in the occurrence ratio of WDS ($P < 0.01$; Mantel-Haenszel test). The occurrence ratio in the F1 1-BP-exposed group at 0.1 mg/kg KA was lower than that in the F1 control group ($P < 0.05$ by Fisher's exact test). The dose of 0.5 mg/kg KA tended to decrease the occurrence ratio in the F1 1-BP-exposed group, but did not reach a significant level. Taken together with the results of the 0.1 and 0.5 mg/kg KA (subclinical doses), the occurrence ratio (6 out of 12 rat pups) in the F1 1-BP-exposed group exhibited a lower value than that in the F1 control group (16 out of 16 rat pups, $P < 0.005$ by Fisher's exact test). This indicates that the effects of prenatal 1-BP exposure can be observed only at the subclinical doses of KA. The duration and the frequency of the scratching and the WDS increased at the dose of 2.0 mg/kg ($P < 0.01$), but we did not find any significant effect of prenatal 1-BP exposure on the duration and frequency of WDS at any of the doses of KA. Our results suggest that prenatal exposure to 1-BP suppresses the occurrence of WDS only at a low dose of KA, possibly due to an effect on mechanisms underlying the generation of WDS.

WDS can be induced by electrical stimulation of limbic structures and by the administration of several chemicals, such as serotonergic compounds [15] and an opioid receptor agonist [16], as well as KA. KA-induced-WDS is depressed by μ-opioid receptor antagonists [16]. An antagonist of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/KA receptors suppresses WDS induced by serotonin receptor agonists [15]. The mechanisms of WDS induction by these chemicals are assumed to be related to each other. Besides those receptors, nitric oxide has also been demonstrated to play a regulatory role in KA- and getting-wet-induced-WDS [9]. These receptors and nitric oxide might be the target of prenatal exposure to 1-BP.

Table 1. The occurrence ratio, duration and frequency of scratching in F1 control and 1-BP-exposed groups

	F1 control			F1 1-BP-exposed		
KA (mg/kg)	S/N	duration (s)	frequency (counts)	S/N	duration (s)	frequency (counts)
PBS	5/5	1.9 ± 0.3	21 ± 6	5/5	2.3 ± 0.3	17 ± 5
0.1	11/11	2.0 ± 0.1	14 ± 2	7/7	2.0 ± 0.1	26 ± 10
0.5	5/5	1.7 ± 0.3	25 ± 4	5/5	2.1 ± 0.5	20 ± 8
2.0	5/5	2.9* ± 0.4	557* ± 164	5/5	3.6* ± 0.2	517* ± 41

F1: first filial generation, 1-BP: 1-bromopropane, KA: kainate, PBS: phosphate buffered saline, S: the number of rats in which scratching was observed, N: the total number of rats used in the experiment, S/N: the occurrence ratio, *: significant effects of KA on the duration or the frequency by two-way ANOVA followed by Scheffe's test ($P < 0.01$), mean ± SEM: mean ± standard error of the mean

Table 2. The occurrence ratio, duration and frequency of WDS in F1 control and 1-BP-exposed groups

	F1 control			F1 1-BP-exposed		
KA (mg/kg)	S/N	duration (s)	frequency (counts)	S/N	duration (s)	frequency (counts)
PBS	4/5	0.3 ± 0.02	1.8 ± 0.6	3/5	0.2 ± 0.03	0.8 ± 0.4
0.1	11/11	0.3 ± 0.03	3.4 ± 0.6	4/7[a]	0.3 ± 0.09	1.0 ± 0.4
0.5	5/5	0.3 ± 0.02	2.6 ± 0.9	2/5	0.2, 0.3	0.8 ± 0.6
2.0	5/5	0.3 ± 0.05	29.2* ± 10.0	5/5	0.4 ± 0.02	54.4* ± 21.1

WDS: wet dog shakes, F1: first filial generation, 1-BP: 1-bromopropane, KA: kainate, PBS: phosphate buffered saline, S: the number of rats in which WDS were observed, N: the total number of rats used in the experiment, S/N: the occurrence ratio, a: a significant difference between F1 control and F1 1-BP-exposed groups at the dose of 0.1 mg/kg in the Fisher's exact test ($P < 0.05$), *: significant effects of KA ($P < 0.01$) on the two-way ANOVA followed by Scheffe's test, mean ± SEM: mean ± standard error of the mean. The data of durations in the F1 1-BP-exposed group administered 0.5 mg/kg of KA are shown in the duration(s) column

There are studies suggesting that the hippocampus is the target of KA. KA receptors have been found in the hippocampus in rat pups [12], and epileptic discharges have been observed when KA-induced seizures occur [17]. Moreover, KA-induced WDS was accompanied by robust electrographic seizures recorded from the hippocampus [18]. On the other hand, Fueta *et al.* have reported that prenatal 1-BP exposure decreases the paired-pulse ratio of population spikes in the CA1 subfield of the dorsal hippocampus in PND14 rats [19]. A decrease in the paired-pulse ratio of the population spike is generally interpreted as an increase in an inhibition [2]. Thus, prenatal 1-BP exposure may disturb the propagation of hyperactivity in the hippocampus, such as electrographic discharges associated with KA-induced WDS. This may account for the suppression of WDS by prenatal exposure to 1-BP. However, it should also be considered that the dentate gyrus (DG) in the ventral hippocampus is thought to be necessary for chemical interventions such as KA-, μ-opioid-, and electrical stimulation-induced WDSs in adult rats [16, 20, 21]. Therefore, further studies are needed to investigate the excitability of the DG in the ventral hippocampus in prenatally 1-BP-exposed rats.

In conclusion, we demonstrate here that prenatal exposure to 1-BP suppresses WDS induced by the administration of a low dose of KA. Our results indicate that prenatal 1-BP exposure may disturb the susceptibility to KA or the functions of neural networks related to the WDS. We also show that it may be advantageous to use pharmacological interventions with convulsants in investigations of the effects of environmental chemicals on behavioral responses in immature rats.

Conflict of Interest

No conflicts of interest to declare.

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1-ブロモプロパンへの胎生期曝露は発達期ラットにおいてカイニン酸で誘導されるWet Dog Shakesを抑制する

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要 旨：1-ブロモプロパン(1-BP)は洗浄やスプレー接着剤の溶剤として用いられている．1-BPの有害性はヒトの事例や成獣を用いた動物で報告されてきた．発達毒性も報告されてはいるが，発達神経毒性についての詳細はわかっていない．我々は，1-BPの胎生期曝露が，発達期ラットへのカイニン酸投与により誘導される行動，すなわちscratching行動やwet dog shake様行動に及ぼす影響を調べた．ウィスター系妊娠ラットの妊娠1日目から20日目まで(6時間/日)，濃度700 ppmの1-BP蒸気を曝露した．生後14日目の対照群と1-BP曝露群にカイニン酸を0.1, 0.5, 2.0 mg/kgで腹腔内投与した．Scratching行動に関しては対照群と1-BP曝露群に違いは見られなかったが，wet dog shake様行動に関しては，低濃度である0.1 mg/kgにおいて発生率の低下が1-BP曝露群で見られた．1-BP胎生期曝露が発達期の神経行動に影響することが示唆された．

キーワード：1-ブロモプロパン，胎生期曝露，発達神経毒性，wet dog shake，ラット．

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ORIGINAL ARTICLE

Ikarisoside A inhibits acetylcholine-induced catecholamine secretion and synthesis by suppressing nicotinic acetylcholine receptor-ion channels in cultured bovine adrenal medullary cells

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Abstract Ikarisoside A is a natural flavonol glycoside derived from plants of the genus *Epimedium*, which have been used in Traditional Chinese Medicine as tonics, antirheumatics, and aphrodisiacs. Here, we report the effects of ikarisoside A and three other flavonol glycosides on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells. We found that ikarisoside A (1–100 μM), but not icariin, epimedin C, or epimedoside A, concentration-dependently inhibited the secretion of catecholamines induced by acetylcholine, a physiological secretagogue and agonist of nicotinic acetylcholine receptors. Ikarisoside A had little effect on catecholamine secretion induced by veratridine and 56 mM K^+. Ikarisoside A (1–100 μM) also inhibited $^{22}Na^+$ influx and $^{45}Ca^{2+}$ influx induced by acetylcholine in a concentration-dependent manner similar to that of catecholamine secretion. In *Xenopus* oocytes expressing α3β4 nicotinic acetylcholine receptors, ikarisoside A (0.1–100 μM) directly inhibited the current evoked by acetylcholine. It also suppressed ^{14}C-catecholamine synthesis and tyrosine hydroxylase activity induced by acetylcholine at 1–100 μM and 10–100 μM, respectively. The present findings suggest that ikarisoside A inhibits acetylcholine-induced catecholamine secretion and synthesis by suppression of nicotinic acetylcholine receptor-ion channels in bovine adrenal medullary cells.

Keywords Adrenal medulla · Catecholamine secretion · *Epimedium* · Flavonoids · Ikarisoside A · Nicotinic acetylcholine receptor

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Introduction

Flavonoids, a group of secondary metabolites with variable phenolic structure, which exist widely in plants (Nijveldt et al. 2001; Ren and Zuo 2012), may exert potential benefits associated with reduced risks of age- and life style-related diseases such as cardiovascular diseases, diabetes, and some cancers (Lu et al. 2013; Yanagihara et al. 2014). Ikarisoside A is one of the flavonol derivatives derived from plants of the genus *Epimedium*, which have been used in Traditional Chinese Medicine as tonics, antirheumatics, and aphrodisiacs (Dou et al. 2006). Previous studies reported that the total flavonoid fraction of *Epimedium* extract suppresses urinary calcium excretion and improves bone properties in ovariectomized mice (Chen et al. 2011), and that other extracts with structures similar to that of ikarisoside A, such as icariin, can stimulate osteogenic activities (Zhou et al. 2013) and have

anti-inflammatory effects (Lai et al. 2013). Furthermore, ikarisoside A also has pharmacological effects such as antioxidant and anti-inflammatory effects (Choi et al. 2008) as well as anti-osteoporosis effects (Choi et al. 2010).

In the human body, the most abundant catecholamines are adrenaline, noradrenaline, and dopamine, all of which are produced from phenylalanine and/or tyrosine. Catecholamines are produced mainly in the chromaffin cells of the adrenal medulla, the postganglionic fibers of the sympathetic nervous system, and the central nervous system. Catecholamines play very important roles in heart rate, blood pressure, blood glucose levels, and the general reactions of the sympathetic nervous system.

Adrenal medullary cells derived from embryonic neural crests are functionally homologous to sympathetic postganglionic neurons. In bovine adrenal medullary cells, catecholamine secretion is associated with the activation of three types of ionic channels: nicotinic acetylcholine receptor (nAChR)-ion channels, voltage-dependent Na^+ channels, and voltage-dependent Ca^{2+} channels (Wada et al. 1985b). ACh induces Na^+ influx via nAChR-ion channels, then, it induces Ca^{2+} influx and subsequent catecholamine secretion (Wada et al. 1985b). On the other hand, stimulation of catecholamine synthesis induced by ACh is associated with the activation of tyrosine hydroxylase in cultured bovine adrenal medullary cells (Yanagihara et al. 1987; Tsutsui et al. 1994). The conversion of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) is the rate-limiting step of catecholamine biosynthesis (Nagatsu et al. 1964). Adrenal medullary cells have provided a good model for the detailed analysis of a drug's actions on catecholamine secretion and synthesis (Kajiwara et al. 2002; Toyohira et al. 2005; Shinohara et al. 2007).

In our previous study, we isolated 20 flavonol glycosides from *Epimedium* species, including ikarisoside A, icariin, epimedoside A, and epimedin C (Mizuno et al. 1988). Ikarisoside A showed neurite outgrowth activity in cultured PC12h cells (Kuroda et al. 2000). There is, however, little evidence regarding ikarisoside A's effects on sympathetic nervous system activity. In the present study, we investigated the effects of four flavonol glycosides on bovine adrenal medullary cell functions and found that ikarisoside A, but not the other three flavonol glycosides, inhibited ACh-induced catecholamine secretion and synthesis by suppression of nAChR-ion channels in the cells.

Materials and methods

Materials

Oxygenated Krebs-Ringer phosphate (KRP) buffer was used throughout unless stated otherwise. Its composition is as follows (in mM): 154 NaCl, 5.6 KCl, 1.1 $MgSO_4$, 2.2 $CaCl_2$, 0.85 NaH_2PO_4, 2.15 Na_2HPO_4, and 10 glucose, adjusted to pH 7.4. Drugs and reagents were obtained from the following sources: Eagle's minimum essential medium (Eagle's MEM) (Nissui Pharmaceutical, Tokyo, Japan); collagenase (Nitta Zerachin, Osaka, Japan); calf serum (Cell Culture Technologies, Gravesano, Switzerland). ACh and veratridine were from Sigma (St. Louis, MO, USA). L-[U-^{14}C]tyrosine was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA); $^{45}CaCl_2$, $^{22}NaCl$, and L-[1-^{14}C]tyrosine from Perkin-Elmer Life Sciences (Boston, MA, USA).

Isolation of flavonol glycosides from the leaves of *Epimedium* species

The leaves of *Epimedium diphyllum* were collected at Miyazaki Prefecture, Japan. Ikarisoside A and other flavonol glycosides were purified by high performance liquid chromatography, as reported previously (Mizuno et al. 1988). Ikarisoside A and other flavonol glycosides were dissolved in 100 % dimethyl sulfoxide (DMSO) and then diluted in a reaction medium before use at a final DMSO concentration not exceeding 0.5 %, unless otherwise specified. DMSO (0.5 %) did not influence the basal and ACh-induced catecholamine secretion in the present study (data not shown).

Primary culture of bovine adrenal medullary cells

Bovine adrenal medullary cells were isolated by collagenase digestion of adrenal medullary slices according to the method as reported previously (Yanagihara et al. 1979, 1996). Cells were suspended in Eagle's MEM containing 10 % calf serum, 3 μM cytosine arabinoside, and several antibiotics, and maintained in monolayer culture at a density of 4×10^6 cells/dish (35 mm dish; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) or 10^6 cells/well (24-well plate; Corning Life Sciences, Lowell, MA, USA) at 37 °C under a humidified atmosphere of 5 % CO_2 and 95 % air. The cells were used for experiments between 2 and 5 days of culture.

Catecholamine secretion from cultured bovine adrenal medullary cells

The secretion of catecholamines was measured as described previously (Yanagihara et al. 1979). Cells (10^6/well) were washed three times with oxygenated KRP buffer, then firstly preincubated with or without ikarisoside A (0.3–100 μM) or other flavonol glycosides (10 μM) at 37 °C for 10 min, and incubated with or without ikarisoside A (0.3–100 μM) or other flavonol glycosides (10 μM) in the presence or absence of various secretagogues (300 μM ACh, 100 μM veratridine or 56 mM K^+) at 37 °C for another 10 min. After the reaction, the incubation medium was transferred immediately to a test tube containing perchloric acid (final concentration, 0.4 M) for the

full stop of the reaction. Catecholamines (noradrenaline and adrenaline) secreted into the medium were adsorbed onto aluminum hydroxide and estimated by the ethylenediamine condensation method (Weil-Malherbe and Bone 1952) using a fluorescence spectrophotometer (F-2500; Hitachi, Tokyo, Japan) with excitation and emission wavelengths of 420 and 540 nm, respectively.

$^{22}Na^{+}$ and $^{45}Ca^{2+}$ influx

The influx of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ was measured as reported previously (Wada et al. 1985a, b). After preincubation with or without ikarisoside A (0.3–100 μM) at 37 °C for 10 min, cells (4×10^6/dish) were incubated with 1.5 μCi of $^{22}NaCl$ or 1.5 μCi of $^{45}CaCl_2$ at 37 °C for 5 min with or without 300 μM ACh and ikarisoside A (0.3–100 μM) in KRP buffer. After incubation, the cells were washed three times with ice-cold KRP buffer, solubilized in 10 % Triton X-100, and counted for radioactivity of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ by a gamma counter (ARC-2005, Aloka, Tokyo, Japan) and a liquid scintillation counter (TRI-CARB 2900TR, PACKARD INSTRUMENT CO., Meriden, CT, USA), respectively.

^{14}C-Catecholamine synthesis from [^{14}C]tyrosine in the cells

After preincubation for 10 min, cells (4×10^6/dish) were incubated with 20 μM L-[U-^{14}C]tyrosine (1.0 μCi) KRP buffer in the presence or absence of various concentrations of ikarisoside A (0.3–100 μM) and 300 μM ACh at 37 °C for 20 min. After removing the incubation medium by aspiration, cells were harvested in 0.4 M perchloric acid and centrifuged at 1600×*g* for 10 min. ^{14}C-Catecholamines were separated further by ion exchange chromatography on Duolite C-25 columns (H^{+}-type, 0.4 × 7.0 cm) (Yanagihara et al. 1987) and counted for the radioactivity by a liquid scintillation counter (TRI-CARB 2900TR, PACKARD INSTRUMENT CO., Meriden, CT, USA). ^{14}C-Catecholamine synthesis was expressed as the sum of the ^{14}C-catecholamines (adrenaline, noradrenaline, and dopamine).

Tyrosine hydroxylase activity in situ

After preincubation with or without ikarisoside A (0.3–100 μM) for 10 min, cells (10^6/well) were exposed to 200 μl of KRP buffer with or without ikarisoside A (0.3–100 μM) and 300 μM ACh, supplemented with 18 μM L-[1-^{14}C]tyrosine (0.2 μCi) for 10 min at 37 °C. Upon addition of the labeled tyrosine, each well was sealed immediately with an acrylic tube capped with a rubber stopper and fitted with a small plastic cup containing 200 μl of NCS-II tissue solubilizer (GE Healthcare UK Ltd. Little Chalfont, Buckinghamshire, UK) to absorb the $^{14}CO_2$ released by the cells and counted for the radioactivity (Bobrovskaya et al. 1998).

Expression of α3β4 nAChRs in *Xenopus* oocytes and electrophysiological recordings

The complementary DNAs (cDNAs) encoding the α3 and β4 subunits of rat neuronal nAChR, subcloned into pcDNAI/Neo (Invitrogen, Carlsbad, CA) vector, were kindly provided from Dr. James W. Patrick (Division of Neuroscience, Baylor College of Medicine, TX, USA). After linearization of cDNA with *NotI*, complementary RNAs (cRNAs) were transcribed using T7 RNA polymerase from the mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA). Adult female *Xenopus laevis* frogs were obtained from Kyudo Co., Ltd. (Saga, Japan). *Xenopus* oocytes and cRNA microinjection were prepared as described previously (Ueno et al. 2004; Horishita and Harris 2008). cRNAs of α3 and β4 subunits were co-injected at a same ratio (10–20 ng/50 nL) into *Xenopus* oocytes, and electrophysiological recordings were performed 2–6 days after injection. Oocytes were placed in a 100 μl recording chamber and perfused at 2 ml/min with extracellular Ringer solution (110 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.8 mM $BaCl_2$, pH 7.5) containing 1.0 μM atropine sulfate. Ca^{2+} in the solution was replaced with Ba^{2+} to minimize the effects of secondarily activated Ca^{2+}-dependent Cl^- channels. Recording electrodes (1–3 MΩ) were filled with 3 M KCl, and the whole-cell voltage clamp was achieved through these two electrodes using a Warner Instruments model OC-725C (Warner, Hamden, CT, USA) at −70 mV. We measured the peak of the transient inward current in response to ACh that was applied for 30 s and examined the effects of ikarisoside A on a concentration of ACh that produced 50 % of the maximal effect (EC_{50}) of ACh. The EC_{50} was determined for each oocyte by 1 mM ACh that produces a maximal current. Ikarisoside A stocks were prepared in 100 % DMSO and diluted in bath solution to a final DMSO concentration not exceeding 0.1 %. Ikarisoside A was preapplied for 2 min to allow an equilibration with its site of interaction before ACh was added and its effect on the cation currents was determined. In all cases, between two currents, there was 10 min interval under washing with normal Ringer solution.

Statistical analysis

All experiments were performed in duplicate or triplicate, and each experiment was repeated at least three times. All values are given as means ± SEM. The significance of differences between means was evaluated using one-way analysis of variance (ANOVA). When a significant *F* value was found by ANOVA, Dunnett's or Scheffe's test for multiple comparisons was used to identify differences among the groups. Values were considered statistically different when *P* was less than

0.05. Statistical analyses were performed using PRISM for Windows version 5.0J software (Abacus Concept, Berkeley, CA, USA).

Results

The structures of four flavonol glycosides isolated from *Epimedium*

The four flavonol glycosides ikarisoside A, icariin, epimedin C, and epimedoside A were isolated from the leaves of *E. diphyllum* as reported previously (Mizuno et al. 1988). The structures of these four flavonol glycosides are shown in Fig. 1.

Effects of the flavonol glycosides on catecholamine secretion induced by various secretagogues in adrenal medullary cells

None of the four flavonol glycosides (ikarisoside A, icariin, epimedin C, and epimedoside A) at 10 μM significantly affected the basal secretion of catecholamines (Fig. 2a). ACh (300 μM), an agonist of nAChRs, caused catecholamine secretion corresponding to 18.90 ± 0.38 % of the total catecholamines in the cells. When the cells were treated with the same four flavonol glycosides at 10 μM for 10 min, ikarisoside A strongly reduced catecholamine secretion induced by ACh, to 6.83 ± 0.51 % of the total, whereas the other three had little effect (Fig. 2a). Veratridine (100 μM), an activator of voltage-dependent Na^+ channels, and 56 mM K^+, which depolarizes cell membranes and then activates voltage-dependent Ca^{2+} channels, also caused catecholamine secretion corresponding to 26.52 ± 0.88 % (Fig. 2b) and 20.51 ± 0.70 % (Fig. 2c) of the total catecholamines, respectively. Treatment of cells with these flavonol glycosides at 10 μM did not affect catecholamine secretion induced by veratridine (Fig. 2b) and 56 mM K^+ (Fig. 2c).

Concentration-inhibition curves for the effects of ikarisoside A on catecholamine secretion, $^{45}Ca^{2+}$ influx, and $^{22}Na^+$ influx induced by ACh

We examined the effects of ikarisoside A on catecholamine secretion, $^{45}Ca^{2+}$ influx, and $^{22}Na^+$ influx induced by ACh. Treatment of cells with ikarisoside A at 1, 3, 10, 30, and 100 μM significantly inhibited ACh-induced secretion of catecholamines (18.22 ± 0.16 % of the total catecholamines in the cells) to 15.36 ± 0.38 %, 12.27 ± 0.40 %, 7.68 ± 0.30 %, 5.33 ± 0.47 %, and 4.95 ± 0.25 % of the total catecholamines in the cells, respectively (Fig. 3a). Ikarisoside A also inhibited ACh-induced $^{45}Ca^{2+}$ influx and $^{22}Na^+$ influx in a concentration-dependent manner (Fig. 3b, c). The half-maximal inhibitory concentration (IC_{50}) of ikarisoside A in catecholamine secretion, $^{45}Ca^{2+}$ influx, and $^{22}Na^+$ influx are 4.00, 9.90, and 2.96 μM, respectively.

Inhibitory mode of ikarisoside A on ACh-induced catecholamine secretion in adrenal medullary cells

To investigate the mechanism by which ikarisoside A inhibits ACh-induced catecholamine secretion, we examined whether or not the inhibitory effect of ikarisoside A on catecholamine secretion is overcome when the ACh concentration is

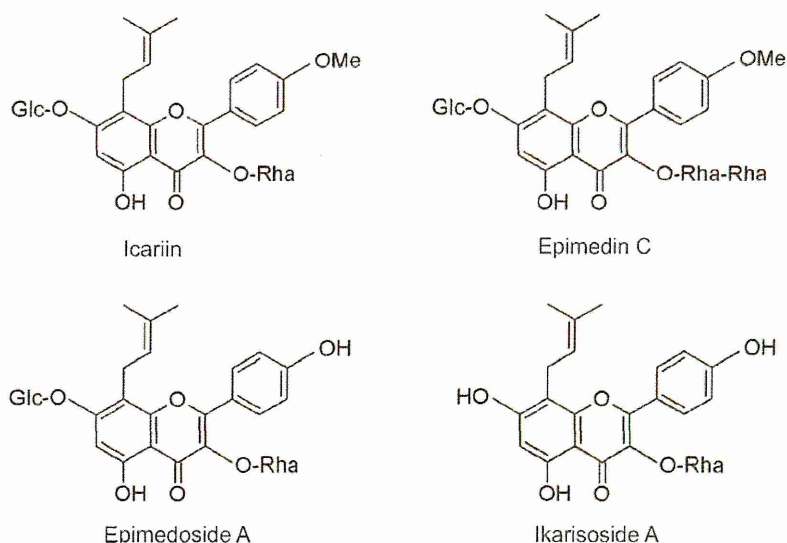

Fig. 1 Chemical structures of icariin, epimedin C, epimedoside A, ikarisoside A. The abbreviations Glc and Rha in the structures are glucose and rhamnose, respectively

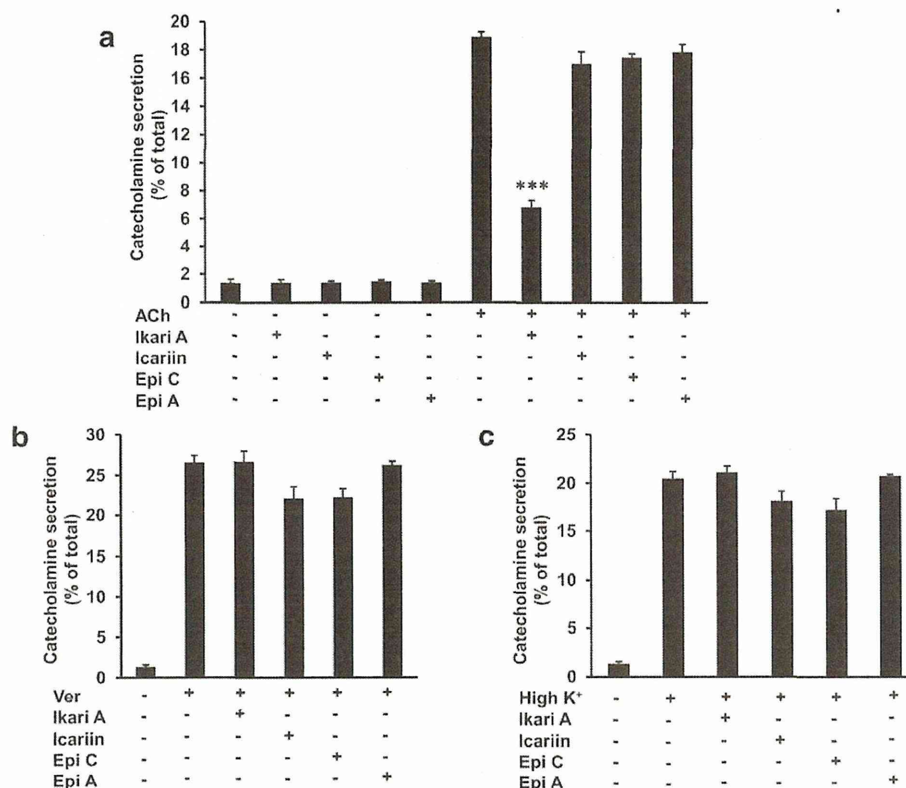

Fig. 2 Effects of ikarisoside A, icariin, epimedin C, or epimedoside A on catecholamine secretion induced by various secretagogues in cultured bovine adrenal medullary cells. After preincubation with cells with or without ikarisoside A (Ikari A) (10 μM), icariin (10 μM), epimedin C (Epi C) (10 μM), and epimedoside A (Epi A) (10 μM) for 10 min, the cells (10^6/well) were incubated with or without these four flavonol glycosides (10 μM), ACh (300 μM) (**a**), veratridine (100 μM) (**b**), or 56 mM K^+ (**c**) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means ± SEM from three separate experiments carried out in triplicate. ***$P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

increased. Even when the ACh concentrations in the incubation medium increased from 3 to 300 μM, they did not overcome the inhibitory effect of ikarisoside A (Fig. 4a). Double-reciprocal plot analysis revealed that ikarisoside A exerts a noncompetitive type of inhibition on ACh-induced secretion of catecholamines (Fig. 4b).

Effects of ikarisoside A on ACh-induced inward current in *Xenopus* oocytes expressing α3β4 nAChRs

We examined the direct effects of ikarisoside A on ACh responses in *Xenopus* oocytes expressing rat α3β4 nAChRs. As shown in Fig. 5a, ikarisoside A reversibly inhibited ACh (0.2 mM)-induced currents. Ikarisoside A inhibited ACh-induced currents concentration dependently. It suppressed those currents to 80 ± 3 %, 69 ± 6 %, 43 ± 6 %, 32 ± 8 %, and 22 ± 5 % of the control at 0.1, 0.3, 1, 3, and 10 μM, respectively, and the inhibitory effects were significant from 0.10 μM onward; the IC_{50} was 0.48 μM (Fig. 5b).

Effect of aglycon of ikarisoside A on ACh-induced secretion of catecholamines

Ikarisoside A is a flavonol glycoside having one rhamnose at the 3 position in the chemical structure. 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-enyl)-4*H*-chromen-4-one is the aglycon of ikarisoside A. We examined the effect of this aglycon on ACh-induced secretion of catecholamines. As shown in Fig. 6, the aglycon of ikarisoside A did not affect basal or ACh-induced secretion of catecholamines.

Effect of ikarisoside A on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine and tyrosine hydroxylase activity

As shown in Fig. 7a, ACh (300 μM) increased the synthesis of ^{14}C-catecholamines from [^{14}C]tyrosine about 3-fold in bovine adrenal medullary cells. The concurrent treatment of cells with ikarisoside A inhibited the stimulatory effect of 300 μM ACh on ^{14}C-catecholamine synthesis in a concentration (1–

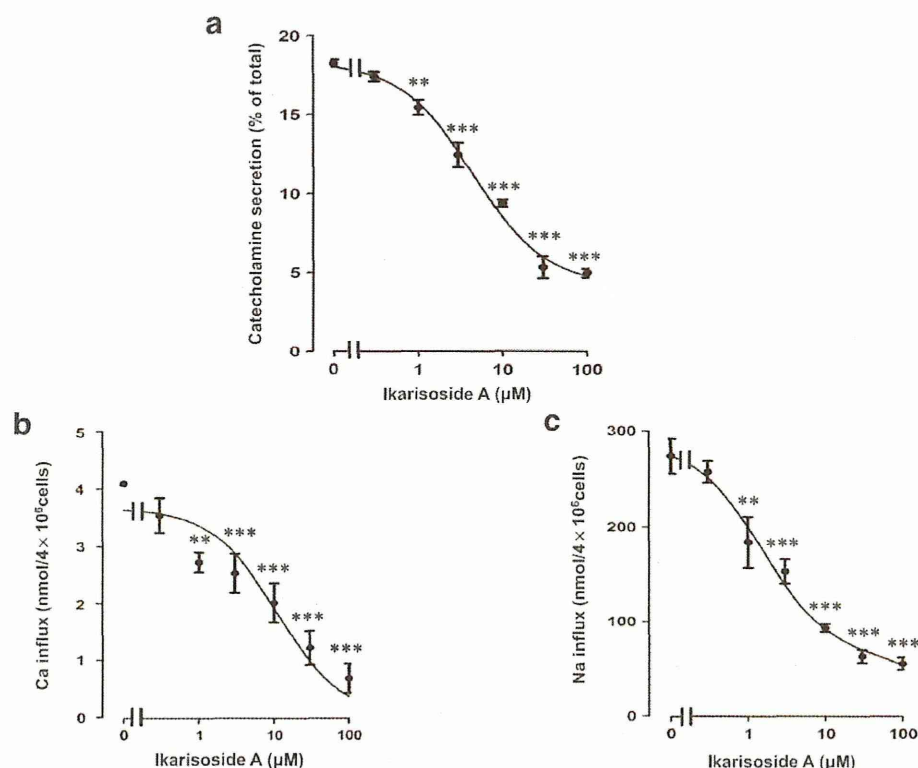

Fig. 3 Effects of ikarisoside A on catecholamine secretion (**a**), $^{45}Ca^{2+}$ influx (**b**), and $^{22}Na^{+}$ influx (**c**) induced by ACh. (**a**) After preincubation for 10 min with or without ikarisoside A (0.3–100 μM), cells were stimulated with ACh (300 μM) in the presence or absence of ikarisoside A (0.3–100 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. **b**, **c** After preincubation for 10 min, cells were stimulated with ACh (300 μM) and 1.5 μCi of $^{45}CaCl_2$ (**b**) or $^{22}NaCl$ (**c**) in the presence or absence of ikarisoside A (0.3–100 μM) for another 5 min at 37 °C. $^{45}Ca^{2+}$ influx and $^{22}Na^{+}$ influx were measured and were expressed as nmol/4 × 10^6 cells. Data are means ± SEM from three separate experiments carried out in triplicate. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

100 μM)-dependent manner (Fig. 7a), yielding an IC_{50} value of 2.85 μM. Ikarisoside A (1–100 μM) had little effect on the basal synthesis of ^{14}C-catecholamines.

We next examined the effect of ikarisoside A on tyrosine hydroxylase activity in the cells. After preincubation with or without ikarisoside A (0.1–100 μM) for 10 min, cells were incubated with 300 μM ACh in the absence or presence of ikarisoside A (0.1–100 μM) for another 10 min at 37 °C. Ikarisoside A (10–100 μM) inhibited the tyrosine hydroxylase activity induced by ACh and tended to inhibit the basal enzyme activity (Fig. 7b). The IC_{50} value of ikarisoside A for its inhibitory effect on the ACh-induced tyrosine hydroxylase activity was 9.13 μM (derived from the curve representing the difference between stimulated and basal tyrosine hydroxylase; not shown).

Discussion

In present study, we investigated the effects of four flavonol glycosides derived from the leaves of the genus *Epimedium*. We demonstrated that ikarisoside A, but not the other three, inhibited the secretion and synthesis of catecholamines induced by ACh in cultured bovine adrenal medullary cells. To our knowledge, this is the first direct evidence of an inhibitory effect of ikarisoside A on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells.

Inhibitory effect of ikarisoside A on catecholamine secretion induced by ACh

The present study demonstrated that ikarisoside A significantly inhibits catecholamine secretion induced by ACh, but not by veratridine or 56 mM K^{+} in adrenal medullary cells. We previously reported that ACh activates nAChR-ion channels, and induces Na^{+} influx, subsequent Ca^{2+} influx, and finally catecholamine secretion. On the other hand, veratridine activates voltage-dependent Na^{+} channels and 56 mM K^{+} depolarizes cell membranes to activate voltage-dependent Ca^{2+} channels (Wada et al. 1984, 1985b). In the present study, ikarisoside A did not inhibit the stimulatory effects of veratridine and 56 mM K^{+} on catecholamine secretion. Therefore,