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Full Paper

Effects of Selective Estrogen Receptor Modulators on Plasma Membrane Estrogen Receptors and Catecholamine Synthesis and Secretion in Cultured Bovine Adrenal Medullary Cells

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Abstract. We previously reported the occurrence and function of plasma membrane estrogen receptors in cultured bovine adrenal medullary cells. Here we report the effects of raloxifene and tamoxifen, selective estrogen receptor modulators, on plasma membrane estrogen receptors and catecholamine synthesis and secretion in these cells. Raloxifene caused dual effects on the specific binding of [^{3}H]17β-estradiol to the plasma membranes isolated from bovine adrenal medulla; that is, it had a stimulatory effect at 1.0 – 10 nM but an inhibitory effect at 1.0 – 10 μM, whereas tamoxifen (1.0 nM – 10 μM) increased binding at all concentrations (except for 100 nM). Tamoxifen at 100 nM caused a significant increase in basal ^{14}C-catecholamine synthesis from [^{14}C]tyrosine, whereas tamoxifen and raloxifene at higher concentrations attenuated basal and acetylcholine-induced ^{14}C-catecholamine synthesis. Raloxifene (0.3, 1.0, and 3 – 100 μM) and tamoxifen (10 – 100 μM) also suppressed catecholamine secretion and $^{45}Ca^{2+}$ and $^{22}Na^{+}$ influx, respectively, induced by acetylcholine. Raloxifene (1.0 μM) inhibited Na^{+} current evoked by acetylcholine in *Xenopus* oocytes expressing $\alpha4\beta2$ neuronal nicotinic acetylcholine receptors. The present findings suggest that raloxifene and tamoxifen at low concentrations allosterically modulate plasma membrane estrogen receptors and at high concentrations inhibit acetylcholine-induced catecholamine synthesis and secretion by inhibiting Na^{+} and Ca^{2+} influx in bovine adrenal medulla.

Keywords: adrenal medulla, catecholamine synthesis and secretion, plasma membrane estrogen receptor, raloxifene, selective estrogen receptor modulator

Introduction

Selective estrogen receptor modulators (SERMs) are compounds that bind to nuclear or classical estrogen receptors (ERs) and exert either estrogenic or anti-estrogenic effects depending on the specific organs (1, 2). At present, at least two SERMs, tamoxifen for the treatment and prevention of breast cancer and raloxifene for the prevention of osteoporosis, are clinically available in Japan (3). Although the precise molecular mechanisms by which SERMs exert their clinical effects are unknown, their estrogenic or anti-estrogenic actions at target tissues are mediated through two ERs, ERα, and ERβ (4). In addition to the genomic ER actions, several lines of evidence have shown that SERMs acutely modulate ionic current through neuronal nicotinic acetylcholine receptors (nAChRs)-ion channels (5, 6) and also modulate functions of the cardiovascular systems (3). Furthermore, estrogens and raloxifene are reported to inhibit catecholamine secretion from rat and bovine

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adrenal medullary cells (7) and PC12 cells (8). These results suggest that SERMs directly affect ion channels and subsequent cellular functions in a non-genomic manner.

Adrenal medullary cells are derived from the embryonic neural crest and share many physiological and pharmacological properties with postganglionic sympathetic neurons. Stimulation of AChRs in the cells increases the synthesis of catecholamines and causes the secretion of catecholamines into the systemic circulation (9, 10). In bovine adrenal medullary cells, at least three distinct types of ionic channels are involved in catecholamine secretion (11): nAChR-ion channels, voltage-dependent Na^+ channels, and voltage-dependent Ca^{2+} channels. In these cells, previous studies have shown that either carbachol (a synthetic derivative of ACh)-induced Na^+ influx via nAChR-ion channels or veratridine-induced Na^+ influx via voltage-dependent Na^+ channels increases Ca^{2+} influx via voltage-dependent Ca^{2+} channels, a prerequisite for the secretion (7, 11) and synthesis (10) of catecholamines. In contrast, high K^+ directly gates voltage-dependent Ca^{2+} channels to increase Ca^{2+} influx without increasing Na^+ influx (11).

Previously, we reported the occurrence and pharmacological characterization of estrogen receptors in the plasma membrane of bovine adrenal medulla (12). Furthermore, phytoestrogens such as daidzein (13) and resveratrol (14) increased catecholamine synthesis through the plasma membrane estrogen receptors. In the present study, we examined the effects of two SERMs, raloxifene and tamoxifen, on [^{3}H]17β-estradiol (17β-E_2) binding to the membrane estrogen receptors, as well as catecholamine synthesis and secretion in cultured bovine adrenal medullary cells. We found that SERMs allosterically modulate [^{3}H]17β-E_2 binding to plasma membrane estrogen receptors and positively or negatively influence catecholamine synthesis and secretion in the cells.

Materials and Methods

Materials

Oxygenated Krebs-Ringer phosphate (KRP) buffer was used throughout. Its composition is as follows: 154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4, and 10 mM glucose, adjusted pH to 7.4. Reagents were obtained from the following sources: Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo); calf serum (Cell Culture Technologies, Zürich, Switzerland); collagenase (Nitta Zerachin, Osaka); raloxifene, tamoxifen, 17β-E_2, ACh, veratridine (Sigma Chemical Co., St. Louis, MO, USA); [2,4,6,7-^{3}H]17β-E_2 (3515 GBq/mmol), [^{22}Na]Cl, [^{45}Ca]Cl_2, and L-[U-^{14}C]tyrosine (Perkin-Elmer, Ltd., Boston, MA, USA). Raloxifene and tamoxifen were dissolved in 100% dimethyl sulfoxide and then diluted in a reaction medium before use at a final concentration of dimethyl sulfoxide not exceeding 0.5% unless otherwise specified.

Isolation and primary culture of bovine adrenal medullary cells

Bovine adrenal medullary cells were isolated by collagenase digestion of adrenal medullary slices according to the previously reported method (15, 16). 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 per dish (35 mm dish; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) or 10^6 cells per well (24-well plate; Corning Life Science, 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.

[^{3}H]17β-E_2 binding to plasma membranes isolated from adrenal medulla

Plasma membranes were isolated from bovine adrenal medulla as described previously (12, 13). The specific binding of [^{3}H]17β-E_2 was determined by incubating plasma membranes (30 μg protein) in Krebs-Ringer HEPES (KRH) buffer (composition: 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, and 25 mM HEPES-Tris, pH 7.4) (final volume of 200 μL) with various concentrations (0.001 – 10 μM) of raloxifene or tamoxifen and [^{3}H]17β-E_2 (5 nM, 0.1 μCi) at 4°C for 30 min. Then [^{3}H]17β-E_2 bound to the membranes was separated from free ligand by filtration through a GF/C glass fiber filter (Whatman, Maidstone, UK), and the filter was washed 3 times with the ice-cold binding buffer. Specific binding of [^{3}H]17β-E_2 was defined as the total binding minus non-specific binding, which was determined in the presence of 17β-E_2 (1.0 μM) (12).

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

After preincubation for 10 min, cells were incubated with 20 μM L-[U-^{14}C]tyrosine (1 μCi) in KRP buffer in the presence or absence of various concentrations of raloxifene or tamoxifen 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 $1600 \times g$ for 10 min. ^{14}C-Labelled catechol compounds were separated further by ion exchange chromatography on Duolite C-25 columns (H^+-type, 0.4 × 7.0 cm) (10) and counted for the radioac-

tivity by a Packard Tri-Carb 2900TR liquid scintillation counter. ^{14}C-Catecholamine synthesis was expressed as the sum of the ^{14}C-catecholamines (adrenaline, noradrenaline, and dopamine).

Catecholamine secretion from cultured bovine adrenal medullary cells

The secretion of catecholamines was measured as described previously (15). After preincubation with or without raloxifene or tamoxifen at 37°C for 10 min, the cells (10^6 per well) were incubated with or without the SERMs in the presence or absence of various secretagogues 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). Catecholamines (noradrenaline and adrenaline) secreted into the medium were adsorbed onto aluminum hydroxide and estimated by the ethylendiamine condensation method using a fluorescence spectrophotometer (F-2500; Hitachi, Tokyo) with excitation and emission wavelengths of 420 and 540 nm, respectively.

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

The influx of $^{22}Na^+$ and $^{45}Ca^{2+}$ was measured as reported previously (11). After preincubation with or without raloxifene or tamoxifen at 37°C for 10 min, the cells (4×10^6 per dish) were incubated with 1.5 μCi of ^{22}NaCl or 1.5 μCi of $^{45}CaCl_2$ at 37°C for 5 min in the presence or absence of 300 μM ACh and various concentrations of the SERMs in KRP buffer. After incubation, the cells were washed 3 times with ice-cold KRP buffer, solubilized in 10% Triton X-100, and counted for radioactivity of $^{22}Na^+$ and $^{45}Ca^{2+}$ by an Aloka ARC-2005 gamma counter and a Packard Tri-Carb 2900TR liquid scintillation counter, respectively.

Expression of nAChRs in Xenopus oocytes and electrophysiological recordings

Isolation and microinjection of *Xenopus* oocytes was performed as described previously (17, 18). In brief, the cDNA encoding the α4 and β2 subunits of rat neuronal nAChR, subcloned into pcDNAI/Neo (Invitogen, Carlsbad, CA, USA) vector, was kindly provided from Dr. James W. Patrick (Division of Neuroscience, Baylor College of Medicine, Houston, TX, USA). Oocytes were injected with cDNAs (1.5 ng/30 nL) and electrophysiological recordings were performed 2 – 3 days after injection. Each oocyte was perfused (2 mL/min) with Ba^{2+}-Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES, pH 7.4) containing 1 μM atropine sulfate, to minimize the effects of secondary activated Ca^{2+}-dependant Cl^- currents and then impaled with 2 glass electrodes (1 – 5 MΩ) filled with 3 M KCl and clamped at −70 mV using the OC-725C Oocyte Clamp Amplifier (Harvard Apparatus, Inc., Holliston, MA, USA). ACh was applied for 30 s to obtain the maximum (peak) current used as a measure of drug response. We examined the effect of raloxifene (1 μM) on Na^+ current induced by ACh at a concentration that produced 50% of the maximal effect (EC_{50}) of ACh (1 mM).

Statistical analyses

All experiments were performed in duplicate or triplicate, and each experiment was repeated at least three times. All values are given as the mean ± S.E.M. 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 the *P*-value was less than 0.05. Statistical analyses were performed using PRISM for Windows version 5.0J software (Abacus Concept, Berkeley, CA, USA).

Results

Effects of raloxifene and tamoxifen on [^{3}H]17β-E_2 binding to plasma membranes

We first examined the effects of raloxifene and tamoxifen on the specific binding of [^{3}H]17β-E_2 to plasma membranes isolated from bovine adrenal medulla. When plasma membranes were incubated with these SERMs at various concentrations, the specific binding of [^{3}H]17β-E_2 was significantly increased by raloxifene and tamoxifen at 1.0 – 10 nM (Fig. 1A) and 1.0 nM – 10 μM (except for 100 nM) (Fig. 1B), respectively, but inhibited by raloxifene at 1.0 – 10 μM (Fig. 1A). These results suggest that the SERMs interact with plasma membrane estrogen receptors to positively or negatively modulate specific [^{3}H]17β-E_2 binding.

Effects of raloxifene and tamoxifen on basal and ACh-induced ^{14}C-catecholamine synthesis from [^{14}C]tyrosine in the cells

Bovine adrenal medullary cells were incubated with 20 μM [^{14}C]tyrosine in KRP buffer in the presence or absence of various concentrations of SERMs at 37°C for 20 min. As shown in Fig. 2B, tamoxifen at 100 nM significantly increased ^{14}C-catecholamine synthesis from [^{14}C]tyrosine, but raloxifene and tamoxifen at higher concentrations (0.1 – 1.0 and 1.0 – 10 μM, respectively) inhibited it (Fig. 2: A and B). Raloxifene (100 nM) and tamoxifen (100 nM and 1.0 μM) had little effect on [^{14}C]tyrosine uptake by the cells (data not shown),

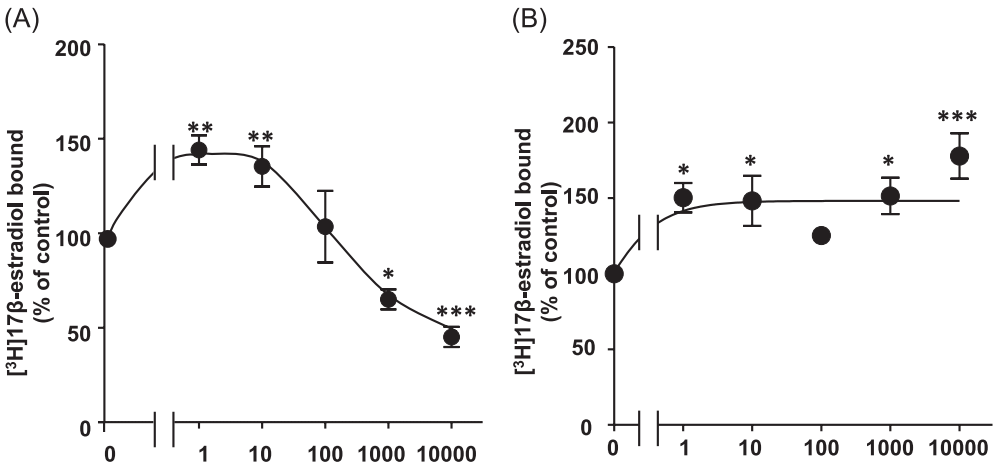

Fig. 1. Effects of raloxifene (A) and tamoxifen (B) on the specific binding of [^{3}H]17β-estradiol (17β-E_2) to plasma membranes isolated from bovine adrenal medulla. Plasma membranes (30 μg/tube) were incubated with [^{3}H]17β-E_2 (5 nM) and various concentrations of raloxifene (A) or tamoxifen (B) for 30 min at 4°C. Non-specific binding of [^{3}H]17β-E_2 was determined in the presence of 200-fold excess concentrations of 17β-E_2, and specific binding was obtained by subtracting non-specific binding from total binding. Control specific binding of [^{3}H]17β-E_2 [150 ± 15 (A) and 208 ± 36 (B) fmol/mg protein] was assigned a value of 100% and the data are expressed as % of control. Values shown are the mean ± S.E.M. of 4 experiments carried out in duplicate. *P < 0.05, **P < 0.01, and ***P < 0.001; compared to each control.

suggesting that the SERMs do not affect tyrosine uptake by the cells. ACh (300 μM) increased ^{14}C-catecholamine synthesis, which raloxifene and tamoxifen suppressed significantly (1.0 μM and 10 – 100 μM, respectively) in a concentration-dependent manner (Fig. 2: C and D).

Effects of pretreatment with raloxifene and tamoxifen on catecholamine secretion induced by ACh in the cells

Raloxifene (1 μM) and tamoxifen (10 μM) did not significantly affect basal secretion of catecholamines (control = 2.85% ± 0.17%, raloxifene = 3.21% ± 0.41%, tamoxifen = 3.47% ± 0.23% of the total catecholamines). Stimulation of nAChR-ion channels by ACh, a physiological secretagogue, caused catecholamine secretion corresponding to 16.79% ± 0.75% of the total catecholamines in the cells (Fig. 3A). Pretreatment of cells with raloxifene (1 μM) (Fig. 3A) and tamoxifen (10 μM) (Fig. 3B) for 0, 5, 10, 20, and 30 min caused a time-dependent decrease in catecholamine secretion induced by ACh for up to 30 min, with a continuously maximal reduced level occurring at 10 min. Therefore, the effect of SERMs on catecholamine secretion was evaluated using cells pretreated with SERMs for 10 min.

We examined the effects of raloxifene (1 μM) and tamoxifen (10 μM) on catecholamine secretion induced by other secretagogues. Veratridine (100 μM), an activator of voltage-dependent Na^+ channels, or 56 mM K^+, an activator of voltage-dependent Ca^{2+} channels, caused catecholamine secretion corresponding to 24.28% ± 1.58% and 19.47% ± 1.11% of the total catecholamines, respectively (Fig. 4A). Raloxifene (1 μM) (Fig. 4A) and tamoxifen (10 μM) (Fig. 4B) had little effect on catecholamine secretion induced by veratridine and high K^+.

Concentration–inhibition curves for the effects of raloxifene or tamoxifen on ACh-induced catecholamine secretion and $^{22}Na^+$ and $^{45}Ca^{2+}$ influx

Pretreatment of cells with raloxifene (0.3, 1, 10, and 100 μM) or tamoxifen (10, 30, and 100 μM) for 10 min reduced ACh-induced secretion of catecholamines to 81.0%, 65.0%, 35.1%, and 33.0% (Fig. 5A) or to 49.0%, 43.1%, and 25.4% (Fig. 6A), respectively, of ACh alone in a concentration-dependent manner. Raloxifene suppressed ACh (300 μM)-induced $^{45}Ca^{2+}$ influx at 1.0 – 100 μM (Fig. 5B) and ACh (300 μM)-induced $^{22}Na^+$ influx at 0.3 – 100 μM (Fig. 5C). Tamoxifen also inhibited ACh-induced $^{45}Ca^{2+}$ influx (Fig. 6B) and $^{22}Na^+$ influx at 10 – 100 μM (Fig. 6C).

Inhibitory mode of raloxifene or tamoxifen on $^{22}Na^+$ influx induced by ACh

We attempted to determine whether either SERM competes with ACh for binding sites on the nAChRs. When the concentration of ACh in the incubation medium increased, the inhibition of $^{22}Na^+$ influx induced by either SERM was not overcome by increasing concentrations (10 – 300 μM) of ACh (Fig. 7: A and B), indicating that neither SERM competes with ACh for the binding sites on nAChRs.

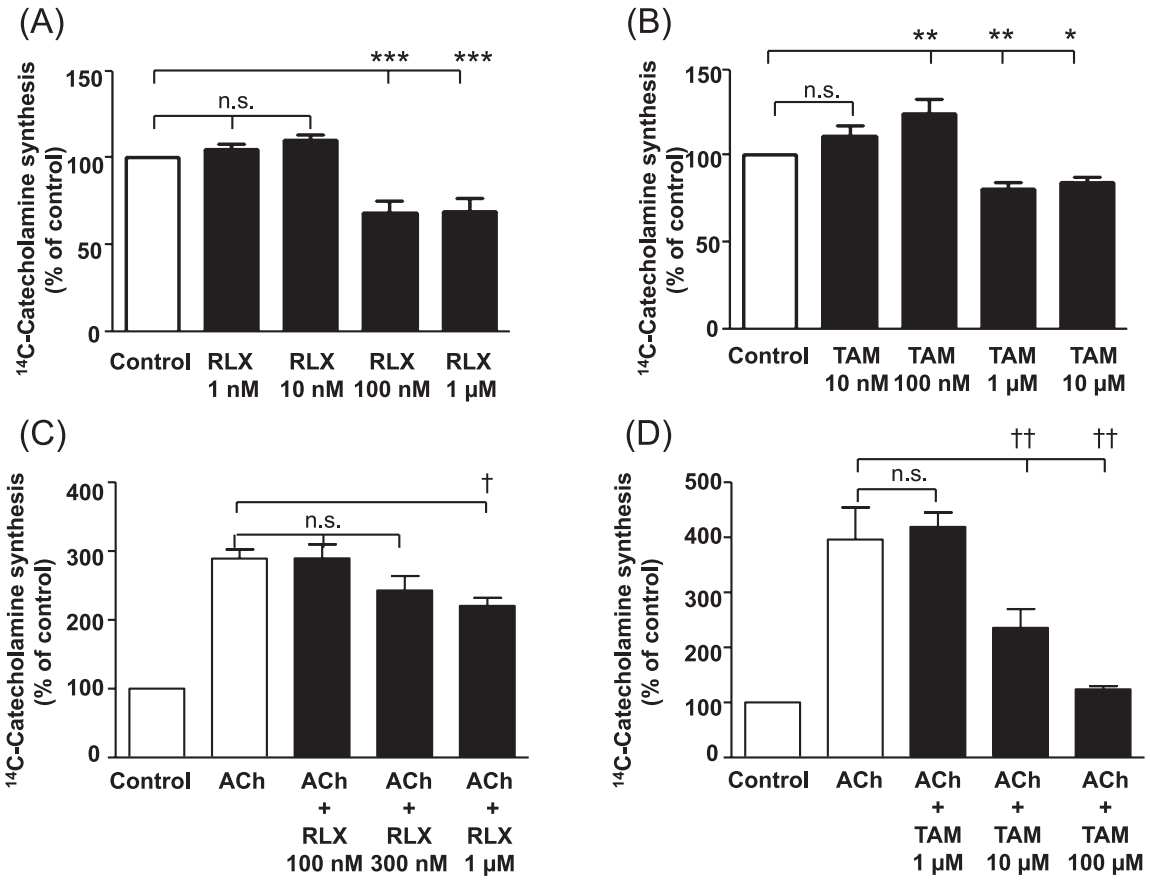

Fig. 2. Effects of raloxifene (A, C) or tamoxifen (B, D) on basal (A, B) and ACh (C, D)-induced ^{14}C-catecholamine synthesis from [^{14}C]tyrosine in the cells. Cells (4×10^6 / dish) were incubated with L-[U-^{14}C]tyrosine (20 μM, 1 μCi) and various concentrations of raloxifene (RLX) (A, C) or tamoxifen (TAM) (B, D) at 37°C for 20 min in the presence (C, D) or absence (A, B) of ACh (300 μM). ^{14}C-Labelled catechol compounds were separated by ion exchange chromatography on Duolite C-25 columns (H^+ type, 0.4×7.0 cm) and counted for radioactivity. Control ^{14}C-catecholamine synthesis [20,500 ± 3,900 (A) and 20,800 ± 6,500 (B) dpm / 4×10^6 cells / 20 min] and ACh-induced synthesis [50,600 ± 3,900 (C) and 112,000 ± 8,000 (D) dpm / 4×10^6 cells / 20 min] were assigned a value of 100% and the data are expressed as % of control or ACh. Values shown are the mean ± S.E.M. of 4 experiments carried out in duplicate. Data are expressed as the mean ± S.E.M. of 4 experiments carried out in triplicate. *$P < 0.05$,**$P < 0.01$, and ***$P < 0.001$; compared with each control. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$; compared with ACh alone.

Effects of raloxifene on ACh responses in Xenopus oocytes expressing nAChRs

The direct effect of raloxifene on ACh responses in *Xenopus* oocytes expressing rat $\alpha 4\beta 2$ nAChRs was examined. As shown in Fig. 8, raloxifene (1.0 μM) reversibly inhibited ACh-induced Na^+ currents.

Discussion

In the present study, we demonstrated the stimulatory or inhibitory effects of two SERMs, raloxifene and tamoxifen, on specific [^{3}H]17β-E_2 binding to plasma membrane estrogen receptors as well as catecholamine synthesis and secretion in bovine adrenal medullary cells.

Raloxifene and tamoxifen are allosteric modulators of plasma membrane estrogen receptors

SERMs are well-known to bind to estrogen-binding sites of classical nuclear ERs to initiate changes in formation on the ER, the dissociation of the ER from heat-shock proteins, and various gene transcriptions (2). In the present study, raloxifene at 1.0 – 10 nM and tamoxifen at 1.0 nM – 10 μM except for 100 nM rather enhanced [^{3}H]17β-E_2 binding to plasma membrane estrogen receptors, whereas raloxifene at higher concentrations (1.0 – 10 μM) inhibited it. This finding suggests that raloxifene and tamoxifen are an allosteric modulator of membrane estrogen receptors and that raloxifene at higher concentrations interferes with the specific binding of [^{3}H]17β-E_2 to membrane estrogen receptors. The

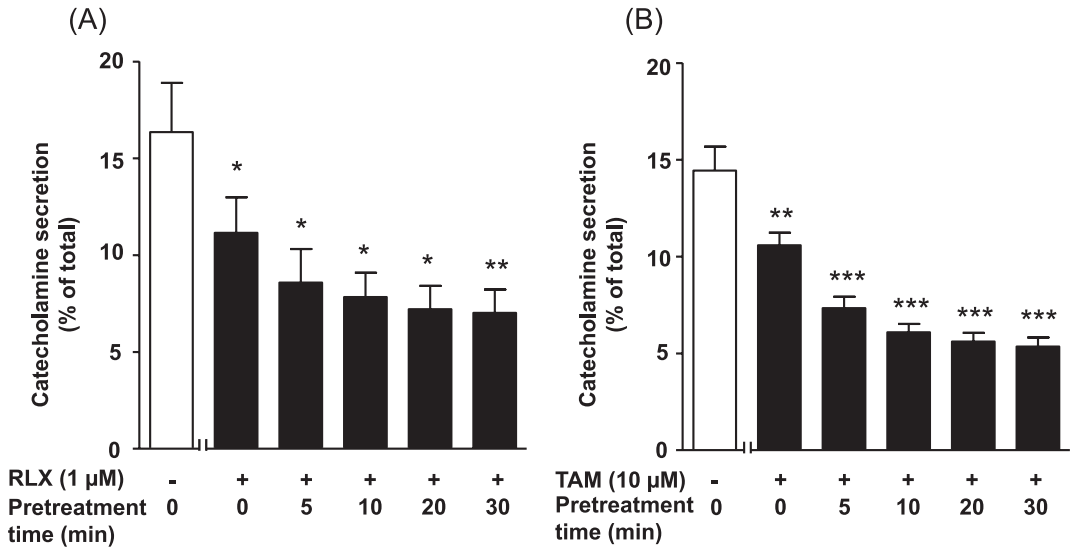

Fig. 3. Time course of pretreatment effect of raloxifene and tamoxifen on ACh-induced catecholamine secretion from the cell. After preincubation with (closed column) or without (open column) 1 μM raloxifene (RLX) (A) or 10 μM tamoxifen (TAM) (B) for the indicated period, the cells (10^6/well) were stimulated with ACh (300 μM) for 10 min at 37°C. Catecholamines secreted into the medium were measured and expressed as a percentage of the total catecholamines [7.19 ± 0.98 μg (A), 7.60 ± 0.82 μg (B)] in the cells. Data are the mean ± S.E.M. of 4 separate experiments carried out in triplicate. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; compared with ACh alone.

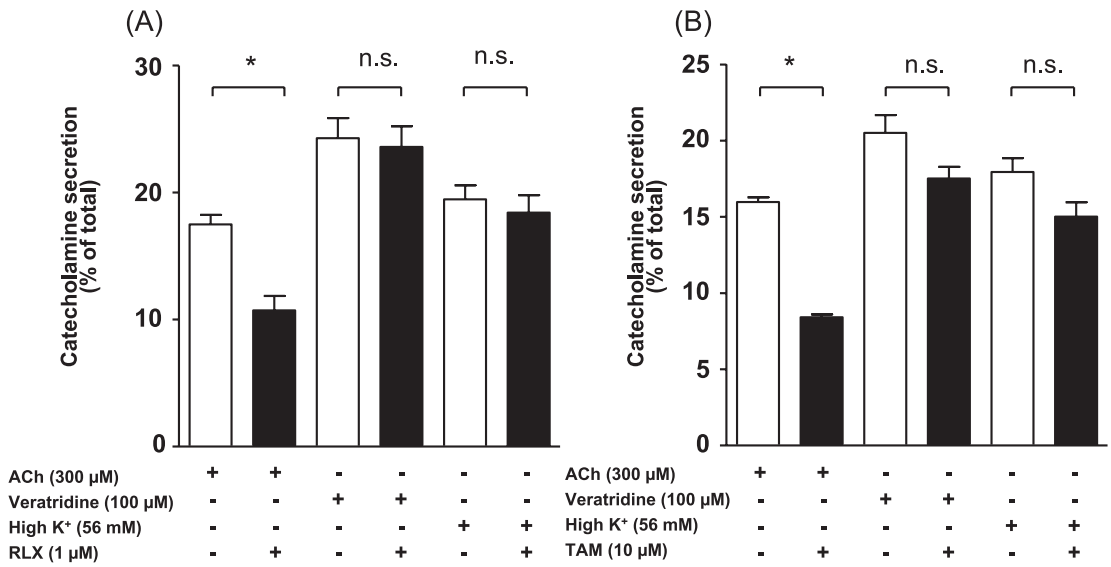

Fig. 4. Effects of raloxifene (A) or tamoxifen (B) on catecholamine secretion induced by various secretagogues. After preincubation of cells with or without raloxifene (RLX) (A) or tamoxifen (TAM) (B) for 10 min, the cells (10^6/well) were incubated with or without ACh (300 μM), veratridine (100 μM), or high concentrations of K^+ (56 mM) for another 10 min at 37°C. Catecholamines secreted into the medium were measured and expressed as a percentage of the total catecholamines [6.21 ± 1.19 μg (A), 7.28 ± 0.59 μg (B)] in the cells. Data are the mean ± S.E.M. of 4 separate experiments carried out in triplicate. *$P < 0.05$, compared with ACh alone.

former result is similar to that of our previous data produced by ICI182,780, a pure antagonist of nuclear ER, and *p*-nonylphenol or bisphenol A, environmental estrogenic pollutants, both of which allosterically enhanced specific [^{3}H]17β-E_2 binding to plasma membrane estrogen receptors (12).

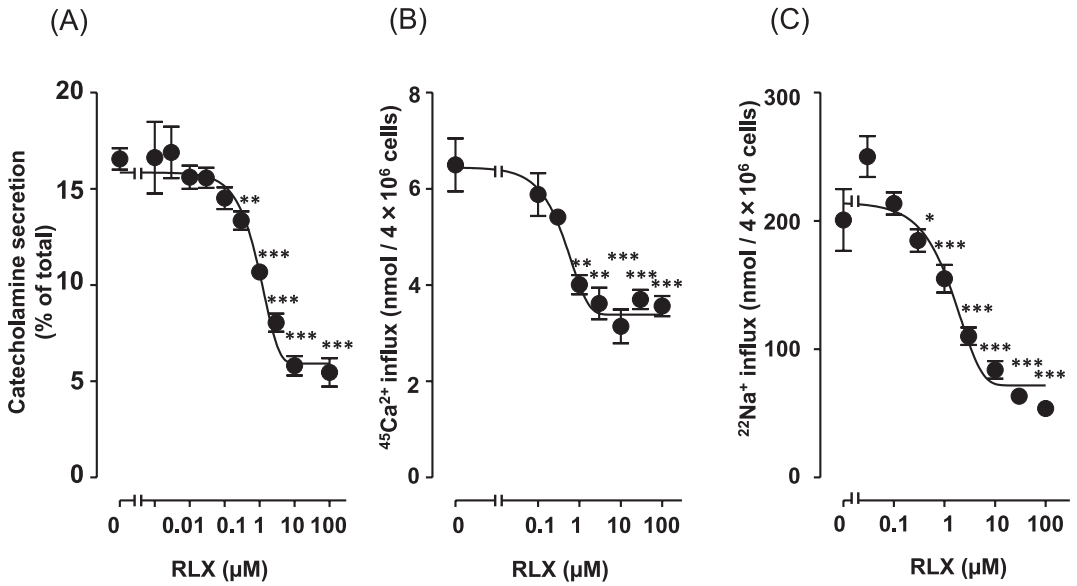

Fig. 5. Effects of various concentrations of raloxifene on ACh-induced catecholamine secretion (A), $^{45}Ca^{2+}$ influx (B), and $^{22}Na^{+}$ influx (C) in the cells. A) After preincubation of cells with various concentrations of raloxifene (RLX) for 10 min at 37°C, cells (10^6/well) were stimulated with ACh (300 μM) in the presence of various concentrations of raloxifene for another 10 min at 37°C. Catecholamines secreted were measured and expressed as a percentage of total catecholamines (5.01 ± 0.37 μg). B and C) After preincubation with various concentrations of raloxifene for 10 min, cells (4 × 10^6 / dish) were incubated in the presence of various concentrations of raloxifene, 300 μM ACh, 1.5 μCi of $^{45}CaCl_2$ (B), or $^{22}NaCl$ (C) for another 5 min at 37°C. $^{45}Ca^{2+}$ influx and $^{22}Na^{+}$ influx were measured, and expressed as nmol / 4 × 10^6 cells. Data are the mean ± S.E.M. of 4 separate experiments carried out in triplicate. *P < 0.05, **P < 0.01, and ***P < 0.001; compared to ACh alone.

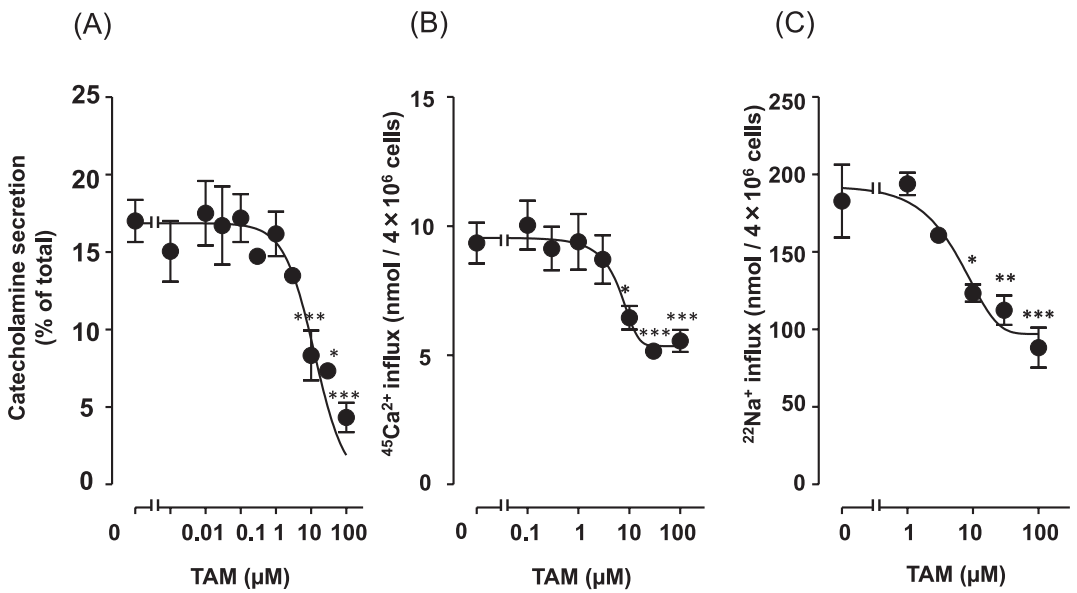

Fig. 6. Effects of various concentrations of tamoxifen on ACh-induced catecholamine secretion (A), $^{45}Ca^{2+}$ influx (B), and $^{22}Na^{+}$ influx (C) in the cells. A) After preincubation of cells with various concentrations of tamoxifen (TAM) for 10 min at 37°C, cells (10^6/well) were stimulated with ACh (300 μM) in the presence of various concentrations of tamoxifen for another 10 min at 37°C. Catecholamines secreted were measured and expressed as a percentage of total catecholamines (5.64 ± 0.49 μg). B and C) After preincubation with various concentrations of tamoxifen for 10 min, the cells (4 × 10^6 / dish) were incubated in the presence of various concentrations of tamoxifen, 300 μM ACh, 1.5 μCi of $^{45}CaCl_2$ (B), or $^{22}NaCl$ (C) for another 5 min at 37°C. $^{45}Ca^{2+}$ influx and $^{22}Na^{+}$ influx were measured, and expressed as nmol / 4 × 10^6 cells. Data are the mean ± S.E.M. of 4 separate experiments carried out in triplicate. *P < 0.05, **P < 0.01, and ***P < 0.001; compared to ACh alone.

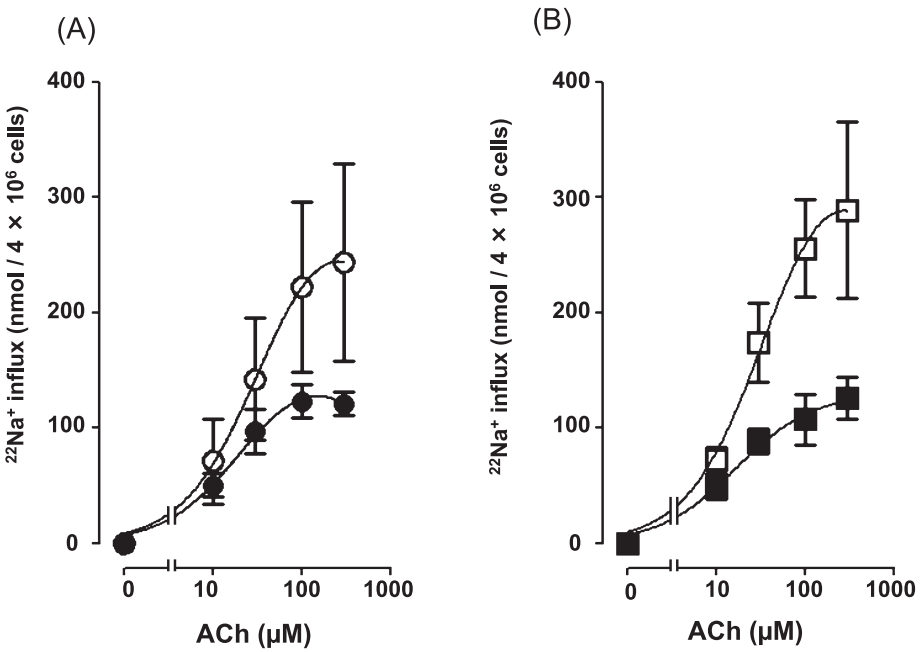

Fig. 7. Inhibitory mode of raloxifene (A) and tamoxifen (B) on $^{22}Na^+$ influx induced by ACh. After preincubation of cells with or without raloxifene (1.0 μM) and tamoxifen (10 μM) for 10 min, the cells were incubated with (closed circle) or without (open circle) raloxifene (1.0 μM) (A) and with (closed square) or without (open square) tamoxifen (10 μM) (B) in the presence of 1.5 μCi of ^{22}NaCl and ACh (3 – 300 μM) for 5 min at 37°C. $^{22}Na^+$ influx was measured and expressed as nmol / 4 × 10^6 cells. Data are the mean ± S.E.M. from 3 separate experiments carried out in triplicate.

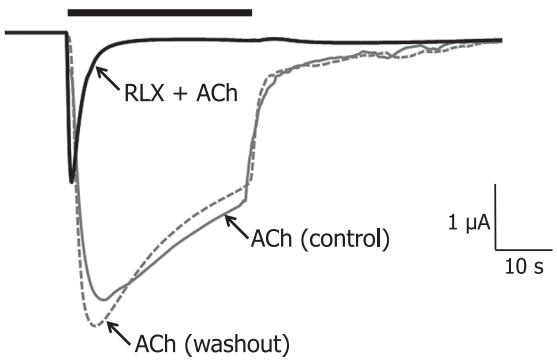

Fig. 8. Effect of raloxifene on ACh-induced response in nAChRs expressed in *Xenopus* oocytes. Representative current tracings obtained from the same *Xenopus* oocyte expressing rat $\alpha4\beta2$ nAChRs were superimposed, demonstrating an inhibitory effect of raloxifene (RLX, 1 μM) on the current induced by the EC_{50} of ACh. Traces represent the responses of ACh (control), in the presence of RLX (RLX + ACh), and 15-min washout [ACh (washout): dotted line]. The bar indicates the time of drug applications (ACh alone or RLX plus ACh), but it should be noted that RLX was pre-applied for 2 min before the coapplication with ACh.

Modulatory effects of raloxifene and tamoxifen on ^{14}C-catecholamine synthesis and catecholamine secretion

In bovine adrenal medullary cells, we previously reported that 17β-E_2 (12), environmental estrogenic pollutants (19), and phytoestrogens, such as daidzein (13) and resveratrol (14), stimulate catecholamine synthesis through plasma membrane estrogen receptors. A previous study reported that activation of membrane estrogen receptors increases intracellular Ca^{2+} concentrations and progesterone synthesis in rat hypothalamic astrocytes (20). In the present study, however, the stimulatory effect of tamoxifen on ^{14}C-catecholamine synthesis may not be mediated through the plasma membrane estrogen receptors because it increased basal synthesis of ^{14}C-catecholamines only at 100 nM. Furthermore, at higher concentrations raloxifene (0.1 – 1.0 μM) and tamoxifen (1.0 – 10 μM) inhibited ^{14}C-catecholamine synthesis. There was no relation between catecholamine synthesis and modulation of [^{3}H]17β-E_2 binding induced by SERMs. From these present results, it seems that the interactions of the SERMs with plasma membrane estrogen receptors are not associated with catecholamine synthesis in the cells.

Both raloxifene (1.0 μM) and tamoxifen (10 – 100 μM) suppressed ACh-induced ^{14}C-catecholamine synthesis in a concentration-dependent manner. We previously reported that ACh activates nAChR-ion channels, thereby inducing Na^+ influx and then Ca^{2+} influx as well as catecholamine synthesis (10) and secretion (11). In the present study, raloxifene and tamoxifen both preferentially inhibited catecholamine secretion mediated through nAChRs but neither did so through voltage-dependent Na^+ channels or voltage-dependent Ca^{2+} channels. The present results were partially consistent with those of a previous report (7) that raloxifene at micromolar concentrations inhibited catecholamine output elicited by ACh or high potassium in perfused rat adrenal glands and cultured bovine adrenal medullary cells. In the present study, both SERMs suppressed ACh-induced $^{45}Ca^{2+}$

influx and $^{22}Na^+$ influx in a concentration-dependent manner, similar to their suppression of catecholamine secretion and synthesis. It is likely that raloxifene and tamoxifen suppress ACh-induced catecholamine synthesis and secretion primarily by inhibiting Na^+ influx through nAChR-ion channels and subsequent Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

We further investigated the inhibitory mechanisms underlying the effects of raloxifene and tamoxifen on nAChR-ion channels. Increased concentrations of ACh did not overcome the inhibitory effects of the SERMs on ACh-induced $^{22}Na^+$ influx, suggesting that the SERMs act on the sites differently than they act on ACh binding sites of nAChR-ion channels. We confirmed that raloxifene directly and reversibly suppressed ACh-induced Na^+ current in *Xenopus* oocytes expressing rat $\alpha4\beta2$ nAChR.

Pharmacological significance of the effects of the SERMs on catecholamine synthesis and secretion

The pharmacokinetic properties of raloxifene and tamoxifen in postmenopausal women and in women with breast cancer showed that the maximum plasma concentrations of raloxifene and tamoxifen were 2 – 3 nM and 20 – 330 nM, respectively, during clinical treatments (21, 22). Tamoxifen, however, is reported to accumulate in tissues, resulting in 100-fold higher concentrations than in plasma after repeated administration of the drug during long-term treatment for breast cancer (23). Therefore, the concentrations of raloxifene and tamoxifen used in the present study should be high, compared to those plasma therapeutic concentrations, but they might be clinically relevant in the tissues.

Several lines of evidence have shown that the SERMs have both potentially adverse (24) and beneficial effects on brain functions such as cognition (25, 26) and neuroprotection (27). Indeed, raloxifene is reported to induce neurite outgrowth in estrogen receptor–positive PC12 cells (28). In the present study, we demonstrated that low concentration of tamoxifen (100 nM) stimulates basal catecholamine synthesis, whereas at high concentrations, it inhibits basal and ACh-induced catecholamine synthesis and secretion. On the basis of the present results, it would be hypothesized that the SERMs negatively or positively modulate the functions of central noradrenergic or dopaminergic neurons, depending on their concentrations, by changing the synthesis and release of each neurotransmitter. To confirm this possibility, further in vivo studies are required in the near future.

In summary, we demonstrated that two SERMs, raloxifene and tamoxifen, allosterically interact with plasma membrane estrogen receptors, whereas at high concentrations each of them inhibits catecholamine synthesis and secretion induced by ACh in adrenal medullary cells and probably in peripheral and central sympathetic neurons.

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Conflicts of Interest

The authors have no conflict of interest to report.

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