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Inhibitory effects of pine nodule extract and its component, SJ-2, on acetylcholine-induced catecholamine secretion and synthesis in bovine adrenal medullary cells

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ABSTRACT

Extract of pine nodules (matsufushi) formed by bark proliferation on the surface of trees of *Pinus tabulaeformis* or *Pinus massoniana* has been used as an analgesic for joint pain, rheumatism, neuralgia, dysmenorrhea and other complaints in Chinese traditional medicine. Here we report the effects of matsufushi extract and its components on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells. We found that matsufushi extract (0.0003–0.005%) and its component, SJ-2 (5-hydroxy-3-methoxy-trans-stilbene) (0.3–100 μM), but not the other three, concentration-dependently inhibited catecholamine secretion induced by acetylcholine, a physiological secretagogue. Matsufushi extract (0.0003–0.005%) and SJ-2 (0.3–100 μM) also inhibited $^{45}Ca^{2+}$ influx induced by acetylcholine in a concentration-dependent manner, similar to its effect on catecholamine secretion. They also suppressed ^{14}C-catecholamine synthesis and tyrosine hydroxylase activity induced by acetylcholine. In *Xenopus* oocytes expressing α3β4 nicotinic acetylcholine receptors, matsufushi extract (0.00003–0.001%) and SJ-2 (1–100 μM) directly inhibited the current evoked by acetylcholine. The present findings suggest that SJ-2, as well as matsufushi extract, inhibits acetylcholine-induced catecholamine secretion and synthesis by suppression of nicotinic acetylcholine receptor-ion channels in bovine adrenal medullary cells.

1. Introduction

Pine nodules (matsufushi) of *Pinus tabulaeformis* or *Pinus massoniana* are formed by pine bark proliferation at places on the trunk or limbs that have undergone damage, either by pests or physical injury. The effective curative components in the matsufushi have been used as an analgesic for joint pain, rheumatism, neuralgia, dysmenorrhea and other complaints in Chinese traditional medicine.[1] Previous studies reported that the oxidation products of oleum terebinthinae have been reported to protect conscious guinea pigs against histamine-induced bronchoconstriction.[2] α-Pinene, a natural compound isolated from pine needle oil, has anti-liver cancer cell growth activity.[3]

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

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central nervous system. Catecholamines play very important roles in modulating heart rate, blood pressure, and blood glucose levels, and in the general reactions of the sympathetic nervous system.[4]

Adrenal medullary cells are derived from embryonic neural crests and share many properties with sympathetic postganglionic neurons. In cultured bovine adrenal medullary cells, there are at least three distinct types of ionic channels involved in catecholamine secretion[5]: nicotinic acetylcholine receptor (nAChR)-ion channels, voltage-dependent Na^+ channels, and voltage-dependent Ca^{2+} channels. ACh induces Na^+ influx via nAChR-ion channels, and then it induces Ca^{2+} influx and subsequent catecholamine secretion.[5] Veratridine and high K^+ stimulate voltage-dependent Na^+ channels and voltage-dependent Ca^{2+} channels, respectively, which also induce catecholamine secretion from the cells. On the other hand, stimulation of catecholamine synthesis induced by ACh is associated with the activation of tyrosine hydroxylase, the rate-limiting step of catecholamine biosynthesis[6] in cultured bovine adrenal medullary cells.[7] Adrenal medullary cells are a good model for the detailed analysis of a drug's actions on catecholamine secretion and synthesis.[8,9]

Previous studies have reported that pine oil extract has analgesic and antitumor effects and a dissolving effect on gallstones.[10] Extract of the oil from Siberian pine (*Pinus sibirica*) has been found to have an anti-inflammatory effect.[11] The pharmacological effects of matsufushi extract, however, have not been studied in the sympathetic nervous system. In the present study, we investigated the effects of matsufushi extract and compounds isolated from matsufushi extract on catecholamine secretion and synthesis. We found that matsufushi extract and SJ-2, but not the other three components, inhibited ACh-induced catecholamine secretion and synthesis by suppression of nAChR-ion channels in the cell.

2. Materials and methods

2.1. Materials

Oxygenated Krebs–Ringer phosphate (KRP) buffer was used throughout. 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 indicated sources as followings: Eagle's minimum essential medium (Eagle's MEM) was from Nissui Pharmaceutical (Tokyo, Japan); collagenase was from Nitta Zerachin (Osaka, Japan); calf serum was from 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).

2.2. Isolation and purification of ethanol extracts from matsufushi

Pine nodules (matsufushi) of *Pinus tabulaeformis carr.* were purchased from Anguoshi Tongli Herbal Medicine CO., Ltd. (Lot No. songje201206) (Anguo, Hebei, China). SJ-2 (5-hydroxy-3-methoxy-trans-stilbene), SJ-3 (Stigmast-5-en-3-ol), SJ-4 (Phenol, 3-[(1E)-2-(4-hydroxyphenyl)ethenyl]-5-methoxy) and SJ-16 (15- hydroxydehydroabietic acid) were purified from matsufushi by high performance liquid chromatography (HPLC) (Waters, Milford, Massachusetts, USA). In brief, baked and crushed, the pine nodule powder was dissolved in pure water and filtered through a fiber membrane to remove macro-impurities. The filtrate was concentrated by gradually extracting with petroleum ether. The final extract was dissolved in 95% ethanol in the rest and used for the present study as matsufushi extract. The extract of matsufushi (100 g) was subjected to silica gel column chromatography (Qingdao Haiyang Chemical Co., Ltd., Qingdao, Shandong, China) [CH_2Cl_2-EtOAc-MeOH (8:1:0.1 → 0:0:100), v/v/v] to give eleven fractions (Fr. A1–11). Fraction A1 (15.4 g) was subjected to silica gel column chromatography [petroleum ether-EtOAc-MeOH (12:1:0.1 → 10:1:0.1, v/v/v)] to yield 5 fractions (Fr. A1-1–5). Fraction A1-3 (2.8 g) was purified by Sephadex LH-20 (MeOH) to yield SJ-2 (5-hydroxy-3-methoxy-trans-stilbene) (500 mg). Fraction A2 (3.5 g) was separated by silica gel column chromatography [petroleum ether-EtOAc-MeOH (12:1:0.1 → 4:1:0.1, v/v/v)] to yield 6 fractions (Fr. A2-1–6). SJ-3 (Stigmast-5-en-3-ol) (11 mg) was obtained from Fr. A2-5 (20 mg) through recrystallization method. Fraction A2-7 (50 g) was subjected to Sephadex LH-20 (MeOH) to yield SJ-4 (Phenol, 3-[(1E)-2-(4-hydroxyphenyl)ethenyl]-5-methoxy) (20 mg). Fraction A5 (11 g) was separated by silica gel column chromatography [petroleum ether-acetone (9:2 → 3:2, v/v)] to yield 12 fractions (Fr. A5-1–12). Fraction A5-12 (45 mg) was subjected to Sephadex LH-20 (MeOH) to yield SJ-16 (15-hydroxydehydroabietic acid) (7 mg). We decided the molecular weights of SJ-2, SJ-3, SJ-4, and SJ-16 by fast atom bombardment mass spectrometry (FAB-MS) (Micromass Co., Manchester, UK), 9.4T Apex Qe high-resolution-electrospray ionization-mass spectrometry (HR-ESI-MS) (Bruker Co., Karlsruhe, GER) and 9.4T Apex Qe quantum field theory-mass spectrometry (Q-FT-MS) (Bruker Co., Karlsruhe, GER). Their chemical structures of SJ-2, SJ-3, SJ-4, and SJ-16 were determined by the nuclear magnetic resonance (NMR) spectra which were recorded with Varian UNITYINOVA 600 at 599.8 (1H) and 150.8 MHz (13C) (Varian, Inc. CA, USA), chemical shifts given in δ [ppm] with tetramethylsilane (TMS) as internal standard. We identified the structures of SJ-2, SJ-3, SJ-4, and SJ-16 as 5-hydroxy-3-methoxy-trans-stilbene. Their structures are shown in Fig. 3. Matsufushi extract, SJ-2, and other compounds 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.

2.3. Isolation and primary culture of bovine adrenal medullary cells

Bovine adrenal glands, the medullary cells were isolated by collagenase digestion according to the method as reported previously.[12] The cells were plated 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).

2.4. Catecholamine secretion from cultured bovine adrenal medullary cells

Catecholamines were measured as described previously.[12] After preincubated with or without matsufushi (0.0001–0.005%), SJ-2 (0.3–100 μM) or other components (10 μM) at 37 °C for 10 min, cells (10^6/well) were incubated with or without matsufushi (0.0001–0.005%), SJ-2 (0.3–100 μM) or other components (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. Catecholamines (noradrenaline and adrenaline) secreted into the medium were measured.[12]

2.5. $^{45}Ca^{2+}$ influx by the cells

After preincubation with or without matsufushi extract (0.0001–0.005%) or SJ-2 (0.3–100 μM) at 37 °C for 10 min, cells (4×10^6/dish) were incubated with 1.5 μCi of $^{45}CaCl_2$ at 37 °C for 5 min with or without 300 μM ACh and matsufushi (0.0001–0.005%) or SJ-2 (0.3–100 μM) in KRP buffer. The influx of $^{45}Ca^{2+}$ was measured, as reported previously.[5]

2.6. ^{14}C-catecholamine synthesis from [^{14}C] tyrosine in the cells

For measurement of ^{14}C-Catecholamine synthesis, cells (4×10^6/dish) were incubated with 20 μM L-[U-^{14}C]tyrosine (1.0 μCi) in KRP buffer in the presence or absence of various concentrations of matsufushi extract (0.0001–0.005%) or SJ-2 (0.3–100 μM) and 300 μM ACh at 37 °C for 20 min after preincubation for 10 min. ^{14}C-Catecholamines in the cells were separated using a Duolite C-25 columns (H^+-type, 0.4 × 7.0 cm) and counted for the radioactivity.[7]

2.7. Tyrosine hydroxylase activity

After preincubation for 10 min, cells with or without matsufushi extract (0.0001–0.005%) or SJ-2 (0.3–100 μM) and 300 μM ACh were exposed to 200 μl of KRP buffer, supplemented with 18 μM L-[1-^{14}C]tyrosine (0.2 μCi) for 10 min at 37 °C. Tyrosine hydroxylase activity was measured to absorb the $^{14}CO_2$ released by the cells, and the radioactivity was counted.[9]

2.8. Expression of α3β4 nAChRs in Xenopus oocytes and electrophysiological recordings

Isolation and microinjection of *Xenopus* oocytes was performed as previous studies.[9] The cDNAs encoding the α3 and β4 subunits of rat neuronal nAChR were kindly provided from Dr. James W. Patrick (Division of Neuroscience, Baylor College of Medicine, TX, USA). Adult female *Xenopus laevis* frogs were obtained from Kyudo Co., Ltd. (Saga, Japan). 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. Each oocytes was 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, as previously reported.[9] We examined the effects of matsufushi or SJ-2 on a concentration of ACh that produced 50% of the maximal effect (EC_{50} = 0.2 mM) of ACh.

2.9. 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, Tukey'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).

3. Results

3.1. Inhibitory effects of matsufushi extract on basal and various secretagogue-induced catecholamine secretion in adrenal medullary cells

ACh (300 μM), an agonist of nAChRs, caused catecholamine secretion corresponding to 17.76 ± 0.22% of total catecholamines in the cells (Fig. 1). When the cells were treated with matsufushi extract at 0.001% for 10 min, the catecholamine secretion induced by ACh was reduced to 14.14 ± 0.62%. 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 27.13 ± 0.41% (Fig. 1) and 14.72 ± 0.60% (Fig. 1) of the total catecholamines, respectively. Matsufushi extract at 0.001% had little effect on basal or veratridine- and 56 mM K^+-induced catecholamine secretion (Fig. 1).

3.2. Effects of various concentrations of matsufushi extract on ACh-induced catecholamine secretion and $^{45}Ca^{2+}$ influx

The effects of matsufushi extract on ACh-induced catecholamine secretion and $^{45}Ca^{2+}$ influx were examined. Matsufushi extract (0.0001%–0.005%) significantly inhibited ACh-induced secretion of catecholamines (Fig. 2A). Matsufushi extract also concentration-dependently inhibited ACh-induced $^{45}Ca^{2+}$ influx (Fig. 2B). The half-maximal inhibitory concentrations (IC_{50} values) of matsufushi in catecholamine secretion and $^{45}Ca^{2+}$ influx were calculated to be 0.0025% and 0.0013%, respectively.

3.3. The structures of four compounds isolated from matsufushi extract

Four compounds, SJ-2, SJ-3, SJ-4 and SJ-16, were isolated from matsufushi extract. The structures of these four compounds are shown in Fig. 3.

3.4. Effects of the four compounds on catecholamine secretion induced by ACh

When the cells were treated with each compound or their mixture at 10 μM for 10 min, SJ-2 and the mixture of four compounds (Mix4) strongly reduced the catecholamine secretion induced by ACh (14.58 ± 0.18% of total catecholamines) to 8.39 ± 0.32% and 6.40 ± 0.15% of the total, respectively, whereas the other three had little effect (Fig. 4).

3.5. Concentration-inhibition curves for the effects of SJ-2 on ACh-induced catecholamine secretion and $^{45}Ca^{2+}$ influx

Treatment of cells with SJ-2 (0.3–100 μM) significantly inhibited ACh-induced secretion of catecholamines (Fig. 5A). SJ-2 also

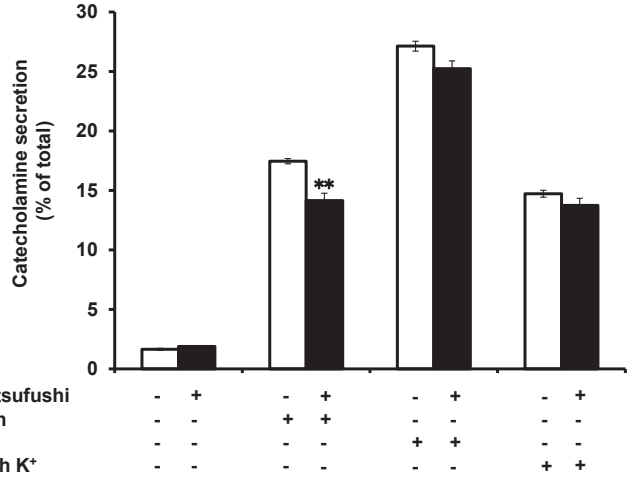

Fig. 1. Effects of matsufushi extract on catecholamine secretion induced by various secretagogues in cultured bovine adrenal medullary cells. After preincubation of cells with or without matsufushi extract (0.001%) for 10 min, the cells (10^6/well) were incubated with or without matsufushi extract (0.001%), ACh (300 μM), veratridine (100 μM) or 56 mM K^+ 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. $^{**}P < 0.01$, compared with ACh alone.

inhibited ACh-induced $^{45}Ca^{2+}$ influx in a concentration-dependent manner (Fig. 5B). The half-maximal inhibitory concentrations (IC_{50} values) of SJ-2 in catecholamine secretion and $^{45}Ca^{2+}$ influx were determined to be 10.3 and 10.0 μM, respectively.

3.6. Effects of matsufushi extract and SJ-2 on ACh-induced response in α3β4 nAChRs expressed in Xenopus oocytes

As shown in Fig. 6A and C, matsufushi extract and SJ-2 reversibly inhibited ACh (0.2 mM)-induced Na^+ currents. Matsufushi extract (0.00003%–0.001%) (Fig. 6C) and SJ-2 (1–100 μM) (Fig. 6D) significantly suppressed those currents. The half-maximal inhibitory concentrations (IC_{50} values) of matsufushi extract and SJ-2 were found to be 0.00019% and 3.12 μM, respectively.

3.7. Inhibitory effect of matsufushi extract and SJ-2 on ^{14}C-catecholamine synthesis and tyrosine hydroxylase activity

ACh (300 μM) increased the synthesis of ^{14}C-catecholamines from [^{14}C]tyrosine about two-fold in bovine adrenal medullary cells. Treatment of cells with matsufushi extract and SJ-2 inhibited the stimulatory effect of ACh on ^{14}C-catecholamine synthesis at concentrations of 0.001% and 0.003%, and 10 and 30 μM, respectively (Fig. 7A). SJ-2, but not matsufushi extract, slightly but significantly inhibited the basal synthesis of ^{14}C-catecholamines.

Matsufushi extract (0.001% and 0.003%) and SJ-2 (10 and 30 μM) inhibited the tyrosine hydroxylase activity induced by ACh (Fig. 7B). SJ-2 also slightly inhibited the basal enzyme activity.

4. Discussion

Matsufushi are the nodules formed by pine bark proliferation at the sites of surface damage to a pine tree. In the present study, we examined the effects of matsufushi extract and its components, SJ-2, SJ-3, SJ-4, and SJ-16, on catecholamine secretion and $^{45}Ca^{2+}$ influx. We demonstrated that matsufushi extract and SJ-2, but not the other three components, inhibited the secretion and synthesis of catecholamines induced by ACh in cultured bovine adrenal medullary cells. To the best of our knowledge, this is the first direct evidence of the inhibitory effects of matsufushi extract and its component SJ-2 on the catecholamine system.

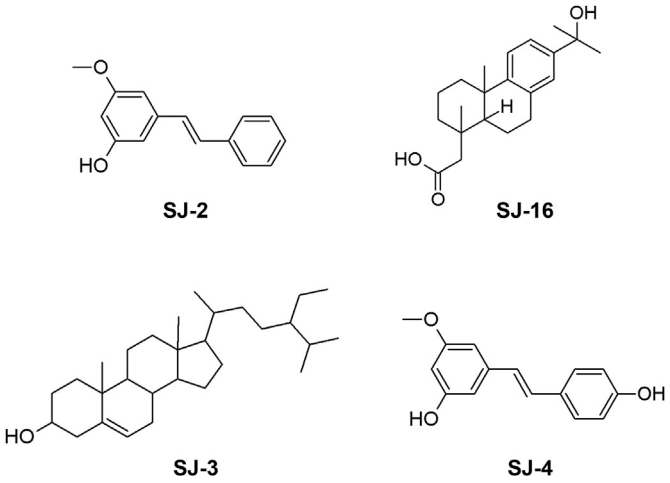

Fig. 3. Chemical structures of SJ-2, SJ-3, SJ-4, and SJ-16.

4.1. Inhibitory effects of matsufushi extract and SJ-2 on ACh-induced catecholamine secretion

Matsufushi extract significantly inhibited the catecholamine secretion induced by ACh, but not that induced by veratridine or 56 mM K^+ in adrenal medullary cells. Our previous study[5] reported that ACh and veratridine activated nAChR-ion channels and voltage-dependent Na^+ channels, respectively, which, in turn, caused Na^+ influx, subsequent Ca^{2+} influx, and finally catecholamine secretion. Addition of 56 mM K^+ depolarizes cell membranes

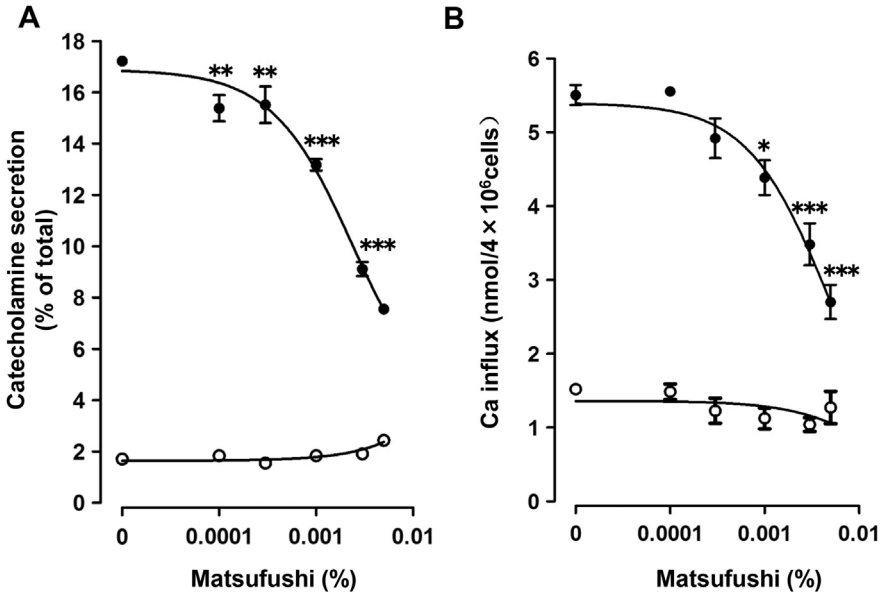

Fig. 2. Effects of matsufushi extract on catecholamine secretion (A) and $^{45}Ca^{2+}$ influx (B) induced by ACh. (A) After preincubation for 10 min with or without matsufushi extract (0.0003–0.005%), cells were stimulated with ACh (300 μM) in the presence or absence of matsufushi extract (0.0003–0.005%) 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) After preincubation for 10 min, cells were stimulated with ACh (300 μM) and 1.5 μCi of $^{45}CaCl_2$ in the presence or absence of matsufushi extract (0.0003–0.005%) for another 5 min at 37 °C. $^{45}Ca^{2+}$ influxwas measured, and was expressed as nmol/4 × 10^6 cells. Data are means ± SEM from three separate experiments carried out in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with ACh alone.

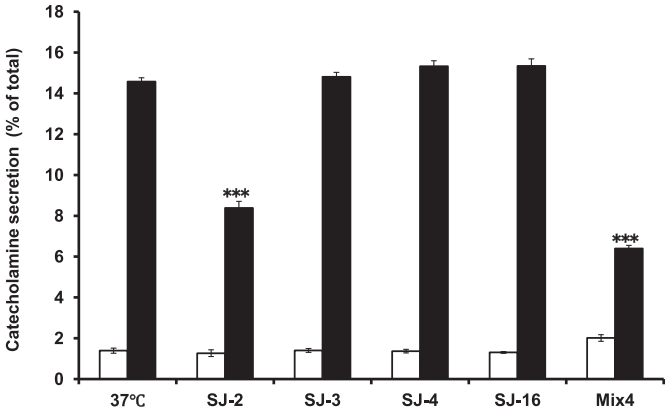

Fig. 4. Effects of SJ-2, SJ-3, SJ-4, SJ-16, and their mixture on catecholamine secretion induced by ACh in cultured bovine adrenal medullary cells. After preincubation of cells with or without SJ-2 (10 μM), SJ-3 (10 μM), SJ-4 (10 μM), C-16 (10 μM) and their mixture (Mix 4) (10 μM) for 10 min, the cells (10^6/well) were incubated with or without each compound and their mixture 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.

and activates voltage-dependent Ca^{2+} channels. In the present study, matsufushi extract had little effect on veratridine- and 56 mM K^+-induced catecholamine secretion. Therefore, matsufushi extract seems to inhibit nAChR-ion channels but not voltage-dependent Na^+ channels or voltage-dependent Ca^{2+} channels. Matsufushi extract and its component SJ-2 inhibited the Ca^{2+} influx induced by ACh in a concentration-dependent manner, which was similar to their effects on catecholamine secretion. Ca^{2+} plays an important role as the coupler in stimulus-secretion coupling.[13] From these findings, it is likely that matsufushi extract and SJ-2 inhibit catecholamine secretion by suppressing the Ca^{2+} influx induced by ACh.

4.2. Structure–activity relationship of SJ-2 for inhibition of nAChR-ion channels

We used four compounds isolated from matsufushi extract. Of these, only SJ-2 inhibited the functioning of nAChR-ion channels. SJ-2 is structurally very similar to SJ-4. SJ-4 has a hydroxyl group at the 4 position, whereas SJ-2 has nothing at this position. Judging from the differences in their structures, this result suggests that the hydroxyl group at the 4 position of SJ-4 may induce stereo-specific interference when the flavonol glycoside interacts with nAChRs. On the other hand, we previously reported that resveratrol (trans-3, 4′, 5-trihydroxystilbene), a grape polyphenol, inhibits catecholamine secretion induced by ACh, veratridine, and 56 mM K^+ (IC_{50} = 20.4, 11.0, and 62.8).[9] The chemical structure of resveratrol (stilbene) is very similar to those of SJ-2 and SJ-4 whereas resveratrol has three hydroxyl groups at the 3, 4′, and 5 positions. More information, however, will be needed to clarify the structural relation between the inhibitory effect of SJ-2 and the function of the ion channels.

4.3. Inhibitory mode of matsufushi extract and SJ-2 on the ACh-induced inward current in Xenopus oocytes expressing α3β4 nAChRs

To study the mechanism by which matsufushi extract and SJ-2 inhibit the ACh-induced catecholamine secretion and ACh-induced $^{45}Ca^{2+}$ influx, we examined their direct effect on ACh-induced currents in *Xenopus* oocytes expressing α3β4 nAChRs. Matsufushi extract and SJ-2 directly inhibited the Na^+ current in a concentration-dependent manner, suggesting that matsufushi and SJ-2 suppress ACh-induced catecholamine secretion and Ca^{2+} influx via inhibiting nAChR in adrenal medullary cells.

4.4. Inhibitory effect of matsufushi extract and SJ-2 on catecholamine synthesis and tyrosine hydroxylase activity

Matsufushi extract and SJ-2 inhibited ACh-induced catecholamine synthesis and ACh-induced tyrosine hydroxylase activity in

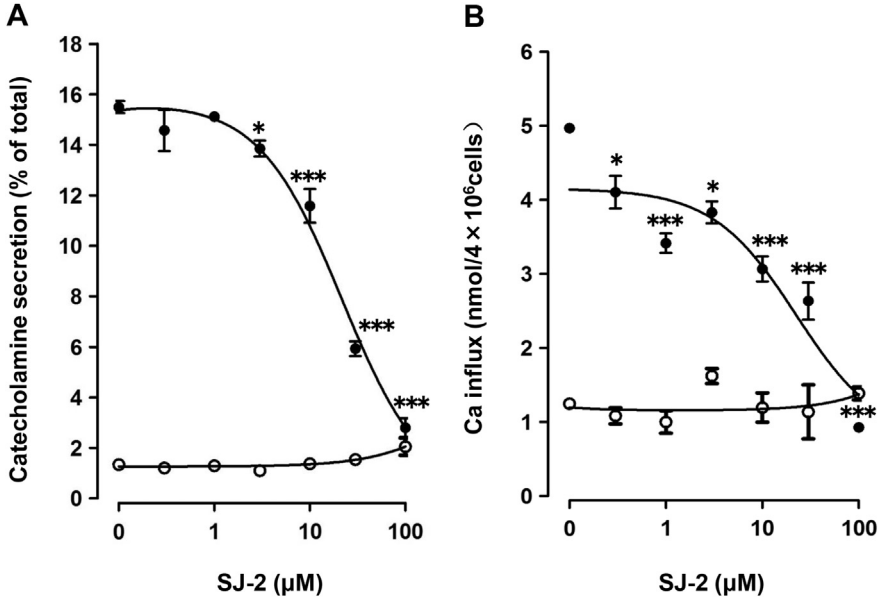

Fig. 5. Effects of SJ-2 on catecholamine secretion (A) and $^{45}Ca^{2+}$ influx (B) induced by ACh. (A) After preincubation for 10 min with or without SJ-2 (0.3–100 μM), cells were stimulated with ACh (300 μM) in the presence or absence of SJ-2 (0.3–100 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. (B) After preincubation for 10 min, cells were stimulated with ACh (300 μM) and 1.5 μCi of $^{45}CaCl_2$ in the presence or absence of SJ-2 (0.3–100 μM) for another 5 min at 37 °C. $^{45}Ca^{2+}$ influx was measured, and was expressed as nmol/4 × 10^6 cells. Data are means ± SEM from three separate experiments carried out in triplicate. $^{*}P < 0.05$ and $^{***}P < 0.001$, compared with ACh alone.

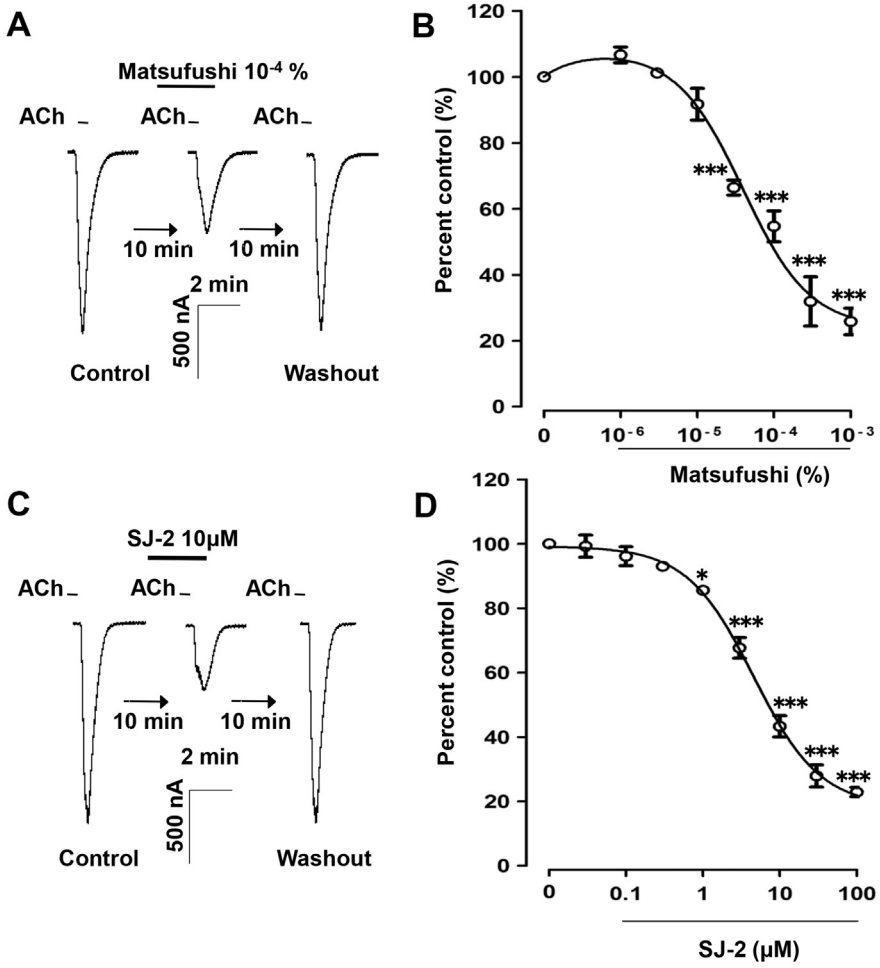

Fig. 6. Effects of matsufushi extract and SJ-2 on peak ACh-induced inward currents in *Xenopus* oocytes expressing rat α3β4 nAChRs. Representative traces from a single *Xenopus* oocyte are shown. The currents of matsufushi extract (Fig. 6A) and SJ-2 (Fig. 6C)-treated oocytes were recorded for 10 min after recording of the control currents, and the washout currents were obtained at 10 min after matsufushi extract and SJ-2 treatment. Matsufushi extract (0.0001%) and SJ-2 (10 μM) suppressed the currents induced by the EC_{50} (0.2 mM) of ACh, and the inhibitory effects were reversible. Concentration-response curve for the inhibitory effects of matsufushi extract (Fig. 6B) and SJ-2 (Fig. 6D) on ACh-induced currents. The peak current amplitude in the presence of matsufushi extract and SJ-2 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. $^{*}P < 0.05$ and $^{***}P < 0.001$, compared to the control. Nonlinear regression analysis was performed and the mean values of IC_{50} for matsufushi and SJ-2 are 0.00019% and 3.116 μM, respectively.

the bovine adrenal medulla cells. It is well-known that Ca^{2+} plays an important role as a coupler in stimulus-synthesis coupling.[7] In the present study, we observed that matsufushi extract and SJ-2 suppressed the $^{45}Ca^{2+}$ influx by inhibiting nAChR-ion channels. Therefore, it is likely that matsufushi extract and SJ-2 inhibit the catecholamine synthesis and tyrosine hydroxylase activity induced by ACh via the suppression of Ca^{2+} influx in cultured bovine adrenal medulla cells.

4.5. Pharmacological significance of the inhibitory effects of matsufushi extract and SJ-2 on the catecholamine system

Although the human serum concentrations of matsufushi extract and SJ-2 have not been reported yet, extracts of *P. massoniana* bark (140 μg/ml = 0.014%) showed an anti-metastatic effect in Hela cells.[14] In addition, ethanol extracts of *Pinus densiflora Sieb. et Zucc.* (100 μg/ml = 0.01%) were shown to alleviate lipogenesis and oxidative stress during oleic acid-induced steatosis in HepG2 cells.[15] In *in vivo* studies, *Pinus sibirica* oil extracts administered orally at a dose of 300 mg/kg showed anti-inflammatory effects.[11] In the near future, it should be clarified whether SJ-2 used in the present study would be relevant as potential supplements for human health.

Although catecholamines play an important role in the regulation of normal function in the central and peripheral sympathetic nervous systems, strong and prolonged stress causes their release in massive amounts, which can lead to cardiovascular diseases such as hypertension, atherosclerosis, coronary heart disease, and heart failure.[4,16] Chronic heart failure is reported to be associated with the activation of the sympathetic nervous system as manifested by increased circulating catecholamines.[4,17] Furthermore, the stress hormone adrenaline stimulates β_2-adrenoceptors, an effect that in turn activates the Gs protein/cyclic AMP-dependent protein kinase and the β-arrestin-mediated signaling pathway, reduces the p53 level, and induces DNA damage.[18]

Our recent review[16] and studies reported that daidzein, a soy isoflavone,[19] nobiletin, a citrus polymethoxy flavones,[20] and ikarisoside A, a natural flavonol glycoside derived from plants of the genus *Epimedium*,[21] suppress the secretion and synthesis of catecholamines induced by ACh in cultured bovine adrenal medullary cells. The present findings would support the idea that matsufushi extract and its constituent SJ-2 suppress the induction of catecholamine system hyperactivity by strong stress or emotional

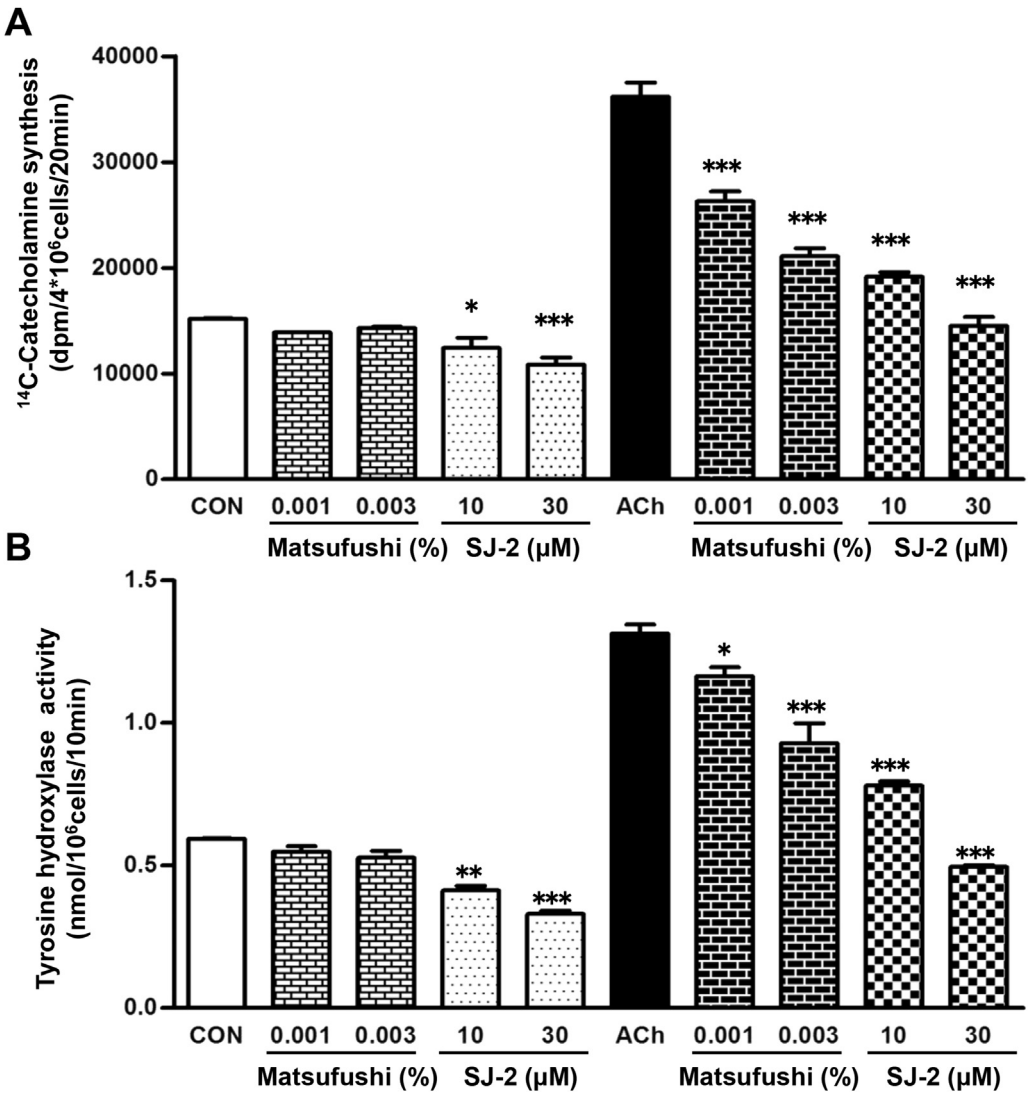

Fig. 7. Effects of matsufushi extract and SJ-2 on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine (Fig. 7A) and tyrosine hydroxylase activity (Fig. 7B) in the cells. After preincubation for 10 min with or without matsufushi extract and SJ-2, cells (4×10^6/dish) were incubated with L-[U-^{14}C] tyrosine (20 μM, 1 μCi) in the presence or absence of matsufushi extract (0.001 and 0.003%) and SJ-2 (10, 30 μM) and with or without 300 μM ACh at 37 °C for 20 min. The ^{14}C-catecholamines formed were measured (Fig. 7A). After preincubation with or without matsufushi extract (0.001 and 0.003%) and SJ-2 (10 and 30 μM) for 10 min, cells (10^6/well) were incubated with L-[1-^{14}C] tyrosine (18 μM, 0.2 μCi) in the presence or absence of matsufushi extracts (0.001 and 0.003%) and SJ-2 (10 and 30 μM) and with or without 300 μM ACh at 37 °C for 10 min, and tyrosine hydroxylase activity was measured (Fig.7B). Data are means ± SEM from three separate experiments carried out in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with ACh alone or control (CON).

excitation which evokes the secretion of ACh from the splanchnic nerves. To confirm this possibility, further *in vivo* experiments will be required in the near future.

In summary, we have demonstrated that matsufushi extract and its component SJ-2 inhibit the catecholamine secretion and synthesis induced by ACh via inhibition of nAChRs-ion channels in the adrenal medulla and probably in the sympathetic neurons.

Conflicts of interest

The authors have conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2017.03.006.

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