**Regular Article**

**Effects of Catecholamine Metabolites on Beta-Adrenoceptor-Mediated Relaxation of Smooth Muscle: Evaluation in Mouse and Guinea-Pig Trachea and Rat Aorta**

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**INTRODUCTION**

Catecholamine (CA) metabolites are generated through oxidative deamination of noradrenaline (NA) and adrenaline (AD) by monoamine oxidase (MAO) and their O-methylation by catechol-O-methyltransferase (COMT).1,2 It is generally recognized that CA metabolites do not show pharmacological activity.3 However, normetadrenaline (NMA) and metadrenaline (MA), COMT metabolites of NA/AD, can act as agonists of adrenoceptors (ARs). For example, NMA and MA have been shown to contract the nictitating membrane smooth muscle via α-AR in cats, and to possess positive chronotropic action in guinea pig (GP) atrial muscle through β-(β1)-AR.4 Extending from this work, we recently found that NMA/MA contracted rat prostate and thoracic aorta through activation of α1A-AR/α1D-AR (our unpublished observation).

In contrast, four CA metabolites, 3,4-dihydomandelic acid (DOMA), 3,4-dihydroxyphenylglycol (DHPG), vanillylmandelic acid (VMA), and 3-methoxy-4-hydroxyphenylglycol (MHPG) were reported not to show β-(β1)-AR stimulatory or antagonistic actions in GP atrial muscle.5 In addition, we found that five CA metabolites (NMA, DOMA, DHPG, VMA, and MHPG) did not show β2-AR agonistic activity, based on observations of relaxant responses in rat urinary bladder preparations.6 However, whether CA metabolites exhibit β-AR-mediated pharmacological actions requires further study.

In order to clarify whether various CA metabolites exert their pharmacological actions through β-ARs, we investigated their possible agonistic/antagonistic effects on β-AR using smooth muscle preparations in which functional β1-AR or β2-AR was dominantly expressed. The smooth muscle preparations used were mouse and GP trachea in which β1-AR-β2-AR-mediated relaxations were exclusively and easily detected.7,8 In addition, rat thoracic aorta (TA) that produces β3-AR-mediated relaxation7,8 was also used. CA metabolites examined in this study were NMA, MA, DOMA, DHPG, VMA, MHPG, and MHPG sulfate (MHPG-S), which have been reported to increase in the blood and urine of patients with pheochromocytoma.9,10 The present pharmacological approaches are clinically significant, since the information about CA metabolites’ β-AR-mediated effects on smooth muscle relaxation may help further understanding of the pathology of pheochromocytoma.

**MATERIALS AND METHODS**

**Drugs** The following drugs were used: dl-normetanephrine (dl-normetadrenaline, or NMA), hydrochloride, dl-metanephrine (dl-metadrenaline, or MA), hydrochloride, dl-3,4-dihydroxymandelic acid (DOMA), dl-3,4-dihydroxyphenyl...
glycol (DHPG), dl-4-hydroxy-3-methoxymandelic acid (VMA), 4-hydroxy-3-methoxyphenylglycol (MHPG) hemipiperazinium salt, 4-hydroxy-3-methoxyphenylglycol sulfate (MHPG-S) potassium salt, (-)-adrenaline (AD) (+)-bitartrate salt, carbamyl choline chloride, (-)-isoproterenol (isoprenaline; ISO) hydrochloride, N-methyl-N-propargyl-3-(2,4-dichlorophenoxy) propylamine (clorgiline) hydrochloride, 3,5-dinitrocatechol, (-)-(R)-noradrenaline (NA) hydrogen tartrate monohydrate, silodosin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), (+,-)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol hydrochloride (ICI-118551) (Tocris Bioscience, Ellisville, MO, U.S.A.), acetylcholine chloride (Daiichi Sankyo Co., Ltd., Tokyo, Japan), and (±)-phentolamine mesylate (Novartis Pharma K.K., Tokyo, Japan). All other chemicals used were commercially available and of reagent grade.

Indomethacin was dissolved in 100% ethanol as a stock solution at 10^-2 M, and 3,5-dinitrocatechol was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 5×10^-3 M. All other drugs were prepared as aqueous stock solutions. All stock solutions were diluted with distilled water to obtain desired concentrations.

**Animals** Male Wistar rats (8–11 weeks old; weight 150–280 g; Sankyo Labo Service Co., Tokyo, Japan), male ddY mice (8–12 weeks old; weight 35–46 g; Sankyo Labo Service Co.), and male Hartley GP (5–8 weeks old; weight 290–465 g; Kyudo Co., Ltd., Saga, Japan) were housed under controlled conditions at a temperature of 21–22°C, relative air humidity of 50±5%, and a fixed 12 h light-dark cycle (08:00–20:00), with food and water available ad libitum. This study was approved by the Toho University Animal Care and User Committee (approval numbers 17-53-294 accredited on May 17, 2017, 18-54-294, accredited on May 7, 2018, and 19-55-294, accredited on April 10, 2019) and was conducted in accordance with the User’s Guidelines for the Laboratory Animal Center of the Faculty of Pharmaceutical Sciences, Toho University.

**Preparation of Trachea and TA Tissues** The mice and GPs were anesthetized with isoflurane by inhalation and euthanized by exsanguination from the carotid arteries. Thereafter, the tracheal tissues were quickly removed and immersed in Locke-Ringer solution of the following composition (mM): NaCl, 154; KCl, 5.6; CaCl2, 2.2; MgCl2, 2.1; NaHCO3, 5.9; D-(+)-glucose, 2.8. The tracheal tissues were cleaned of unnecessary adipose and connective tissues under a dissecting microscope. Subsequently, tracheal cartilage containing smooth muscles was cut into pieces about 2 mm long. In this series of experiments, the intimal surface of tracheal tissue was gently rubbed with moistened filter paper to remove tracheal epithelium as much as possible.

The rats were anesthetized with isoflurane by inhalation and euthanized by exsanguination from the carotid arteries. Thereafter, the TA was quickly removed and immersed in normal Tyrode’s solution of the following composition (mM): NaCl, 158.3; KCl, 4.0; CaCl2, 2.0; MgCl2, 1.05; NaH2PO4, 0.42; NaHCO3, 10.0; D-(+)-glucose, 5.6. After removing the surrounding adipose and connective tissue under a dissecting microscope, the isolated TA was then cut into spiral segments (approximately 2 mm in width and 20 mm in length), and the endothelium was removed by gentle rubbing of the intimal surface with filter paper.

**Recordings of Isometric Tension Changes of Smooth Muscle Tissues** Tracheal tissues were suspended with stainless steel hooks (outer diameter 200μm) in a 5-mL organ bath (UC-5; UFER Medical Instrument, Kyoto, Japan) filled with Locke-Ringer solution. Rat TAs were suspended with cotton thread in a 5-mL organ bath containing normal Ty-
rode’s solution. Both solutions were aerated with mixed gas (95% O₂ + 5% CO₂) and kept at 35 ± 1°C (pH = 7.4). Tension changes were isometrically recorded with a force displacement transducer (tracheas: T7-8-240; Orientec Co., Ltd., Tokyo, Japan; rat TAs: TB-612T, Nihon Koden, Tokyo, Japan) connected to a signal conditioner (MSC-2; Primtech Co., Tokyo, Japan), and were acquired in a Windows PC computer via data acquisition software (Chart 7 for Windows; ADInstruments) through an A/D converter (PowerLab/4sp; ADInstruments). The preparations were incubated for 60 min while changing the bath solution every 20 min so that the final passive tension was 0.5 g for mouse tracheas, 2 g for GP tracheas, or 1 g for rat TAs. After these procedures, the tissues were incubated for a further 10 min and the subsequent experiments were then started in the presence of indomethacin (3 × 10⁻⁶ M).

Evaluation of Agonistic and Antagonistic Effects of CA Metabolites in Mouse and GP Tracheas After the procedures shown in Recordings of Isometric Tension Changes of Smooth Muscle Tissues, carbachol (10⁻⁶ M) was applied to produce a contraction, and when the tension level became constant, ISO was gradually applied in concentrations to produce about 20% relaxation. Thereafter, the preparation was washed with a fresh bath solution and when the tension was recovered to the level observed before carbachol application, the preparation was washed again and incubated for 20 min. Subsequently, the same procedures (application of carbachol, cumulative application of ISO, and wash-out) were repeated using ISO in concentrations to produce about 70% relaxation. Then, the preparation was contracted by carbachol (10⁻⁸ M) in the presence of phenotolamine (10⁻⁶ M). When the contraction reached a steady-state level, ISO was gradually (10⁻¹⁰–10⁻⁶ M) applied to obtain a concentration–response curve, which was used as a control. After the maximum relaxation response to 10⁻⁶ M ISO was obtained, the preparation was washed with a fresh bath solution and incubated further until the next carbachol application (10⁻⁸ M). Following recovery of the preparation in fresh bath solution, it was contracted again by carbachol (10⁻⁶ M) in the presence of phenotolamine (10⁻⁶ M). When the contraction reached a steady-state level, any CA metabolites or CA (NA and AD) (10⁻⁶ M) were applied and tension level changes were observed for 40 min. Thereafter, ISO was gradually (10⁻¹⁰–10⁻⁶ M) applied to obtain a concentration–response curve and the results were compared with the control. For testing the effects on CA metabolites’ actions of a selective β₂-AR antagonist ICI-118551, a MAO-A inhibitor clorgiline, and a COMT inhibitor 3,5-dinitrocatechol, IC-118,551 (3 × 10⁻⁶ M), clorgiline (10⁻⁵ M) and/or 3,5-dinitrocatechol (2 × 10⁻⁶ M) were applied together with carbachol (10⁻⁶ M). The concentrations of CA metabolic inhibitors (clorgiline and 3,5-dinitrocatechol) were determined based on reports from Sturza et al. and Martel et al.

CA (NA and AD) and CA metabolites are used as diagnostic markers for pheochromocytoma. When blood concentrations higher than or equal to 7.7 nmol/L (NA), 1.2 nmol/L (AD), 1.4 nmol/L (NMA), or 0.42 nmol/L (MA), they indicate the presence of pheochromocytoma. At the same time, MA concentration in pheochromocytoma tissue has been reported to be over 10000-fold higher than its plasma concentration. Therefore, the possible concentrations of CA metabolites released from tumor tissue (pheochromocytoma) will reach µM-order concentration ranges in the tissue-surrounding areas. In view of the above reports, the concentration of CA metabolites was determined to be 10⁻⁶ M.

Evaluation of the Antagonistic Effects of CA Metabolites in Rat TA Experimental protocols for our rat TA experiments were substantively the same as for our tracheal studies, except that we used phenylephrine (≥10⁻⁷ M) as a pre-constructor. Initially, a control curve was obtained by gradually applying ISO (10⁻⁶–10⁻⁴ M), the β₁,β₂-AR agonist salbutamol (10⁻⁶–3 × 10⁻⁵ M), and the β₁,β₂-AR partial agonist CGP-12177 A (10⁻⁷–3 × 10⁻⁵ M) against the third phenylephrine-induced contraction. When the third phenylephrine-induced contraction was reduced as compared to the second contraction, phenylephrine concentration was increased so that the contraction levels were almost the same. To examine the effects of CA metabolites (DHPG, DOMA, VMA, MHPG, and MHPG-S) on the relaxation responses to ISO, salbutamol, and CGP-12177 A, we used a fresh preparation, and CA metabolites were applied 20 min before application of phenylephrine. The concentration response curves obtained in the presence of CA metabolites were compared with the control. Other chemicals applied were the COMT inhibitor 3,5-dinitrocatechol (2 × 10⁻⁶ M), applied 20 min before phenylephrine, and the β₁,β₂-AR antagonist propranolol (3 × 10⁻⁷ M), applied with phenylephrine.

RT-PCR Analysis of β-AR Subtype mRNA Expression in Mouse and GP Tracheas and Rat TA The mouse and GP tracheas and rat TA were immediately frozen in liquid nitrogen after removing moisture with filter paper. The frozen mouse and rat tissues were homogenized in RNAiso Plus™ (TaKaRa Bio Inc., Shiga, Japan) and total RNA was extracted according to the manufacturer’s protocol. Total RNA was extracted from frozen GP trachea using the APGC-method. A 18S: 28S ribosomal RNA (rRNA) ratio of >1:2 in the extracted total RNA samples was confirmed using electrophoresis. The First-strand cDNA was synthesized with 1 µg of total RNA by reverse transcription using ReverTraAce® qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer’s protocol. PCR was performed using the GoTaq® Green Master Mix (Promega Corp., Madison, WI, U.S.A) with 0.5 µL of cDNA solution per 10 µL of reaction mixture in TaKaRa PCR Thermal Cycler Dice® Touch (TaKaRa Bio Inc.). The PCR reaction mixtures were heated for 2 min at 95°C, and then amplified with n cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension phase of 5 min at 72°C. The sequence of specific primers for target genes (synthesized by Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.), the number of cycles, and the product size are shown in Table 1. The PCR products were separated with 2% agarose gel electrophoresis. After electrophoresis, the agarose gel was stained with ethidium bromide (0.5 µg/mL) for 10 min and visualized under UV irradiation.

Data Analysis The extent of relaxation induced by all relaxants, including the CA metabolites, was calculated relative to the tension level before the application of carbachol or phenylephrine (100% relaxation) and to the steady-state tension level prior to the application of each relaxant (0% relaxation). The potencies of the relaxants ISO, salbutamol, and CGP-12177 A were calculated as pD₂ (pEC50) values. These represent the negative logarithm of the effective agonist con-
centration producing a response that is 50% of the maximum response. The data were plotted as a function of relaxant concentration and fitted to the equation:

\[ E = \frac{E_{\text{max}}}{(EC_{50} + A)^n} \]

where \( E \) is the % relaxation at a given concentration, \( E_{\text{max}} \) is the maximum response, \( A \) is the relaxant concentration, \( n \) is the Hill coefficient, and \( EC_{50} \) is the relaxant concentration producing a 50% response. Curve-fitting was carried out using GraphPad Prism™ (Version 6; GraphPad Software, Inc., San Diego, CA, U.S.A.).

**Statistical Analysis** All values in the text and illustrations are presented as means ± standard error of the mean (S.E.M.) of the data obtained from different numbers (n) of preparations. GraphPad™ Prism 6 software (GraphPad Software Inc., San Diego, CA, U.S.A.) was used for statistical analysis. Differences between values were evaluated using an

Table 1. RT-PCR Primer Sequences, Number of Cycles, and Product Sizes

| Species, tissue | Target gene | Primer sequence (5′–3′) | Number of PCR cycles | PCR product size (base pair) |
|----------------|-------------|-------------------------|----------------------|-----------------------------|
| **Mouse, trachea** | Adrb1 (F) | GCCCTTTTCGCTACCAGAGTT | 31 | 158 |
| | Adrb1 (R) | ACTTGGGGTCTGTTAGACAG | | |
| | Adrb2 (F) | AATAGCAACGGGAGAAGCAGG | | |
| | Adrb2 (R) | CTTCCTGGAAGTCAACGCT | | |
| | Adrb3 (F) | CAGTCCCTGCTATGTTTGGT | | |
| | Adrb3 (R) | GTGAGGAGACAGGGATGAAAC | | |
| **Guinea pig, trachea** | Adrb1 (F) | ACGTTCTCTTCTGTGCAAG | 36 | 207 |
| | Adrb1 (R) | GAATGGGAGTCTATGCGCTG | | |
| | Adrb2 (F) | TTCATGATGTGCTGTCG | | |
| | Adrb2 (R) | ATGTAAGGGGAGCACAACC | | |
| | Adrb3 (F) | CGCCAAGACTCAGGACCAC | | |
| | Adrb3 (R) | CACAGACGCATCAGCAAG | | |
| **Rat, thoracic aorta** | Adrb1 (F) | GATCTGGTCATGGGACTGCT | 32 | 337 |
| | Adrb1 (R) | AGCACTTGGGGTCTGAGCAG | | |
| | Adrb2 (F) | ACCAGAGAATAAGGCCGAGT | 32 | 386 |
| | Adrb2 (R) | GTCTTGAGGGCTTTGCT | | |
| | Adrb3 (F) | TCCCTGAGAGATTCTGCT | | |
| | Adrb3 (R) | GAGGCAAGATTGAGT | | |

![Fig. 2.](image-url) Relaxant Effects of Catecholamine (CA) Metabolites on Mouse (A–C) and Guinea Pig (GP) (D, E, G) Tracheas Contracted by Carbachol in the Absence (A, D) and Presence of Clorgiline (10⁻⁵ M) (B, E) or 3,5-Dinitrocatechol (2 × 10⁻⁶ M) (C, G) and Effect of the Selective β₂-AR Antagonist ICI-118551 (3 × 10⁻⁸ M) on MA-Induced Relaxation of GP Trachea in the Presence of Clorgiline (10⁻⁵ M) (F)

Relaxations were expressed as percent inhibitions by each CA metabolite (10⁻⁴ M) vs. carbachol (10⁻⁶ M)-induced contraction. Data are presented as means ± S.E.M. Numbers of experiments are n = 5–9 for A–G. **p < 0.01 vs. control (one-way-ANOVA followed by Dunnett’s multiple comparison test). ***p < 0.01 vs. control (unpaired *t*-test).
unpaired t-test, or one-way ANOVA followed by Dunnett’s multiple comparison test, or two-way ANOVA followed by Šidák’s multiple comparison test. A $p$ value $<0.05$ was considered significant.

**RESULTS**

**Evaluation of Relaxant Effects of CA Metabolites and CA on Trachea** Figure 2 shows effects of tested CA metabolites ($10^{-4}$M) on mouse (Figs. 2A–C) and GP (Figs. 2D–G) tracheas contracted by carbachol ($10^{-6}$M). In mouse trachea, DOMA had a weak relaxant effect, though this was not significantly different from the time-matched control (Fig. 2A). NMA and MA did not show substantial relaxant effects even in the presence of the MAOA inhibitor clorgiline ($10^{-5}$M) (Fig. 2B). DOMA and DHPG showed no substantial relaxant effects even in the presence of the COMT inhibitor 3,5-dinitrocatechol ($2 \times 10^{-6}$M) (Fig. 2C). In GP trachea, MA showed a significant relaxant effect (Fig. 2D), and this was also significant in the presence of clorgiline ($10^{-5}$M) (Fig. 2E). MA ($10^{-4}$M)-induced relaxation was significantly suppressed by a selective $\beta_2$-AR antagonist, ICI-118,551 ($3 \times 10^{-8}$M), in the presence of clorgiline ($10^{-5}$M) (Fig. 2F). DOMA and DHPG did not show significant relaxant effects even in the presence of 3,5-dinitrocatechol ($2 \times 10^{-6}$M) (Fig. 2G). In contrast, NA and AD (each $10^{-4}$M) showed potent relaxant effects in both mouse (NA: $94.3 \pm 1.8\%$, $n=5$; AD: $95.8 \pm 1.1\%$, $n=6$) and GP (NA: $97.7 \pm 1.4\%$, $n=5$; AD: $98.0 \pm 0.7\%$, $n=6$) tracheas.

**Effects of CA Metabolites on ISO-Induced Relaxation in Mouse Trachea** None of the seven CA metabolites (each $10^{-4}$M) had any apparent effect on ISO-induced relaxation (Figs. 3A–G). In contrast, in the presence of clorgiline ($10^{-3}$M), NMA and MA (each $10^{-4}$M) significantly suppressed ISO-induced relaxation (Figs. 4A, B). These suppressive effects were also indicated by significant decreases in the ISO’s $pD_2$ values due to these CA metabolites in the presence of clorgiline. In the case of NMA, the $pD_2$ values of ISO were $8.02 \pm 0.09$ (control) vs. $7.84 \pm 0.10$ (NMA) ($n=7$, $p<0.05$)

**Fig. 3. Effects of CA Metabolites ($10^{-4}$M) on Isoprenaline (ISO)-Induced Relaxation in Mouse Trachea Contracted by Carbachol** CA metabolites studied were: NMA (A), MA (B), DOMA (C), DHPG (D), VMA (E), MHPG (F), and MHPG-S (G). Data are presented as means $\pm$ S.E.M. Numbers of experiments are $n=6$ for each.

**Fig. 4. Effects of NMA, MA, DOMA, and DHPG on ISO-Induced Relaxation in Mouse Trachea Contracted by Carbachol** Data are presented as means $\pm$ S.E.M. Numbers of experiments are $n=7$ for A, and $n=6$ for B–F. *$p<0.05$, **$p<0.01$ vs. control (two-way ANOVA followed by Šidák’s multiple comparison test).
in the presence of clorgiline (Fig. 4A), and 8.31 ± 0.11 (control) vs. 8.09 ± 0.11 (NMA) (n = 6, p > 0.05) in the absence of clorgiline (Fig. 3A). In the case of MA, the pD2 values of ISO were 8.09 ± 0.17 (control) vs. 7.89 ± 0.16 (MA) (n = 6, p < 0.01) in the presence of clorgiline (Fig. 4B), and 8.11 ± 0.08 (control) vs. 8.01 ± 0.10 (MA) (n = 6, p > 0.05) in the absence of clorgiline (Fig. 3B). In the presence of 3,5-dinitrocatechol (2 × 10^-5 M), DOMA (10^-4 M) did not significantly affect ISO-induced relaxation (Fig. 4C), whereas DHPG (10^-4 M) significantly suppressed ISO-induced relaxation (Fig. 4D). DHPG also significantly decreased the pD2 value of ISO only in the presence of 3,5-dinitrocatechol. The pD2 values of ISO were 8.26 ± 0.11 (control) vs. 8.10 ± 0.11 (DHPG) (n = 6, p < 0.01) in the presence of 3,5-dinitrocatechol (Fig. 4D), and 8.36 ± 0.10 (control) vs. 8.29 ± 0.11 (DHPG) (n = 6, p > 0.05) in the absence of 3,5-dinitrocatechol (Fig. 3D).

Effects of Clorgiline and 3,5-Dinitrocatechol on ISO-Induced Relaxation in Mouse Trachea Neither clorgiline (10^-3 M) (Fig. 4E) nor 3,5-dinitrocatechol (2 × 10^-5 M) (Fig. 4F) significantly affected ISO-induced relaxation.

Effects of CA Metabolites on ISO-Induced Relaxation in GP Trachea NMA (Fig. 5A), MA (Fig. 5B), DOMA (Fig. 5C), and DHPG (Fig. 5D) significantly enhanced ISO-induced relaxation. VMA (Fig. 5E), MHPG (Fig. 5F), and MHPG-S (Fig. 5G) did not affect ISO-induced relaxation.

The degree of NMA-induced potentiation of ISO relaxation was greatly reduced in the presence of clorgiline (10^-3 M), though the potentiation was still significant (Fig. 6Aa). However, in the presence of 3,5-dinitrocatechol (2 × 10^-5 M) (Fig. 6Ab) or in the combined presence of clorgiline (10^-3 M) and 3,5-dinitrocatechol (2 × 10^-5 M) (Fig. 6Ac), the potentiation induced by NMA was replaced by significant suppression of ISO-induced relaxation. NMA also significantly decreased the pD2 values of ISO in the presence of clorgiline plus 3,5-dinitrocatechol as follows: 8.25 ± 0.05 (control) vs. 7.82 ± 0.04 (NMA) (Fig. 6Ac, n = 4, p < 0.01). MA did not significantly affect the relaxation response to ISO in the presence of clorgiline (10^-5 M) (Fig. 6Ba). In the presence of 3,5-dinitrocatechol (2 × 10^-5 M) (Fig. 6Bb), or in the combined presence of clorgiline (10^-5 M) and 3,5-dinitrocatechol (2 × 10^-6 M) (Fig. 6Bc), MA again significantly suppressed ISO-induced relaxation. MA also significantly decreased the pD2 values of ISO in the presence of clorgiline plus 3,5-dinitrocatechol as follows: 8.33 ± 0.07 (control) vs. 7.97 ± 0.05 (MA) (Fig. 6Bc, n = 5, p < 0.01). In the presence of 3,5-dinitrocatechol (2 × 10^-5 M), DOMA did not affect the relaxation response to ISO (Fig. 6C), but DHPG significantly suppressed this response (Fig. 6D). DHPG also significantly decreased the pD2 values of ISO in the presence of 3,5-dinitrocatechol as follows: 8.46 ± 0.11 (control) vs. 8.12 ± 0.07 (DHPG) (Fig. 6D, n = 6, p < 0.01).

Effects of Clorgiline and 3,5-Dinitrocatechol on ISO-Induced Relaxation in GP Trachea Clorgiline (10^-5 M) slightly but significantly enhanced ISO-induced relaxation (Fig. 7A). 3,5-Dinitrocatechol (2 × 10^-5 M) significantly enhanced the relaxation response to ISO (Fig. 7B).

Effects of CA Metabolites on β-AR Agonist-Induced Relaxation in Rat TA DOMA (Fig. 8A), VMA (Fig. 8C), MHPG (Fig. 8D), and MHPG-S (Fig. 8E) did not affect ISO-induced relaxation. DHPG (10^-4 M) suppressed the relaxation response to ISO, although the suppression was not statistically significant (Fig. 8B).

DOMA did not significantly affect the relaxation response to ISO even in the presence of 3,5-dinitrocatechol (2 × 10^-5 M) (Fig. 8F). DHPG significantly suppressed the relaxation response to ISO in the presence of 3,5-dinitrocatechol (2 × 10^-5 M) (Fig. 8G). DHPG also significantly decreased the pD2 values of ISO in the presence of 3,5-dinitrocatechol as follows: 7.91 ± 0.08 (control) vs. 7.23 ± 0.21 (DHPG) (Fig. 8G, n = 6, p < 0.01). DHPG did not affect salbutamol-induced relaxation in the presence of 3,5-dinitrocatechol (2 × 10^-6 M) (Fig. 8H), but significantly suppressed the relaxation response to CGP-12177 A (a β2-AR partial agonist) in the presence of 3,5-dinitrocatechol (2 × 10^-6 M) plus propranolol (3 × 10^-7 M) (Fig. 8I). By itself, 3,5-dinitrocatechol (2 × 10^-6 M) did not affect the relaxation response to ISO (Fig. 8I).

Evaluation of mRNA Expression Figure 9 shows representative images of agarose gels for Adrb1 (β1-AR), Adrb2 (β2-AR), and Adrb3 (β3-AR) PCR products in mouse trachea.

Fig. 5. Effects of CA Metabolites (10^-4 M) on ISO-Induced Relaxation in GP Trachea Contracted by Carbachol

CA metabolites studied are: NMA (A), MA (B), DOMA (C), DHPG (D), VMA (E), MHPG (F), and MHPG-S (G). Data are presented as means ± S.E.M. Numbers of experiments are n = 8 for MA, n = 7 for NMA and DOMA, and n = 6 for DHPG, VMA, MHPG, and MHPG-S. *p < 0.05, **p < 0.01 vs. control (two-way ANOVA followed by Šidák’s multiple comparison post-test).
GP trachea, and rat TA. In all tissues, mRNAs for all three β-ARs (β1, β2, and β3) were detected. In the mouse trachea, Adrb1, Adrb2 (158 base pairs (bp) each) and Adrb3 (202 bp) mRNAs were clearly detected. In contrast, Adrb3 mRNA (168 bp) was less expressed in guinea pig trachea compared to those of Adrb1 (207 bp) and Adrb2 (164 bp). Similarly, in rat TA, Adrb3 (352 bp) mRNA was less expressed compared to those of Adrb1 (337 bp) and Adrb2 (386 bp). No bands were observed in the absence of reverse transcription (RT(−)).

DISCUSSION

This study examined the possibility that noradrenaline/adrenaline metabolites show stimulatory or antagonistic effects on β-AR distributed in smooth muscles. Our principal results were: 1) MA shows a β2-AR agonistic action; 2) NMA and MA augment β2-AR-mediated tracheal relaxation in the absence of CA metabolic inhibitors, while themselves possessing β1-, β2-AR antagonistic action (β2 > β1); 3) DHPG exhibits β1-, β2-AR antagonistic action, and this is particularly marked for β3-AR.

Tracheal Relaxant Actions of CA Metabolites Of the seven types of CA metabolites studied, MA showed a significant relaxant effect on GP trachea (Fig. 2D). This suggests that MA has a β2-AR stimulatory effect, a view that is supported by our previous observations that the functional β-AR subtype in GP trachea is primarily β2-AR,6) although mRNAs for all three β-ARs are expressed (Fig. 9). In addition, the relaxant effect of MA in GP trachea was significantly attenuated by ICI 118,551 (Fig. 2F). Furthermore, MA did not induce significant relaxation in mouse trachea, in which the functional β-AR subtype is β1-AR,5) although mRNAs for all three β-ARs are expressed (Fig. 9). We also confirmed that isoprenaline-induced relaxation in mouse trachea is elicited via primarily β1-AR (our unpublished observation). Therefore,
MA-induced relaxation of GP trachea is unlikely to be mediated through β1-AR stimulation or anticholinergic action. In addition, since the relaxation response to MA was significant even in the presence of a MAO A inhibitor (clorgiline) (Fig. 2E), relaxation due to MA is likely to be caused by MA itself rather than its metabolites. This result was consistent with the finding by Langer et al.3) that MA showed positive chronotropic action in GP atrial muscle via β-AR. We found that DOMA had a slight relaxant effect on mouse trachea, which appeared even in the presence of a COMT inhibitor (3,5-dinitrotochecol), suggesting that DOMA itself might have a β1-AR-stimulating action, but the effect was not significant (Figs. 2A, C). NA and AD almost completely relaxed both mouse and GP tracheas, whereas their metabolites showed negligible or slight relaxant effects in both tracheas. These results indicate that the relaxant effects of NA and AD via β1-, β2-AR are markedly attenuated by COMT/MAO metabolism.

Effects of CA Metabolites on β-AR-Mediated Tracheal Relaxation

The seven CA metabolites examined did not significantly affect ISO-induced relaxation in mouse trachea (Fig. 3), but four CA metabolites (NMA, MA, DOMA, and DHPG) significantly augmented ISO-induced relaxation in GP trachea (Fig. 5). Since the main β-ARs that trigger ISO relaxation are β1-AR in mouse and β2-AR in GP trachea, we inferred that the four CA metabolites (NMA, MA, DOMA, and DHPG) potentiated β2-AR-mediated relaxation.

Regarding NMA and MA, their stimulatory effects on ISO-induced relaxation were remarkably attenuated (NMA) (Fig. 6Aa) or disappeared (MA) (Fig. 6Ba) in the presence of a MAO A inhibitor (clorgiline). These results suggest that MAO metabolites of NMA/MA were responsible for the enhancement of ISO-induced relaxation by NMA/MA. However, VMA and MHPG did not augment ISO-induced relaxation (Figs. 5E, F), which eliminates the possibility that VMA/MHPG produced via MAO-mediated metabolism of NMA/MA cause the enhancement. Furthermore, the degree of potentiation of ISO-induced relaxation by the MAO A inhibitor clorgiline11) was marginal, though statistically significant (Fig. 7A). This finding suggests that ISO is not easily metabolized by MAO. Therefore, even if NMA and MA themselves have MAO inhibitory actions, their participation in the potentiation of ISO-induced relaxation is presumed to be negligible.

In view of the above considerations, the MAO-generated intermediate metabolite 3-methoxy-4-hydroxymandelaldehyde (MHMA) (Fig. 1) may be a candidate metabolite responsible for the potentiation of ISO-induced relaxation by NMA/MA. Details including the potential roles of this intermediate me-
tabolite, and their underlying specific mechanisms, remain to be studied. Interestingly however, ISO-induced relaxation was significantly potentiated by a COMT inhibitor (Fig. 7B), suggesting that the relaxant activity of ISO is decreased by COMT-mediated metabolism. Therefore, if an intermediate metabolite produced by MAO from NMA/MA is responsible for the enhancement of ISO-induced relaxation, its possible inhibition of COMT activity might partly explain the underlying mechanism.

NMA slightly enhanced ISO-induced relaxation (Fig. 6Aa), while MA had little effect (Fig. 6Ba) in the presence of a MAO_A inhibitor. In contrast, in the combined inhibition of a MAO_A inhibitor plus a COMT inhibitor, NMA and MA greatly suppressed ISO-induced relaxation (Figs. 6Ac, Bc). These findings suggest that NMA and MA themselves have \( \beta_2 \)-AR antagonistic effects. On the other hand, since the inhibitory effects of NMA and MA on ISO-induced relaxation were detected in the presence of inhibitors of both MAO_A and COMT (Figs. 6Ac, Bc), NMA and MA themselves may have COMT inhibitory effects that counteract their \( \beta_2 \)-AR antagonistic effects. It should be noted that NMA and MA, and their MAO metabolites, were speculated to co-exist under the experimental conditions shown in Fig. 6 (Ab and Bb) where MAO was not inhibited, and thus, interpretation of these data was complicated.

Based on the agonistic/antagonistic actions of MA, it is reasonable to conclude that MA is a partial agonist of \( \beta_2 \)-AR. However, in addition to these effects, MA may possess a strong COMT inhibitory action, which would mask the former \( \beta_2 \)-AR agonistic/antagonistic actions under physiological conditions. Therefore, MA is expected to enhance the physiological actions of sympathetic nerve-derived NA and adrenal medulla-secreted AD/NA through inhibition of COMT activity, which supersedes \( \beta_2 \)-AR agonistic/antagonistic actions. More specifically, MA could enhance AD-induced, \( \beta_2 \)-AR-mediated tracheal relaxation, and NA/AD-induced, \( \alpha_1 \)-AR-mediated vasconstriction.

DOMA and DHPG also enhanced ISO-induced relaxation of GP trachea (Figs. 5C, D). Of these, enhancement of DOMA disappeared in the presence of a COMT inhibitor (Fig. 6C). However, VMA, a COMT metabolite of DOMA, did not enhance ISO-induced relaxation (Fig. 5E). These findings suggest that one of the mechanisms for the potentiating action of DOMA on ISO-induced relaxation may be COMT inhibition by DOMA itself. This inference is consistent with the result that ISO-induced relaxation was enhanced by a COMT inhibitor (Fig. 7B). However, CA metabolites and a COMT inhibitor did not show significant stimulatory effects on the mouse trachea (Figs. 3A–G, Fig. 4F) or rat TA relaxation induced by ISO (Figs. 8A–E; 8I), the pharmacological activities of which were shown to be reduced through the COMT pathway. These results suggest that COMT activity is low in mouse tracheas and rat TAs, but high in GP tracheas. Therefore, GP trachea-specific enhancement of ISO-induced relaxation by CA metabolites (Figs. 5A–D) could be explained by high COMT activity in this smooth muscle tissue. In contrast, the effects of DHPG seemed to be more complicated. First, DHPG significantly enhanced ISO-induced relaxation (Fig. 5D). However, DHPG suppressed the relaxation in the presence of a COMT inhibitor (Fig. 6D). In addition, MHPG, a COMT metabolite of DHPG, did not affect the relaxation response to ISO (Fig. 5F). These findings implicate DHPG in two effects: a COMT inhibitory action and a \( \beta_2 \)-AR antagonistic action. Of these two actions, COMT inhibition was presumed to be greater than \( \beta_2 \)-AR antagonism, since DHPG potentiated the relaxation response to ISO in the absence of COMT inhibitor.

In mouse trachea, NMA and MA (each \( 10^{-4} \) M) and DHPG (\( 10^{-4} \) M) showed small but significant inhibitory effects vs. ISO-induced relaxation in the presence of a MAO_A inhibitor (NMA/MA) (Figs. 4A, B) or a COMT inhibitor (DHPG) (Fig. 4D). These findings suggest that these CA metabolites (NMA, MA, and DHPG) themselves have a \( \beta_2 \)-AR antagonistic effect. In order to compare the apparent antagonistic potencies of NMA, MA, and DHPG against \( \beta_1 \)-AR and \( \beta_2 \)-AR, we obtained the concentration ratios (CRs) for the ISO’ EC50 values in the absence vs. in the presence of CA metabolites. For NMA, the CRs were 1.59 (mouse trachea; \( \beta_1 \)-(AR) (Fig. 4A) vs. 2.79 (GP trachea; \( \beta_2 \)-AR) (Fig. 6Ac). For MA, the CRs were 1.64 (mouse trachea; \( \beta_1 \)-AR) (Fig. 4B) vs. 2.39 (GP trachea; \( \beta_2 \)-AR) (Fig. 6Bc). For DHPG, the CRs were 1.45 (mouse trachea; \( \beta_1 \)-AR) (Fig. 4D) vs. 2.26 (GP trachea; \( \beta_2 \)-AR) (Fig. 6D). Therefore, these results indicated that NMA, MA, and DHPG possess \( \beta_2 \)-AR antagonistic actions, of which \( \beta_2 \)-AR antagonism actions was more pronounced.

In rat TA, among the five CA metabolites examined (DOMA, DHPG, VMA, MHPG, and MHPG-S), DHPG showed an inhibitory effect vs. ISO-induced relaxation (Fig. 8B). This inhibitory effect was significant and more conspicuous in the presence of a COMT inhibitor (Fig. 8G). This inhibitory effect of DHPG vs. ISO-induced relaxation was speculated to be partly caused by \( \beta_2 \)-AR antagonistic action, since DHPG also largely inhibited \( \beta_2 \)-AR agonist (CGP-12177) (Fig. 8I). The effect of DHPG on ISO-induced relaxation in rat TA (Fig. 8G) seems to be greater than the effects observed in mouse (\( \beta_1 \)) and GP (\( \beta_2 \)) tracheas (Figs. 4D, 6D). For DHPG, the CRs were 1.45 (mouse trachea; \( \beta_1 \)-(AR) (Fig. 4D), 2.26 (GP trachea; \( \beta_2 \)-AR) (Fig. 6D), and 7.88 (rat TA; \( \beta_1 \)-AR and \( \beta_2 \)-AR) (Fig. 8G). The antagonistic actions of DHPG against ISO in rat TA included both \( \beta_2 \)-AR and \( \beta_2 \)-AR. However, if we assume that the CR for \( \beta_2 \)-AR is 2.26, the remaining CR of 5.62 can be estimated to belong to \( \beta_2 \)-AR. Therefore, \( \beta_2 \)-AR antagonistic actions of DHPG may be exerted preferentially on \( \beta_2 \)-AR. In this study, we found that DHPG did not affect \( \beta_2 \)-AR agonist (salbutamol)-induced relaxation (Fig. 8H). However, we could not find any reasonable explanation for this observation, though DHPG itself inhibited ISO-induced relaxation in GP trachea, and this should be elucidated in future.

**Possible Clinical Significance of the Findings of This Study**  CA metabolites examined in this study increase in patients with pheochromocytoma. Therefore, the results of this study may explain some of the pathologies associated with this illness. Specifically, if our findings are reproduced in patients with pheochromocytoma, then increased NMA, MA, DOMA, and DHPG in the blood inhibit COMT activity, and thus inhibit the metabolism of NA/AD to NMA/MA, thus increasing their blood levels. Therefore, these metabolites may exacerbate pathologies, such as hypertension, caused by NA/AD. In addition, the \( \beta_2 \)-AR antagonistic action of DHPG may cause pollakuria by the inhibition of the relaxation reaction in urinary bladder smooth muscle via \( \beta_2 \)-AR, caused by NA. To verify this hypothesis, further study of the effects of
these metabolites on the contraction or relaxation responses induced by AD and NA is required.

**Conflict of Interest** The authors declare no conflict of interest.

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