Table 1. Experimental groups and ages of adult and fetal rats exposed to 700 ppm of 1-BP and of pups on sampling day

Groups (n)		Age (n) on sampling day
Virgin female	Exposure (5)	GD21 (2)
Mother	Exposure (11) Control (5)	GD20 (3)
Fetus	Exposure	GD20 (13)
Pup†	Exposure Postnatal exposure Gestation exposure Control	PND1 (10), PND3 (10), PND5 (5), PND7 (5) PND2 (5), PND4 (5), PND8 (5) PND4 (5), PND8 (5) PND3 (5)

1-BP: 1-bromopropane, GD: gestation day, PND: postnatal day, †: Exposure = 1-BP exposed pups were raised by their birth mother exposed to 1-BP, Postnatal exposure = control pups were raised by 1-BP exposed mother, Gestation exposure = 1-BP exposed pups were raised by control mother, Control = control pups were raised by control mother

Table 2. Parameters of the one-compartment model

Groups		$T_{1/2}$ (days)	ρV (g)	R (μg/h)	Results
GD	Virgin female	7.0	271.1	2853	Fig. 2
	Mother	7.0	271.1+27w	2853	Fig. 2
	Fetus	3.1	w	22w	Fig. 2
PND	Gestation exposure	3.1			Fig. 3
	Postnatal exposure	3.1	w	$388e^{-0.126(t-32)}$	Fig. 3
	Exposure	Gestation exposure + Postnatal exposure			Fig. 3
	Mother	7.0			Text†

†: the concentration in mother brain corresponding to PND1 (486.2 μg/g-brain),
$w=0.00028T^{3.31}$ by equation (6)

Figure Captions

Fig. 1. The average body weight of mothers (*W*) exposed to 700 ppm of 1-BP up to GD20 and that of pups (*w*) after exposure. 1-BP: 1-bromopropane; GD: gestation day; PND: postnatal day

Fig. 2. Change in bromine ion concentration in rat brain exposed to 700 ppm of 1-BP on GDs. Symbols represent experimental data: ●, fetus; ▲, mother; △, virgin female. Solid, broken, and dotted lines indicate calculated lines for fetuses, mothers, and virgin females, respectively. 1-BP: 1-bromopropane; GD: gestation day

Fig. 3. Change in bromine ion concentration in pup brain during PNDs. Symbols represent experimental data: ●, exposure group (1-BP exposed pups were raised by their birth mother exposed to 1-BP); ◇, postnatal group (control pups were raised by 1-BP exposed mother); □, gestation exposure (1-BP exposed pups were raised by control mother). Solid, broken, and dotted lines indicate calculated lines for exposure, postnatal exposure, and gestation exposure groups, respectively. 1-BP: 1-bromopropane; PND: postnatal day

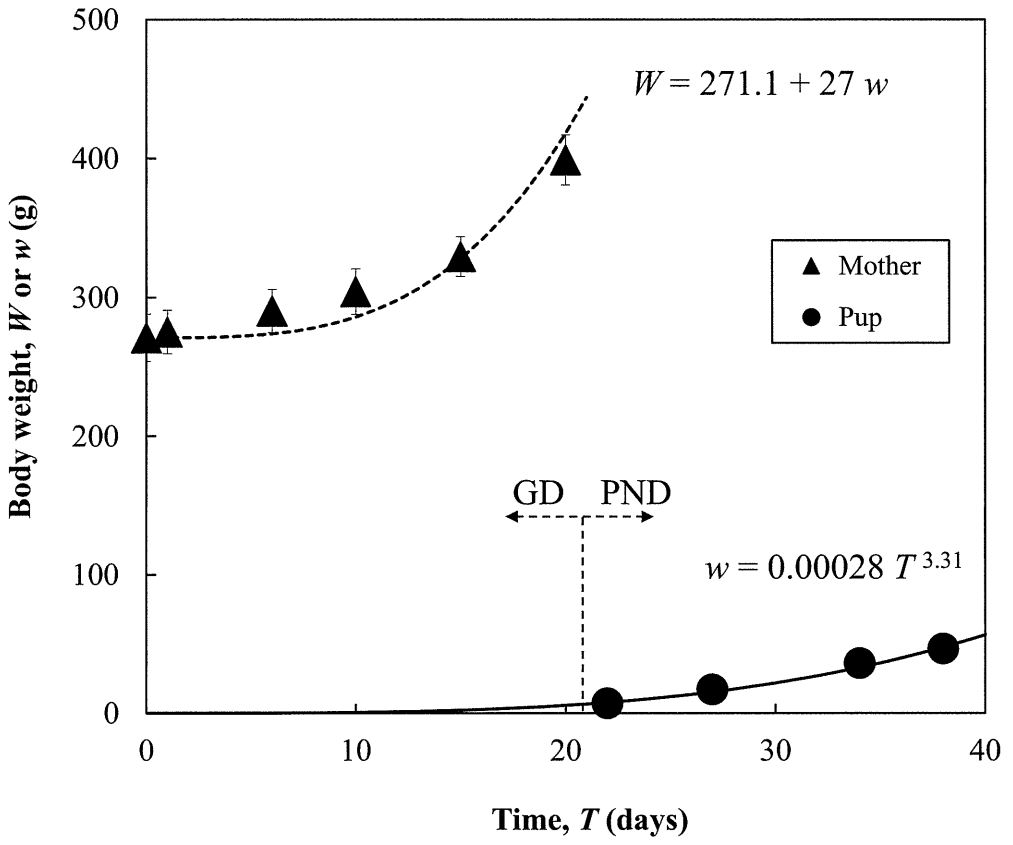
500
400
300
200
100
0
0
10
20
30
40
Body weight, W or w (g)
Time, T (days)
W = 271.1 + 27 w
w = 0.00028 T 3.31
GD
PND
Mother
Pup

Fig. 1.

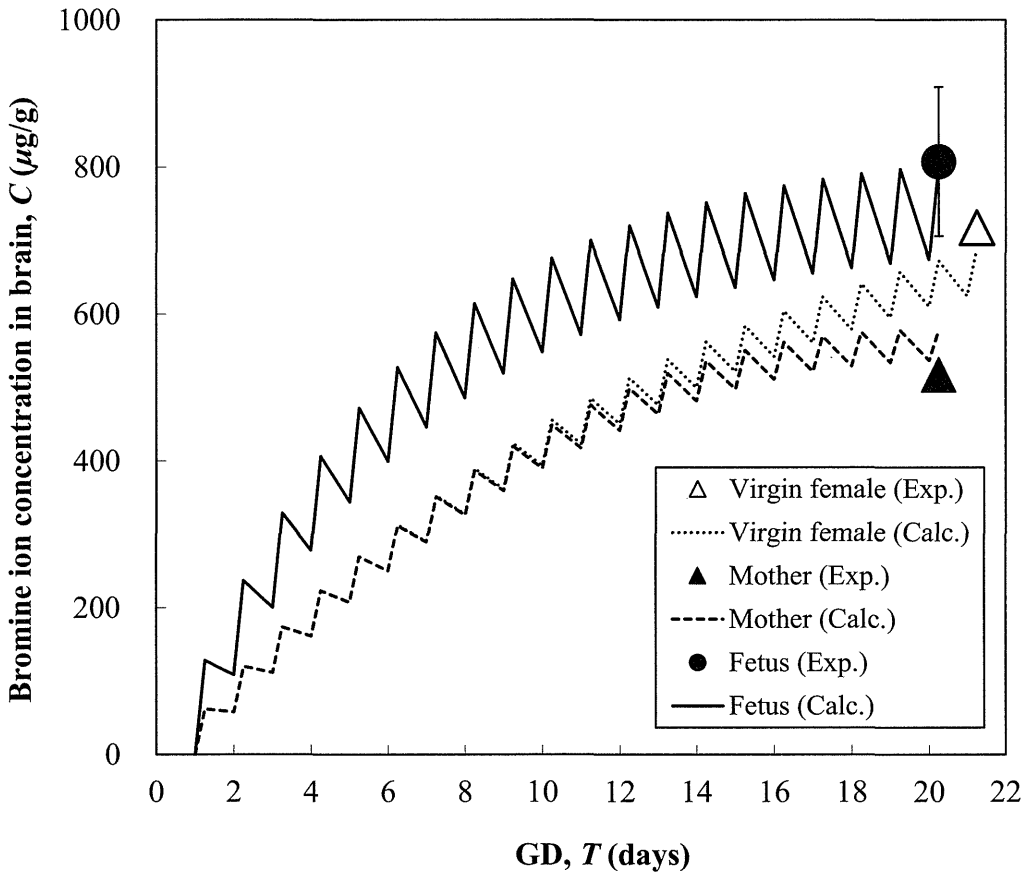
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
22
Bromine ion concentration in brain, C (μg/g)
GD, T (days)
Virgin female (Exp.)
Virgin female (Calc.)
Mother (Exp.)
Mother (Calc.)
Fetus (Exp.)
Fetus (Calc.)

Fig. 2.

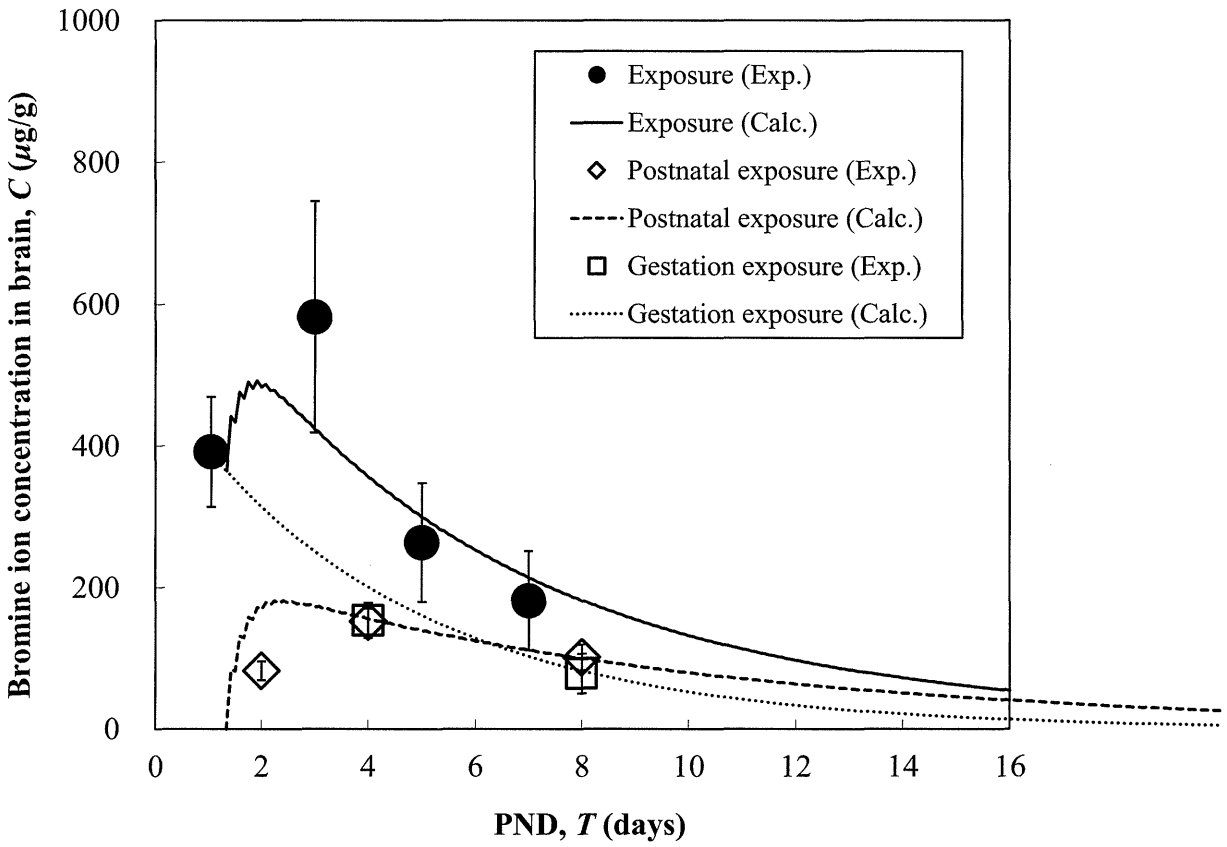
Bromine ion concentration in brain, C (μg/g)
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
PND, T (days)
Exposure (Exp.)
Exposure (Calc.)
Postnatal exposure (Exp.)
Postnatal exposure (Calc.)
Gestation exposure (Exp.)
Gestation exposure (Calc.)

Fig. 3.

[Research Note]

Prenatal Exposure to 1-Bromopropane Suppresses Kainate-Induced Wet Dog Shakes in Immature Rats

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Abstract : 1-Bromopropane (1-BP) is used in degreasing solvents and spray adhesives. The adverse effects of 1-BP have been reported in human cases and adult animal models, and its developmental toxicity has also been reported, but its effects on developmental neurotoxicity have not been investigated in detail. We evaluated the effects in rat pups of prenatal exposure to 1-BP on behaviors such as scratching and wet dog shakes (WDS), which were induced by injection of kainate (KA). Pregnant Wistar rats were exposed to vaporized 1-BP with 700 ppm from gestation day 1 to day 20 (6 h/day). KA at doses of 0.1, 0.5, and 2.0 mg/kg were intraperitoneally injected into a control group and a 1-BP-exposed group of pups on postnatal day 14. There was no significant difference in scratching between the control and the prenatally 1-BP-exposed groups, while suppression of the occurrence ratio of WDS was observed at the low dose of 0.1 mg/kg of KA in the prenatally 1-BP-exposed pups. Our results suggest that prenatal exposure to 1-BP affects neurobehavioral responses in the juvenile period.

Keywords : 1-bromopropane, prenatal exposure, developmental neurotoxicity, wet dog shake, rats.

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Introduction

The volatile organic compound 1-bromopropane (CH_3-CH_2-CH_2Br; 1-BP), a substitute for specific chlorofluorocarbons, is mainly used in degreasing solvents and spray adhesives. It has been reported that occupational exposure to 1-BP causes neurotoxicity, such as numbness, gait disturbance, prolongation of distal latency and memory dysfunction [1].

Animal models exposed to 1-BP have also shown central neurotoxicity, including ataxic gait, prolongation of distal latency, alteration of mRNA levels of neurotransmitter receptors [1], and hippocampal disinhibition [2]. *In vitro* studies have revealed that the direct application of 1-BP enhanced the currents mediated by the activation of A type γ-aminobutyric acid ($GABA_A$) receptors, suppressed the currents mediated by neuronal nicotinic acetylcholine receptors, and potentiated feedback inhibition in the cornu ammonis 1 (CA1) subfield of hippocampal slices [3]. The gene expression of the B-cell lymphoma-extra large molecule (Bcl-xl), and the activity of nuclear factor-kappa B (NF-κB), were suppressed in *in vitro* and *in vivo* studies [4]. The developmental effects of 1-BP have also been investigated [5], but little is known about the developmental neurotoxicity in offspring.

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In our previous study of the developmental neurotoxicity of 1-BP, prenatal exposure to 1-BP altered hippocampal excitability and the gene expression of the Na^+ channel [6] and glutamate receptor subunits on postnatal day (PND) 14 [7]. These results raised the possibility that prenatal exposure to 1-BP affects brain development and its related behaviors. However, conventional behavioral tests for rodents are difficult to apply to pups. Thus, we focused on the particular behaviors of scratching and wet dog shakes (WDS), which can be observed in pups.

Scratching is defined as repetitive and quick flexion-extension movements of the hind limbs toward the neck or the head region. This behavior has been shown to be spontaneously induced in normal as well as pathological conditions and is used as an itch model in rodents [8], although the behavior in pups remains to be analyzed. WDS is characterized as brief and fierce shaking of the head, neck, and trunk, appearing when rodents are wet, as the name suggests [9]. Interestingly, it has been reported that both scratching and WDS can be induced by electrical stimulation of limbic structures [10], and by several pharmacological interventions, such as kainate (KA) [9, 11] and pentylenetetrazole. KA is the agonist of ionotropic glutamate receptors, which mediate excitatory neurotransmission and are predominantly distributed in the hippocampus, inner lamina of the neocortex, and ventral thalamus [12]. Thus, scratching and WDS induced by KA could be useful indices of changes in the excitatory neurotransmission of neuronal networks in pup brains. In this study, we examined the effect of prenatal exposure to 1-BP on behaviors in pups by evaluating the incidences of scratching and WDS induced by KA.

Materials and Methods

Animals and 1-BP inhalation

Thirty-two female and 16 male Wistar rats (designated the parental (P) generation) purchased from Kyudo *Co.* (Tosu, Japan) at 11 weeks of age were housed in plastic cages with paper-made chips (ALPHA-dri, Shepherd Specialty Papers, Milford, USA) on a 12 h light/dark cycle (light period: 07:00-19:00). The room temperature was kept at 23 ± 1°C. The relative humidity was about 70%. The animals had free access to food and water. Proestrus stage was verified with an impedance checker (MK-10B, Muromachi Kikai *Co., Ltd.*, Tokyo, Japan). When the impedance was over three kΩ, the F0 female rats were mated with male rats. In the morning of the following day, the existence of sperm in the vaginal smear or vaginal plug was verified as the gestation day (GD) 0. Fourteen dams from the colony were used in the experiment. The pregnant rats of the P generation were randomly divided into two groups (7 rats in each): one group as the control and the other for exposure to 1-BP.

1-BP was purchased from Kanto Chemical *Co., Ltd.* (Tokyo, Japan). Seven dams were exposed to 1-BP vapor at a concentration of 700 ppm (6 h/day) for 20 days from GDs 1 to 20 in an exposure chamber [13], whereas the other seven dams were provided fresh air in the same type of chamber. Both P generation groups were not allowed access to food and water during the inhalation period. Four weeks of 1-BP inhalation (700 ppm) resulted in apparent effects on the hippocampus in the adult rats [2]. Therefore, we first chose the concentration of 700 ppm to study the possible underlying mechanism of developmental neurotoxicity in prenatally 1-BP-exposed rats. The concentration of 1-BP was monitored with a gas chromatograph (GC353B FSL, GL Sciences *Inc.*, Japan) equipped with a flame ionization detector.

All the pregnant rats gave birth to offspring (termed the first filial (F1) generation) on GD 21. The day of birth was defined as PND 0. We randomly gathered 26 F1 rats from the 7 control litters and 22 F1 rats from the 7 1-BP-exposed litters. All the F1 pups were bred with their mother rats during the lactation period. In this study, 24 female and 3 male F1 rats were obtained from the 7 dams in the control group, and 18 female and 4 male F1 rats were obtained from the 7 dams in the 1-BP-exposed group, respectively. We examined the F1 rats for the general toxicity of 1-BP inhalation exposure, such as litter size, sex ratio, testicular descent, vaginal opening, ear opening, and survival rate. The body weight of the F1 rats was measured on PND 14.

KA administration and behavioral observation

KA was obtained from Wako Pure Chemical Industries, *Ltd.* (Osaka, Japan). KA (0.1, 0.5, and 2.0 mg/kg) was dissolved in phosphate buffered saline (PBS).

PBS or KA was intraperitoneally injected to the F1 rats at PND 14, after which the F1 rats were placed in a clear plastic cage, and the scratching and WDS were observed by video-recording for 180 min in a room for the behavioral observation. The room temperature was kept at about 25°C. The behavioral observation was conducted for 180 min between 09:30 and 15:30. The number of F1 rats that showed the scratching and the WDS behavior was counted and then the occurrence ratio was calculated. The duration and frequency of scratching and WDS were also measured. This experiment was approved by the Ethics Committee for Animal Care and Experimentation in accordance with the University of Occupational and Environmental Health, Japan.

All the chemicals used in this study were a reagent grade and purchased from commercial sources.

Statistical analysis

The difference in bodyweight between the F1 control and F1 1-BP-exposed groups was analyzed by Student *t*-test. The Mantel-Haeinzel procedure was utilized to see the whole effect of the prenatal inhalation of 1-BP on the occurrence ratio of scratching and WDS. When appropriate, Fisher's exact test determined significant differences. A two-way analysis of variance (ANOVA) was performed to clarify the effects of prenatal exposure to 1-BP and/or a dose of KA on the frequency and the duration of scratching and WDS. When appropriate, *post hoc* analysis by Scheffe's test determined significant differences, respectively. The criteria of significant difference was $P < 0.05$ in all the statistical analyses. Data represent mean ± standard error of the mean (SEM).

Results and Discussion

General toxicity of 1-BP inhalation exposure in F0 and F1 generations

There were no outward pathological signs related to 1-BP in the F0 rats. The body weights of the P generation dams treated with 1-BP were not significantly different from those in the control (fresh air) group (data not shown). None of the F1 rats died during the experimental period, indicating that the exposure seemed to cause little stress on the dams in this study. There was no difference in the sex ratio, survival rate, or other clinical signs between the F1 control and F1 1-BP-exposed groups, with the exception of body weight. The body weight in the female F1 1-BP-exposed group (32.5 ± 0.5 g) was significantly lower ($P < 0.01$, Student *t*-test) than that in the female F1 control group (35.0 ± 0.4 g). The 1-BP-exposed male F1 rats also had a lower body weight (33.5 ± 0.3 g) compared to the male F1 control group (37.0 ± 0.8 g) ($P < 0.01$, Student *t*-test). Our results were consistent with previous studies showing that prenatal exposure to 1-BP has no effects on postnatal survival rate, excluding the body weight [5].

Effect of prenatal exposure to 1-BP on behavioral responses

KA administration elicits immobilization, followed by scratching, WDS, forelimb clonus, and status epilepticus (continuous chronic-tonic posturing of all 4 limbs) [11]. It is also known that a low dose less than 3 mg/kg of KA elicits scratching and WDS but hardly ever elicits epileptic convulsions. Our preliminary study also showed that doses of KA higher than 4 mg/kg induced convulsive behaviors as well as scratching and WDS, thus we chose doses of 0.1, 0.5, and 2.0 mg/kg of KA.

Behavioral data obtained from both genders is combined in Tables 1 and 2, because it has been reported that there are no sex differences in KA induced-behaviors in pups [14].

In the F1 control group, all of the tested pups showed scratching during the 180 min after injection of PBS or KA. The frequency and duration of the scratching was significantly higher only at the dose of 2.0 mg/kg (Table 1). WDS were observed in 80% of the PBS-injected control pups and in all of the KA-injected control pups. A significantly higher frequency of WDS was observed at the dose of 2.0 mg/kg (Table 2). The behavioral changes induced by the KA doses of 0.1 and 0.5 mg/kg were similar to those of PBS, thus it can be said that these two doses are subclinical.

Spontaneous scratching and WDS were also observed in the F1 1-BP-exposed group. The occurrence ratio of scratching was 100% at all doses of KA (Table 1), whereas that of WDS was 40 to 60% in 0 to 0.5 mg/kg and 100% in 2.0 mg/kg of KA (Tables 2). The effect of prenatal exposure to 1-BP was observed

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.

Acknowledgments

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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 *Not*I, 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

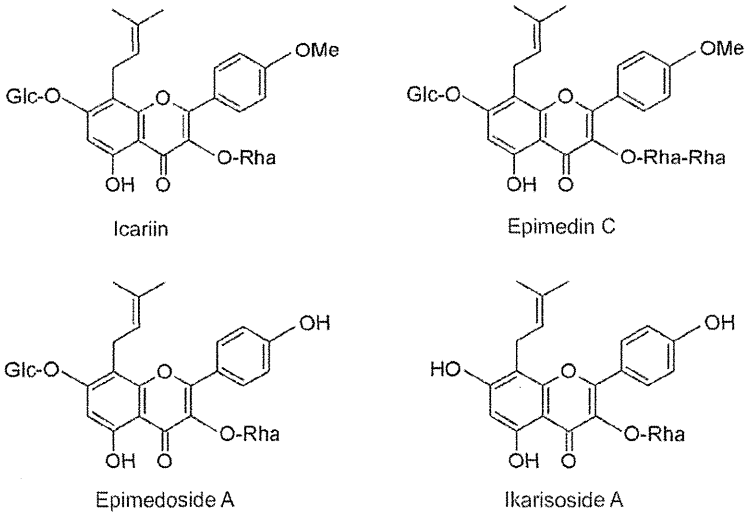

Fig. 1 Chemical structures of icariin, epimedin C, epimedoside A, ikarisoside A. The abbreviations Glc and Rha in the structures are glucose and rhamnose, respectively

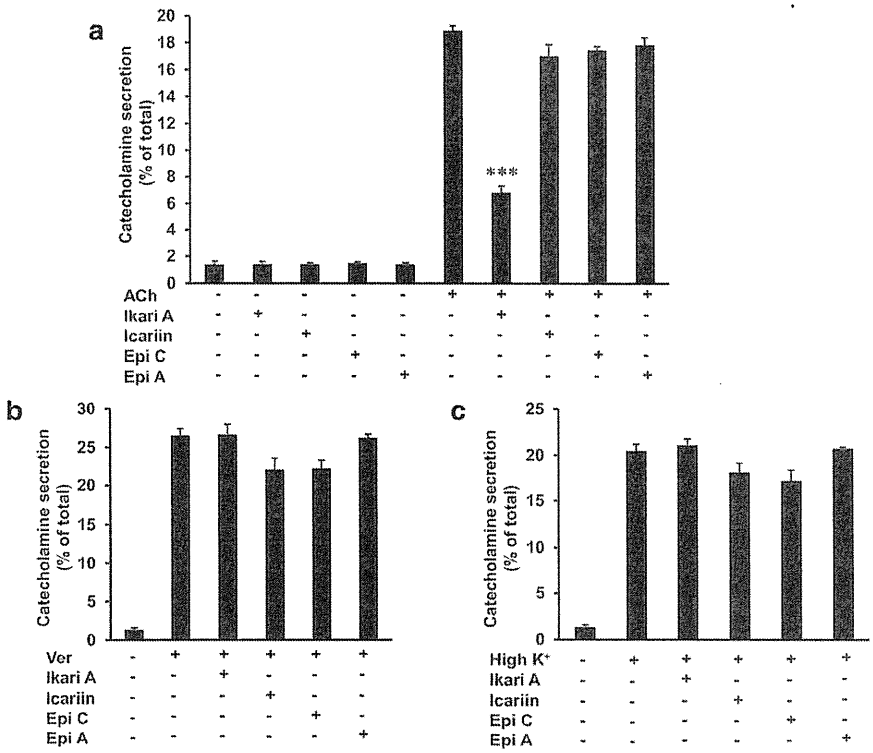

Fig. 2 Effects of ikarisoside A, icariin, epimedin C, or epimedoside A on catecholamine secretion induced by various secretagogues in cultured bovine adrenal medullary cells. After preincubation with cells with or without ikarisoside A (Ikari A) (10 μM), icariin (10 μM), epimedin C (Epi C) (10 μM), and 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–

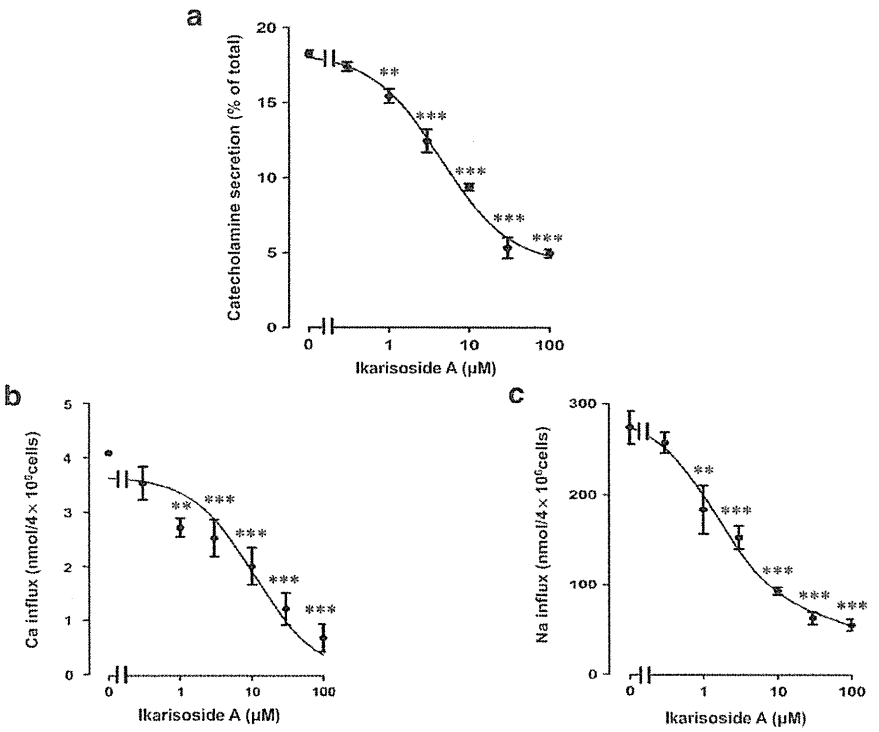

Fig. 3 Effects of ikarisoside A on catecholamine secretion (**a**), $^{45}Ca^{2+}$ influx (**b**), and $^{22}Na^{+}$ influx (**c**) induced by ACh. (**a**) After preincubation for 10 min with or without ikarisoside A (0.3–100 μM), cells were stimulated with ACh (300 μM) in the presence or absence of 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,

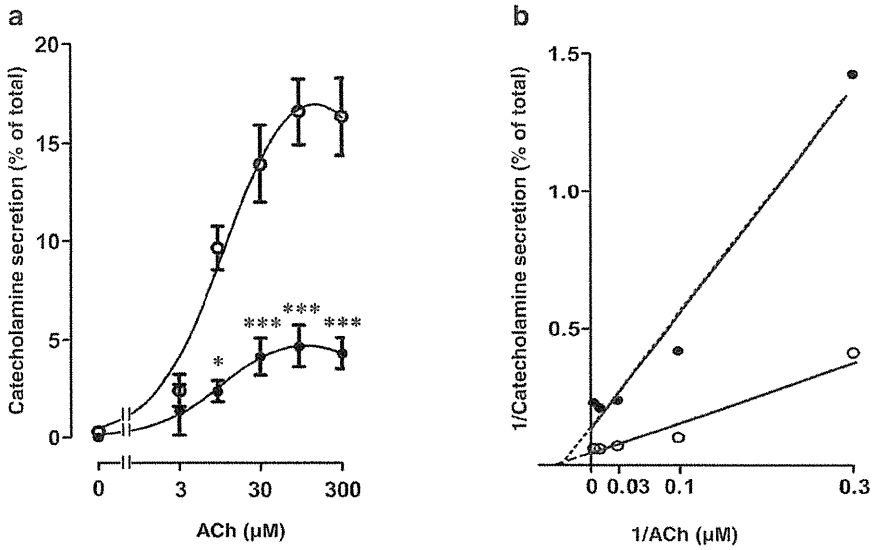

Fig. 4 Inhibitory mode of ikarisoside A on catecholamine secretion induced by ACh. **a** After preincubation for 10 min, cells were stimulated with (*black circle*) or without (*white circle*) ikarisoside A (10 μM) in the presence or absence of ACh (1–300 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means + SEM from three separate experiments carried out in triplicate. The data of ACh plus ikarisoside A are shown by subtracting basal secretion obtained in the presence of ikarisoside A. $^{*}P < 0.05$ and $^{***}P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test). **b** Double-reciprocal plot analysis of the data in (**a**)

ikarisoside A seems to inhibit nAChR-ion channels but not voltage-dependent Na^+ channels or voltage-dependent Ca^{2+} channels. Ikarisoside A inhibited Ca^{2+} influx and Na^+ influx induced by ACh in a concentration-dependent manner similar

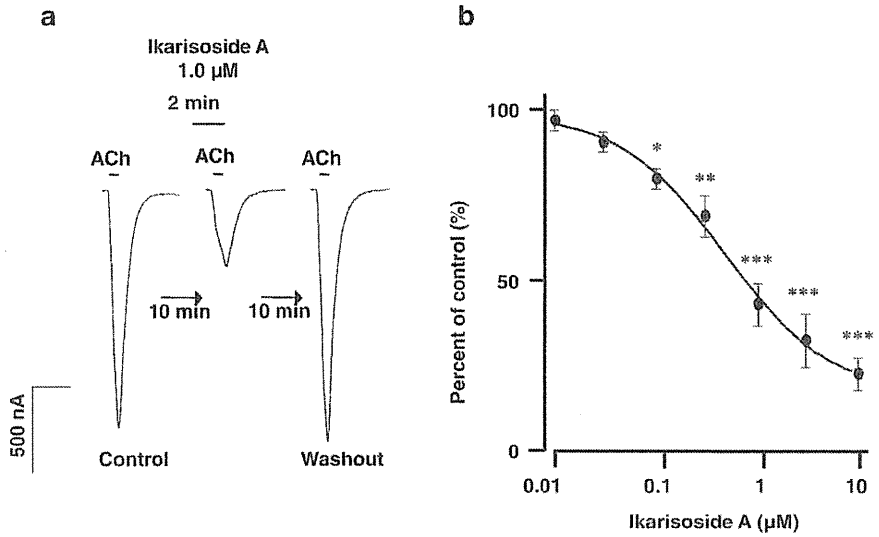

Fig. 5 Effects of ikarisoside A on peak ACh-induced inward currents in *Xenopus* oocytes expressing rat α3β4 nAChRs. **a** Representative traces from a single *Xenopus* oocyte are shown. The currents of ikarisoside A-treated oocytes were recorded 10 min after recording of the control currents, and the washout currents were obtained 10 min after ikarisoside A treatment. Ikarisoside A (1 μM) suppressed the currents induced by the EC_{50} (0.2 mM) of ACh, and the inhibitory effects were reversible. **b** Concentration-response curve for the inhibitory effects of ikarisoside A on ACh-induced currents. The peak current amplitude in the presence of ikarisoside A was normalized to that of the control and the effects are expressed as percentages of the control. Data are presented as means + SEM from four separate experiments carried out in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared to the control (based on one-way ANOVA with Dunnett's multiple comparison post hoc test). Nonlinear regression analysis was performed and the mean value of IC_{50} for ikarisoside A is 0.48 μM