nAChRs by steroids (Uki et al., 1999; Inoue and Kuriyama, 1989). On the same principle, cimicifugoside seems to act like other steroids because it has a similar inhibitory potency of 17β-estradiol against intracellular sodium increase induced by DMPP in our condition (data not shown).

The mechanism of action of steroid hormones can be classified as genomic and nongenomic, depending on whether the hormone binds to intracellular receptors and causes transcriptional activation. Nongenomic effects do not conclude gene expression but result in modulation of cellular event such as exocytosis (Machado et al., 2002). Whereas at least 30min are required for the genomic response to estrogen
to occur, the inhibition of the stimulant-induced $[\text{Ca}^{2+}]_i$ rise following cimicifugoside treatment occurs within 5 min. In consideration of effective incubation time, the action of cimicifugoside to nAChRs could be the nongenomic action of phytoestrogen. In several reports, nongenomic effects of estrogen were investigated, for example, on voltage-sensitive calcium channels (VSCCs), purinergic receptors, ionotropic receptors, and Maxi-K$^+$ channels, but in case of phytoestrogen, little attention has been paid to their role as bioactive materials in cellular signal transduction system.

The specific inhibition on nAChR-mediated effects by cimicifugoside may contribute to the understanding of the basic mechanism of phytoestrogen effects as medicine, and may be classified as the general action of steroid on membrane receptors reported previously. Furthermore, its specific action implicates cimicifugoside as a potential candidate for therapeutic agents. Clinical agents such as local anesthetics, products that alleviate symptoms of nicotine withdrawal or agent that release hyperactivation of sympathetic nerves were shown to inhibit functions of nAChRs, although the correlation between the inhibitory effect and its clinical function is not yet clear. In addition, the relationship between nAChR inhibition of cimicifugoside and the alleviation of climacteric/menopausal symptoms is still unclear. The determination of cimicifugoside’s inhibition mechanism on nAChR and its potential clinical application are subjects of interest for further study and
experimentation.
Acknowledgements

We thank Mr. Byung-Soon Kang in Kyung-Buk Packers Company Inc. (Pohang, South Korea) for kindly providing the bovine adrenal gland. We are also grateful to Ms. Yeoul Kang for critical editing the manuscript.
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Footnotes

This work was supported by the IMT-2000 Program of the Korea Ministry of Information and Communication and the Korea Ministry of Health and Welfare. This work was also supported by the Brain Korea 21 Program of the Korea Ministry of Education.

Request for offprints should be addressed: Dr. Kyong-Tai Kim, Department of Life Science, POSTECH, San 31, Hyoja Dong, Pohang, 790-784, Korea.

Tel: 82-54-279-2297, Fax: 82-54-279-2199, E-mail: ktk@postech.ac.kr
Legends for figures

Figure 1 Structure of cimicifugoside (CF)

Figure 2 Inhibitory effect of cimicifugoside on catecholamine secretion in bovine chromaffin cells. Bovine chromaffin cells were treated with 10µM DMPP(A) or 60mM KCl(B) in the presence of the various concentration of cimicifugoside (CF, filled boxes) for 10min. Secretion of catecholamines (NE) induced by DMPP in the absence of cimicifugoside was presented (open box). Open triangles represented no stimulation by agonists. The secreted catecholamines were measured as described Section 2. The experiments were performed three times independently, and the results were reproducible. Data were the means ± SEM (n=3) values.

Figure 3 Inhibitory effect on catecholamine secretion in single bovine chromaffin cell. (A, B) Chromaffin cells were stimulated with 10µM DMPP, or 60mM KCl for 20 s in the absence or presence of 60µM CF, respectively. (C, D) Total amperometric currents induced by the 20 s DMPP or KCl pulse in (A and B, respectively) were integrated and represented as percentage of the average currents by DMPP or KCl pulses. The experiments were performed three times independently, and the results were reproducible. Data were the means ± SEM (n=8) values.

Figure 4 Inhibitory effect of Cimicifugoside in [Ca^{2+}]_i elevation in bovine chromaffin
cells. (A) Trace of intracellular calcium increase by DMPP. The intracellular [Ca$^{2+}$]$_i$ rise induced by 10µM DMPP was measured in the absence (trace a) or presence (trace b, c) of cimicifugoside. Cells were incubated with CF for 5min before stimulation with DMPP. The experiments were performed five times independently, and the typical Ca$^{2+}$ traces were presented. (B) The calcium increase induced by 10µM DMPP was measured 5min after preincubation with the indicated concentration of cimicifugoside (filled boxes). The peak height of respective stimulation was compared to that of the control calcium increase caused by DMPP alone (open box). Data were the means ± SEM (n=5) values. (C) The calcium increase induced by the indicated concentration of DMPP was measured in the absence (open boxes) or presence (filled boxes) of 30µM cimicifugoside. Data were the means ± SEM (n=4) values. (D) Chromaffin cells were preincubated for the indicated time with 60µM cimicifugoside and then stimulated with 10µM DMPP (filled boxes). Incubation with zero time means that cimicifugoside and DMPP were treated simultaneously. The peak height of each stimulation was compared to that of the control calcium increase caused by DMPP alone (open box). Data were the means ± SEM (n=3) values.

Figure 5 Inhibitory effect of Cimicifugoside in [Ca$^{2+}$]$_i$ elevation in bovine chromaffin cells. The intracellular [Ca$^{2+}$]$_i$ rise induced by 10µM DMPP (A) or 60mM KCl (B) was measured via multiphoton confocal microscope using calcium sensitive dye Fluo-4/AM. Cells were stimulated by 10µM DMPP or 60mM KCl in the absence (ii) or presence (iv, 5min preincubation) of 60µM cimicifugoside. Left lower pictures (iii) were
conditioned by 60μM cimicifugoside alone. In the bar graphs of each panel, filled bars represented induction by DMPP or KCl alone (i,ii), and open bars induction by each stimulants in the 5min preincubation of 60μM cimicifugoside (iii,iv), respectively. The experiments were performed three times independently, and the results were reproducible. Typical sets of pictures were presented. Values were represented average fluorescence intensity(ROI) ± SEM in selected circled three areas.

**Figure 6** The effect of cimicifugoside on [Ca^{2+}]_{i} rises induced by other reagents. Chromaffin cells were incubated with 20μM cimicifugoside for 5min. then the cells were stimulated with 50mM KCl, 5μM bradykinin (BK), and 100μM veratridine (VT). Net increase in [Ca^{2+}]_{i} was obtained by subtracting the basal level of [Ca^{2+}]_{i} from the peak height after stimulation at each case. The experiments were performed three times independently, and the results were reproducible. Data were the means ± SEM (n=3) values.

**Figure 7** Inhibitory effect of cimicifugoside on sodium increase in bovine chromaffin cells. (A) The intracellular sodium increase induced by 10μM DMPP was measured in the absence (trace a) or presence (trace b) of 60μM cimicifugoside. The experiments were performed three times independently, and the results were reproducible. Typical Na^{+} traces were presented. (B) The sodium increase induced by 10μM DMPP was measured 5min after preincubation with the indicated concentration of cimicifugoside (filled boxes). The peak height of each stimulation was compared to that of the
control sodium increase caused by DMPP alone (open box). (C) The intracellular sodium current induced by 10µM DMPP was recorded through whole-cell patch clamp in the absence or presence of indicated cimicifugoside concentrations. Inductions by DMPP were performed after the 5min preincubation of cimicifugoside and washed out for 3min to get back the control response. (D) Total peak currents induced by DMPP were integrated and represented as percentage of average currents by DMPP. Data were the means ± SEM (n=6) values. (E) The traces by voltage ramp application from -120mV to +50mV were presented in each case of DMPP stimulation with or without cimicifugoside.

**Figure 8** Effect of cimicifugoside on [³H] nicotine binding. Chromaffin cells were incubated with 40nM [³H] nicotine and various concentrations of cimicifugoside (filled box) for 40min at 25°C. Specific binding of [³H] nicotine was presented. Total binding was presented by open box. Nonspecific binding was determined in the presence of 1mM unlabeled nicotine. The experiments were performed three times independently, and the results were reproducible. Data were the means ± SEM (n=3) values.

**Figure 9** Comparison of the inhibitory effects of cimicifugoside and other antagonists. The calcium increase induced by 10µM DMPP(open box) was measured 5min after preincubation with the indicated concentrations of cimicifugoside, lidocaine or mecamylamine. The peak height of respective stimulation was compared to that of the control calcium increase caused by DMPP alone. The experiments were
performed three times independently, and the results were reproducible. Data were the means ± SEM (n=3) values.

**Figure 10** Comparison of the inhibitory effects of cimicifugoside on calcium increase by nAChR subtype selective agonists. (A) The calcium increase induced by indicated concentrations of subtype selective agonists UB165 (rectangular) and 5-lodo A-85380 (triangle) for α3β2 and α4β2 subtype, respectively. The peak height of respective stimulation was compared in each concentration. (B) The maximum induction of calcium by each agonist was inhibited by 5min pretreatment of cimicifugiside with indicated concentration. UB165 (rectangular), 5-lodo A-85380 (triangle): 1μM; DMPP(diamond), nicotine(circle): 10μM
Fig. 2

A

NE secretion, (µM/5×10^5 cells)

[Cimicifugoside(CF)], M

B

NE secretion, (µM/5×10^5 cells)

[Cimicifugoside(CF)], M
Relative exocytosis by DMPP (% of control)

DMPP 10μM
CF 60μM pretreatment
DMPP 10μM

Relative exocytosis by KCl (% of control)

KCl 60mM
CF 60μM pretreatment
KCl 60mM
Fig. 4

A

$[\text{Ca}^{2+}]_{i}(\text{nM})$

$1\text{min}$

CF

DMPP 10$\mu$M

B

Net calcium increase by DMPP, [nM]

[CF], M

$10^{-3}$

$10^{-4}$

$10^{-5}$

$10^{-6}$

C

Calcium increase by DMPP$_i$[nM]

$[\text{DMPP}], \text{M}$

$10^{-7}$

$10^{-6}$

$10^{-5}$

$10^{-4}$

D

Net calcium increase (% of control)

Preincubation time (min)

$0$ 1 2 3 4 5 6 7 8 9 10
**Fig. 5**

A

NT

DMPP

CF

DIC

B

NT

KCI

CF

DIC

**Relative Intensity**

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Peak</th>
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<tbody>
<tr>
<td>DMPP</td>
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<td>300</td>
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<tr>
<td>CF-DMPP</td>
<td>50</td>
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**Experiments:**

1. **NT**
2. **DMPP**
3. **CF**
4. **DIC**

**Results:**

- **NT**
- **KCI**

**Significance:** ****
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Fig.6

Net calcium increase (of control)
Fluorescence ratio (arbitrary unit)

A

1min

DMPP

10^{-6}

[CF], M

B

Net sodium increase by DMPP (% of control)

Fig. 7

10^{-5}

10^{-4}

10^{-3}

10^{-2}

10^{-1}

10^0

C

CF 3 μM

DMPP 10 μM

CF 60 μM

DMPP (wash-out)

1 nA

1 min

D

peak current induced by DMPP (% of control)

Control

CF 3 μM

CF 60 μM

E

V(mV)

CF 60 μM

CF 1 μM

No CF

I(pA)
Fig. 8

Specific binding of [3H]nicotine (% of total binding)

[CF], M
Fig. 9

Net calcium increase by DMPP (% of control) vs. [Concentration], M

- DMPP 10μM
- MMA+DMPP
- CF+DMPP
- Lido+DMPP
Fig. 10

A

Net calcium increase [nM]

[Concentration], M

UB 165

A-85380

B

Net calcium increase by agonist (% of control)

[CF], µM

UB 165

A-85380

DMPP

Nicotine