5-HT release from chemoreceptor cells in the chicken thoracic aorta

（ニワトリ胸部大動脈の化学受容器細胞からの

5-HT 放出機構に関する研究）

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Contents

Chapter II

Effects of H_2S on 5-HT-containing chemoreceptor cells in chicken thoracic aorta

Abbreviations

AADC	Aromatic L-amino acid decarboxylase
ACh	Acetylcholine
ATP	Adenosine triphosphate
CBS	Cystathionine –β-synthase
CHO cells	Chinese hamster ovary cells
CNS	Central nervous system
CLM	Clomipramine
CSE	Cystathionine-γ-lyase
HPLC	High performance liquid chromatography
5-HT	5-Hydroxytriptamine
5-HIAA	5-Hydroxyindoleacetic acid
5-HTP	5-Hydroxytriptophan
MAO	Monoamine oxidase
MET	Methamphetamine
3-MST	3-Mercaptopyruvate sulfurtransferase
PBS	Phosphate buffered saline
PCA	para-Chloroamphetamine
SERT	Serotonin transporter
SRI	Serotonin reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TPH	Tryptophan hydroxylase

Trp	Tryptophan
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1
VMAT	Vesicular monoamine transporter
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration

Preface

5-Hydroxytriptamine (5-HT, serotonin) is a neurotransmitter. 5-HT is localized in three essential pools in the body. First, a large amount of 5-HT is found in the enterochromaffin cells of the mucosal epithelium, which are scattered along the gastrointestinal tract. Second, about 10% of 5-HT is concentrated in blood platelets, which do not synthesize 5-HT but incorporate it from blood. Third, a small amount of 5-HT is found in the raphe nuclei neurons of the central nervous system (CNS), the axons of which innervate various regions of the CNS including the spinal cord. In addition, 5-HT is also stored in the rodent mast cells along with histamine and heparin. 5-HT is synthesized from the dietary tryptophan with enzymatic reaction (Walther et al., 2003) (Fig. 1), and stored into secretory vesicles via vesicular monoamine transporter (VMAT) in neuronal cells. 5-HT is released by Ca^{2+}-dependent exocytotic and then incorporated into cells by serotonin transporters (SERT) or metabolized to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO) (Fig. 2). In addition to the exocytotic release, the impairments of these transports and/or metabolic processes could evoke the outflow of 5-HT.

The epithelioid cells in the luminal wall of the chicken thoracic aorta also contain 5-HT (Yamamoto et al., 1989). These cells aggregate into clusters and form a band of about 1 mm in width (Fig. 3), which has been advocated as the term “chemoreceptive ring” because of their similar morphological and functional characteristics to mammalian carotid chemoreceptor cells (Miyoshi et al., 1995; Ito et al., 2001). In mammals, there are two primary chemoreceptor organs in the peripheral

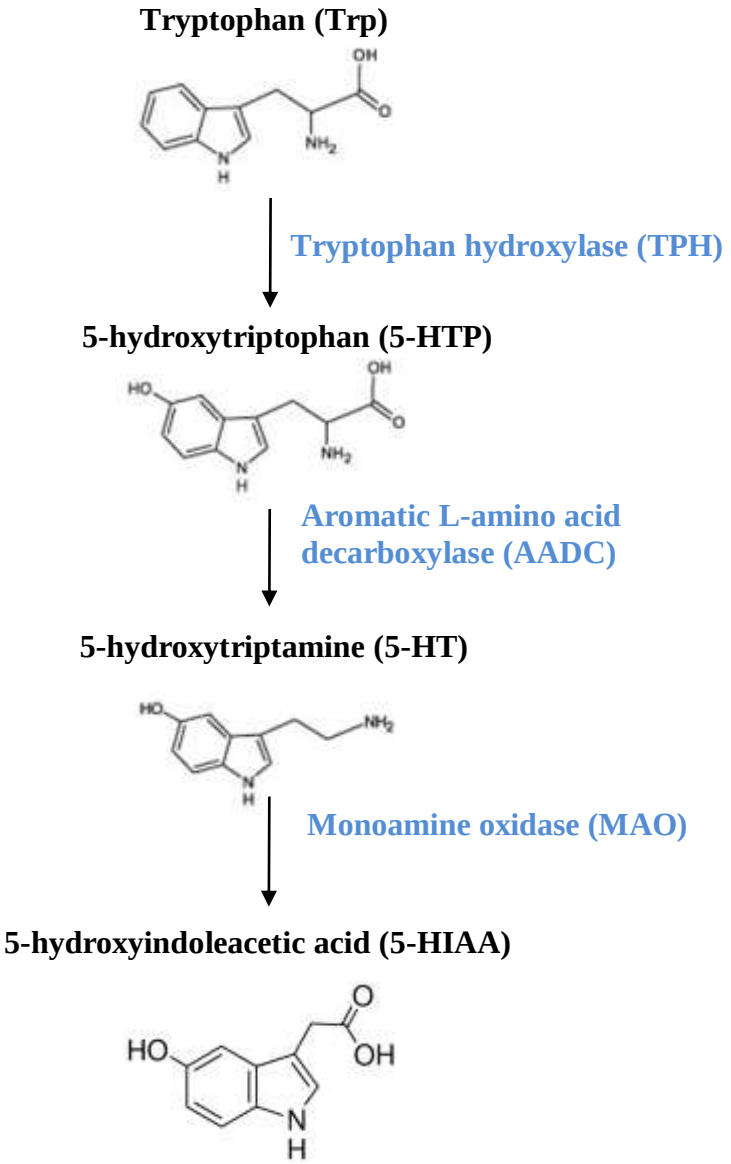

Fig. 1. 5-HT synthesis and metabolism

Tryptophan (Trp) is hydroxylated to 5-hydroxytriptophan (5-HTP) by tryptophan hydroxylase (TPH). 5-Hydroxytriptamine (5-HT) is produced from 5-HTP by aromatic L-amino acid decarboxylase (AADC). 5-HT is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO).

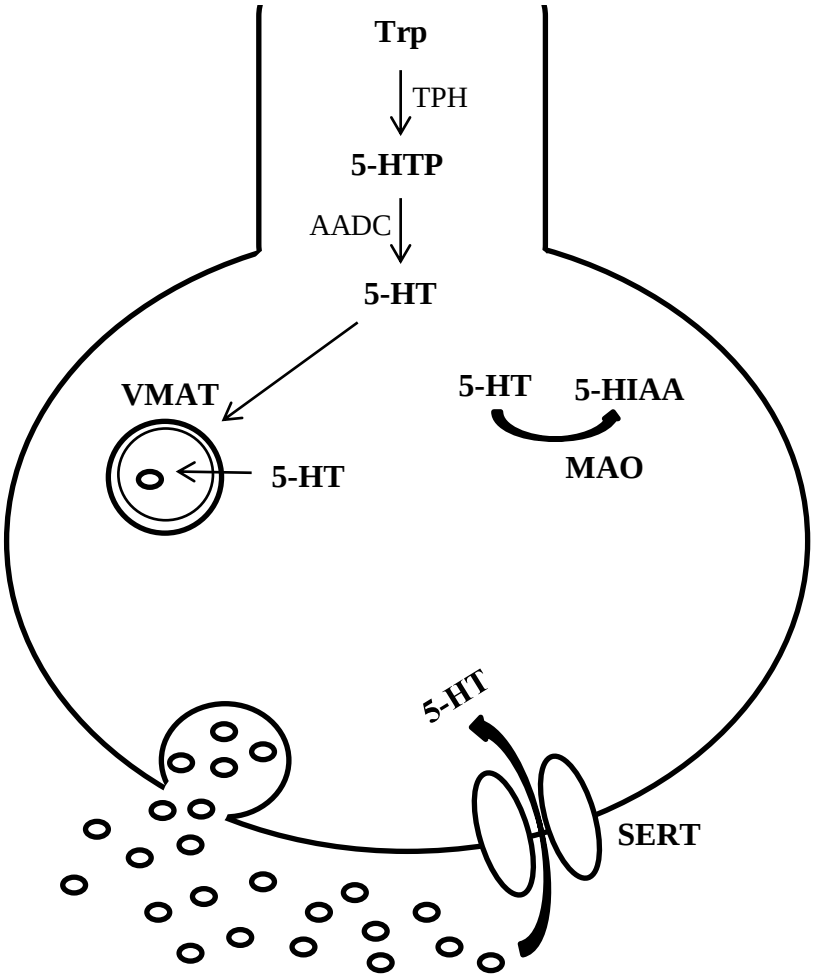

Fig. 2. Pathways of 5-HT synthesis, metabolism and transport in the neuron

5-HT is synthesized from Trp with an enzymatic reaction and transported from the cytoplasm into the secretory vesicles and stored by VMAT. 5-HT is released by exocytosis and then incorporated into the cells by SERT or metabolized to 5-HIAA by MAO.

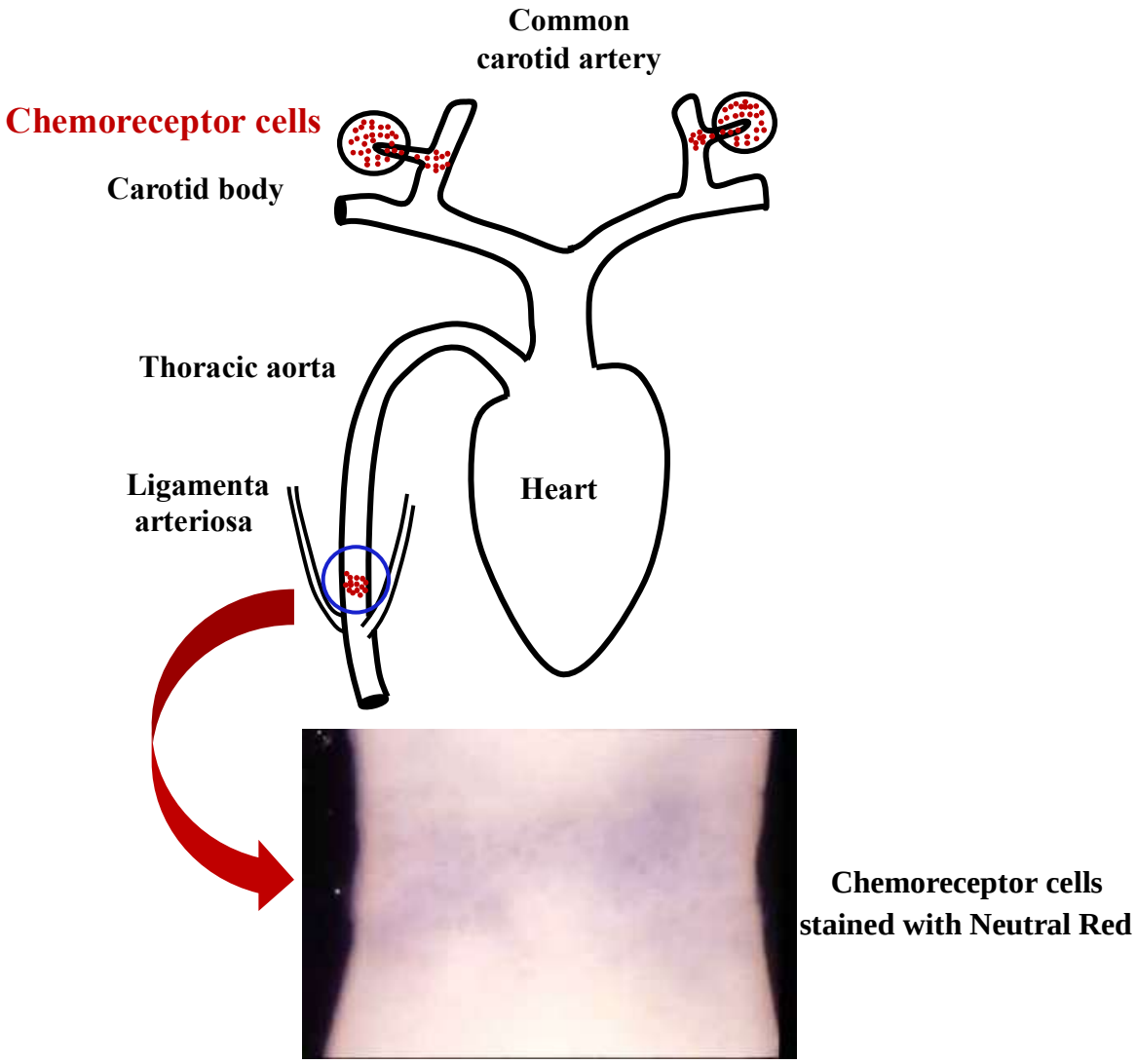

Fig. 3. Location of chemoreceptor cells in the chicken aorta

Schematic illustration shows the localization of chemoreceptor cells in the chicken aorta (red dot). Chemoreceptor cells in the thoracic aorta are located near the ligamenta arteriosa. Carotid bodies are located near the carotid bifurcation of common carotid artery. Lower image shows the wall of the thoracic aorta stained with Neutral Red. Acidic secretory vesicles containing 5-HT are red-coloured.

tissues, i.e., carotid and aortic bodies. These bodies are mainly composed of two types of cells: glomus or type I cells, which exhibit a neuronal phenotype, and the sustentacular or type II cells, which resemble glial cells of the nervous system. The glomus cells of carotid body are closely associated with cuplike endings of afferent nerves. They sense changes in plasma P_{O2}, P_{CO2} and pH, and then release some transmitters such as dopamine and noradrenaline, which activate the sensory nerve endings (Gonzalez et al., 1994). In the chicken carotid bodies, 5-HT has been reported to be a dominant transmitter (Yamamoto et al., 1989). In mammals, the structure of parenchymal chemoreceptor glomus or type I cells in aortic body and their innervation are indistinguishable from those of the carotid body (Piskuric and Nurse, 2012; McDonald and Blewett, 1981) and in both cases, the type I cells express multiple neurotransmitters, acetylcholine (ACh), 5-HT and ATP (Dvorakova and Kummer, 2005; Piskuric and Nurse, 2013). However, the physiological function of aortic body remains controversial compared with carotid body in mammals.

Our laboratory has previously reported that the epithelioid cells in the chicken thoracic aorta release 5-HT in response to various stimuli such as depolarization and hypoxia. In addition, these 5-HT-containing cells express neuronal nicotinic ACh receptors and voltage-dependent Na^+, K^+, and Ca^{2+} channels (Ito et al., 1997, 1999, 2001). Therefore, these data suggest that 5-HT-containing chemoreceptor cells in the chicken aorta have similar mechanisms responsible for 5-HT transport, storage and release to those in 5-HT-containing neurons.

In Chapter I, to reveal whether the chicken aortic chemoreceptor cells had a 5-HT transporter and storage activity like 5-HT-containing neurons in the CNS, the effects of 5-HT uptake inhibitors and amphetamine derivatives (5-HT transporter

inhibitors and VMAT inhibitors, respectively) on 5-HT outflow from these cells were examined.

Recently, it has been reported that hydrogen sulfide (H_2S) is one of the key factors for hypoxia sensing in rat carotid glomus cells (Makarenko et al., 2012). In rodent carotid glomus cells, hypoxia triggers the synthesis of H_2S, which increases catecholamine release (Makarenko et al., 2012). Exogenous H_2S also activates carotid body type 1 cells in rat, cat, and rabbit (Buckler, 2012; Jiao et al., 2015).

In Chapter II, thus, the effect of H_2S on 5-HT release from the chemoreceptor cells in the chicken thoracic aorta was examined.

Most of the materials contained in this thesis have been already published in the following papers.

1. Delgermurun D, Ito S, Ohta T, Yamaguchi S, Otsuguro K (2016). Endogenous 5-HT outflow from chicken aorta by 5-HT uptake inhibitors and amphetamine derivatives. *J Vet Med Sci* 78: 71-76.
2. Delgermurun D, Yamaguchi S, Ichii O, Kon Y, Ito S, Otsuguro K (2016). Hydrogen sulfide activates TRPA1 and releases 5-HT from epithelioid cells of the chicken thoracic aorta. *Comp Biochem Physiol C Toxicol Pharmacol* 187: 43-49.

Chapter I

Effects of 5-HT uptake inhibitors and amphetamine derivatives on 5-HT-containing chemoreceptor cells in chicken thoracic aorta

1. Introduction

5-HT is a monoamine neurotransmitter which plays many important roles in physiological and pathological processes such as sleep, sensory perception, cognition, motor activity, appetite, mood and behavior (Adams, 2009). Abnormalities in 5-HT production or metabolism make some behavioral problems or diseases. For example, lower level of 5-HT in the CNS is involved in aggressive behavior (Reisner et al., 1996).

Epithelioid cells containing 5-HT aggregate into clusters and form a band of about 1 mm in width in the wall of the chicken thoracic aorta (Yamamoto et al., 1989; Miyoshi et al., 1995). These 5-HT containing cells are chemoreceptors because oxygen-sensitive K^+ currents and the release of 5-HT by hypoxia have been observed (Ito et al., 1997, 1999). Like neuron, the neuronal nicotinic ACh receptors and voltage-dependent Na^+, Ca^{2+} and K^+ channels are present on these chemoreceptor cells (Ito et al., 1999, 2001). From these facts, it is likely that the chicken aortic chemoreceptor cells have similar mechanisms of 5-HT transport activity to those in 5-HT-containing neurons in mammals. However, these issues have not been addressed.

In 5-HT-containing neurons, cytosolic 5-HT is transported into the secretory vesicles by VMAT, and then released by exocytosis. 5-HT is mainly degraded by MAO or incorporated into the cells by 5-HT transporters. 5-HT uptake inhibitors or

serotonin reuptake inhibitors (SRIs) inhibit cellular membrane 5-HT transporters and are typically used as antidepressants in the treatment of depression and other neurological disorders such as aggression, obsessive-compulsive disorder and anxiety disorders (Dodman et al., 1996; Seksel and Lindeman, 2001; Fitzgerald and Bronstein, 2013). Fluvoxamine and fluoxetine are selective serotonin reuptake inhibitors (SSRIs). SSRIs increase extracellular level of 5-HT by inhibiting the reuptake of 5-HT in the neurons (Bel and Artigas, 1996; Tatsumi et al., 1997; Hiemke and Hartter, 2000; Nagayasu et al., 2013). Clomipramine (CLM) is a tricyclic antidepressant (TCA), which inhibits the reuptake of 5-HT and norepinephrine. CLM is the most potent 5-HT uptake inhibitor among the TCAs (Sherman and Papich, 2009). Amphetamine derivatives such as PCA and MET inhibit VMAT and MAO, resulting in a rise in cytosolic 5-HT and then elicitation of 5-HT outflow from them into synaptic regions (Sulzer et al., 2005; Fleckenstein et al., 2009; Heal et al., 2013).

The aim of this study is to examine whether 5-HT-containing cells in the chicken aorta had a 5-HT transport activity like 5-HT-containing neurons in the CNS. For this purpose, the effects of 5-HT uptake inhibitors (fluvoxamine, fluoxetine and CLM) and amphetamine derivatives (PCA and MET) on the outflow of 5-HT from the chicken thoracic aorta were examined.

2. Materials and Methods

2.1. Animals

Newly-hatched chickens (White Leghorn, Julia) were purchased from Iwamura Poultry (Yuni, Japan). They were housed in a room with 12/12 h light/dark cycle at 22 ± 4ºC, and were given food and water ad libitum. In addition, a heat lamp was used to warm newborn chicks. All experiments were performed under the regulation of the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University, Japan (Approval ID: 13-0022). The animal facilities and animal care programs are accredited by AAALAC international in the USA.

2.2. Preparation of aortic tissue

Male chickens (14–28 days after hatching) were deeply anesthetized by placing them in a small chamber in which ether or isoflurane was vaporized, and then decapitated. The 5-HT-containing cells aggregate in clusters in the inner wall of the chicken thoracic aorta as a "chemoreceptive ring" (Miyoshi et al., 1995). The thoracic aorta was isolated and freed from surrounding tissues under a stereomicroscope. Aortic strips (about 5 mm in length) with a chemoreceptor ring were cut longitudinally to open them and kept in oxygenated Hepes-buffered saline solution on ice until use. Hepes-buffered saline with the following composition was used (mM): NaCl 140, KCl 6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, Hepes 10, and glucose 10. The pH was

adjusted to 7.3 with NaOH. In a Ca^{2+}-free solution, $CaCl_2$ was removed and 0.5 mM EGTA was added. In a low NaCl solution, NaCl was iso-osmotically replaced with sucrose.

2.3. Measurement of 5-HT outflow

The aortic strip was placed in a sample tube containing ice-cold Hepes-buffered saline solution (0.1 ml) with or without drugs and then incubated at 37°C for 10 min. In some experiments, temperature or incubation time was altered. The secretory response of 5-HT was terminated by placing the tubes on ice. After stopping the secretory response, the aortic tissue was transferred to another sample tube containing 0.4 N perchloric acid (0.2 ml) to extract 5-HT and 5-HIAA that remained in the tissue. To measure the amounts of 5-HT and 5-HIAA in the incubation medium, 4 N perchloric acid (10 µl) was added to the medium to obtain a final concentration of 0.4 N. After centrifugation of the sample tube containing tissue extract or incubation medium, K_2HPO_4 was added to the supernatant to obtain a final concentration of 580 mM (pH 5‑6). After removal of potassium percolate by centrifugation, the clear supernatant was subjected to high-performance liquid chromatography (HPLC). The mobile phase consisted of a citric acid buffer (0.1 M citric acid, 0.1 M sodium acetate; pH 3.5), 19% methanol, 5 mg/l EDTA-2Na, and 190 mg/l 1-octanesulfonic acid. The mobile phase was degassed using DG-350 (EICOM, Kyoto, Japan), at a flow rate of 0.5 ml/min. The samples (50 µl) were applied using an autosampler (System Instruments model 33, Tokyo, Japan) to an

octadecylsilane-column (EICOMPAK SC-50DS, 3.0 × 150 mm, EICOM) kept at 30ºC, and 5-HT and 5-HIAA were detected at +450 mV with an electrochemical detector (ECD-300, EICOM).

The retention times were about 7 and 11 min for 5-HIAA and 5-HT, respectively (Fig. 4). The standard solutions (100, 200, 300, 400 and 500 nM) of 5-HT and 5-HIAA were applied to HPLC before and after the sample injection. The averages of 5-HT and 5-HIAA peak areas were used in order to construct a calibration curve (Fig. 5). The area under the curve of the peak was measured to calculate the amount of 5-HT and 5-HIAA.

5-HT outflow was expressed as a percentage of total 5-HT content in the aortic strip. The sum of the amounts of 5-HT and 5-HIAA in the tissue and incubation medium (supernatant) was regarded as the amount of 5-HT. The ratio of 5-HT outflow (%) was determined by dividing the amount of 5-HT in the supernatant by the total 5-HT (in the tissue and supernatant) as follows.

Amount of 5-HT = 5HT + 5HIAA

$$\textbf{Outflow of 5-HT (\% of content)} = \frac{\text{Supernatant}}{\text{Total 5-HT (Supernatant + tissue)}} \times 100$$

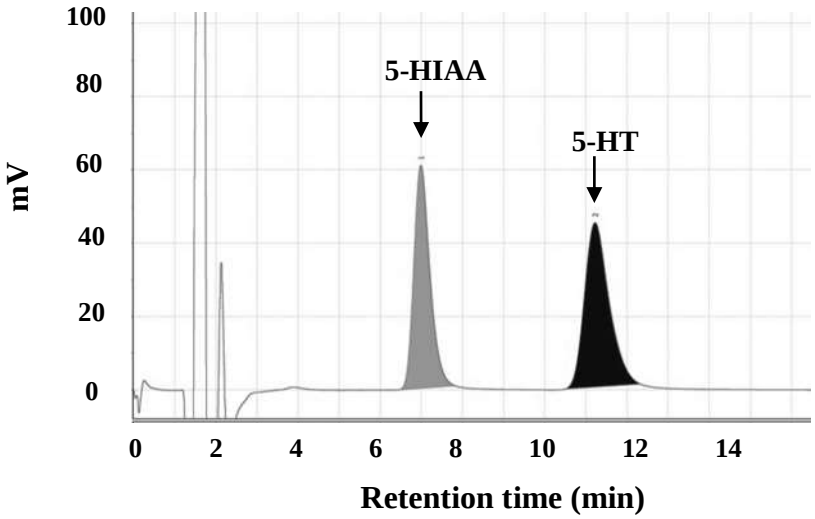

Fig. 4. Chromatograms for 5-HT and 5-HIAA

Chromatograms of 5-HT and 5-HIAA standard solution. A 50 µl aliquot of a standard mixture of 5-HT (100 nM) and 5-HIAA (100 nM) was injected. The retention times for 5-HIAA and 5-HT were 7 min and 11 min, respectively.

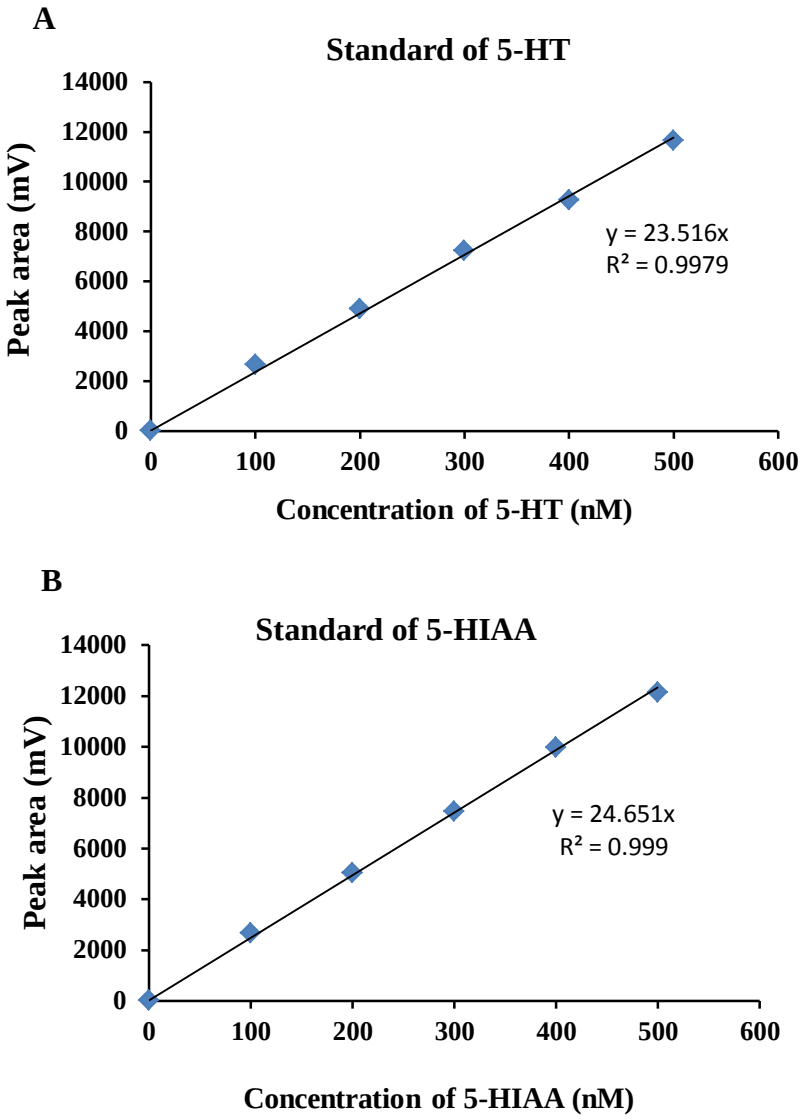

Fig. 5. Calibration curves for 5-HT and 5-HIAA.

Calibration curves of 5-HT (A) and 5-HIAA (B). The standard solutions (50 µl) of 5-HIAA (100, 200, 300, 400 and 500 nM) were injected to HPLC. The area under the curve of the peak was measured.

2.4. Drugs

Following drugs were used. Methamphetamine (MET) (Sumitomo Dainihon Pharma, Osaka, Japan), *p*-chloroamphetamine (PCA), clomipramine (CLM) (Sigma, St. Louis, MO, U.S.A.), fluvoxamine, fluoxetine (Tocris, Bristol, UK), ω-conotoxin GVIA (Peptide Institute, Osaka, Japan), and nifedipine (Wako Pure Chemical Industries, Osaka, Japan) were prepared from 0.1 M stock solutions and dissolved in Hepes-buffered saline solution.

2.5. Data analysis

All data were expressed as means ±S.E.M. Statistical comparisons between two groups were performed by unpaired Student's *t*-test. For multiple comparisons, one-way ANOVA, followed by Dunnett's test, was used. A P value of less than 0.05 was considered significant.

3. Results

3.1. Time-dependent outflow of 5-HT by PCA

The effect of PCA on 5-HT outflow from the chicken thoracic aorta with a chemoreceptor ring was first examined. PCA has been known as the most potent 5-HT releaser among the amphetamine derivatives (Wichems et al., 1995).

The aortic tissues were incubated at 37°C for various incubation times. The resting outflow of 5-HT (control) gradually increased, and about 5% of 5-HT content in the tissue was effluxed during 20-min incubation (Fig. 6). In the presence of PCA (1 mM), the outflow of 5-HT was around 20% at 20-min incubation. Under this condition, the 10-min incubation was enough to substantially increase 5-HT outflow in response to PCA. In the following experiments, therefore, aortic tissues were incubated for 10 min in the presence of various drugs.

3.2. Temperature-dependent outflow of 5-HT by PCA and CLM

If 5-HT outflow from the chemoreceptor cells induced by PCA and CLM resulted from biological activities such as transporters and metabolic enzymes, it would be dependent on incubation temperature. Therefore, the effect of temperature on 5-HT outflow in the presence of PCA and CLM, a 5-HT reuptake inhibitors, was examined. The resting outflow of 5-HT increased with the increase in temperature from 5 to 37ºC (Fig. 7). PCA (1 mM) and CLM (0.1 mM) significantly increased

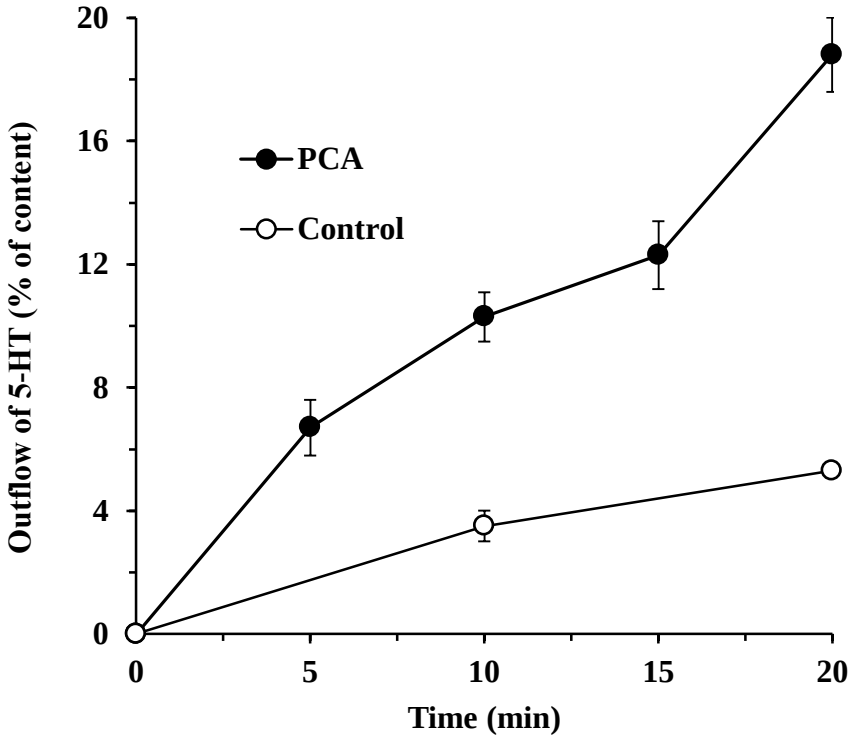

Fig. 6. Time-dependent outflow of 5-HT from the chicken aorta with a chemoreceptor ring induced by PCA.

The aortic tissues were incubated at 37ºC for 5, 10, 15 and 20 min in the presence of PCA. The time-course of the resting outflow (control, n = 4‑5) and PCA (1 mM, n = 4‑5)-induced outflow of 5-HT. Data are means ± S.E.M.

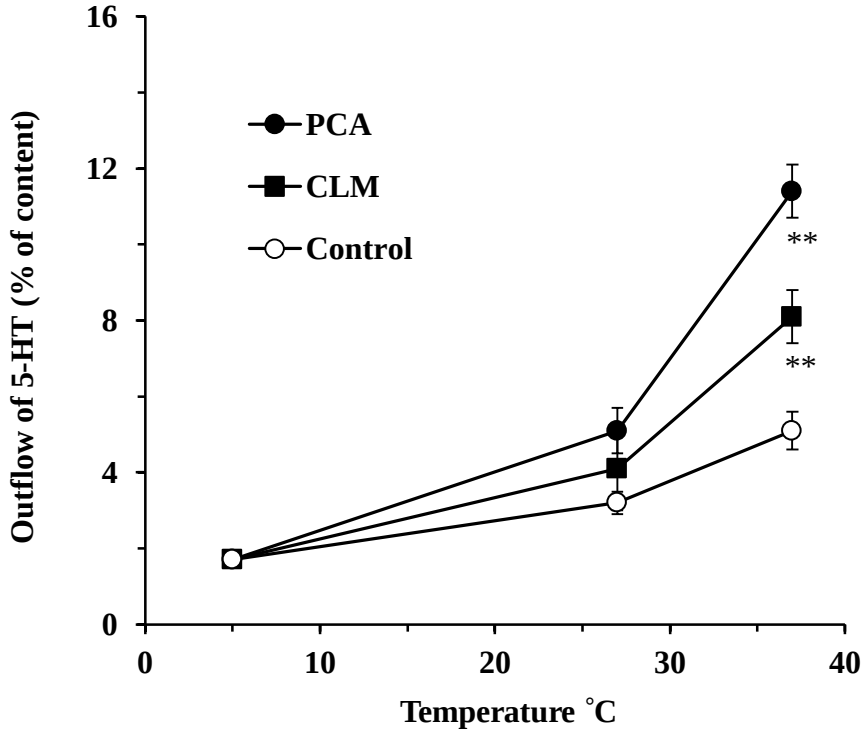

Fig. 7. Temperature-dependent outflow of 5-HT from the chicken aorta with a chemoreceptor ring induced by PCA and CLM.

The aortic tissues were incubated for 10 min at 5, 27 and 37^{O}C. Temperature -dependence of resting 5-HT outflow (control, n = 4‑8) and PCA (1 mM, n = 4‑7) and CLM (0.1 mM, n = 4)-induced 5-HT outflow. Data are means ± S.E.M. **P < 0.01 vs. control (Dunnett's test).

5-HT outflow at 37ºC as compared to the control. However, at lower temperatures (5ºC and 27ºC), both drugs failed to increase 5-HT outflow. These data indicate that 5-HT outflows by PCA and CLM were due to the biological activities but not to the leakage from damaged cells. In addition, it was assumed that 37ºC was convenient condition to measure 5-HT outflow.

3.3. Concentration-dependent outflow of 5-HT by amphetamine derivatives and 5-HT uptake inhibitors

To investigate whether the chemoreceptor cells in the chicken aorta had a 5-HT transport and uptake system similar to the 5-HT-containng neurons, the effects of 5-HT uptake inhibitors, fluoxetine, fluvoxamine and CLM and amphetamine derivatives, MET and PCA on 5-HT outflow from chicken thoracic aorta were examined. It has been reported that antidepressants such as fluvoxamine inhibit 5-HT reuptake, and elicit a significant increase in extracellular 5-HT in the vicinity of the dorsal and median raphe nuclei in the CNS (Bel and Artigas, 1992; Fuller, 1994). Amphetamine derivatives also increase extracellular levels of neurotransmitters including 5-HT by promoting reverse transport (efflux) of them through plasma membrane monoamine transporters (Robertson et al., 2009).

The aortic tissues were incubated at 37ºC for 10 min with MET, PCA, CLM, fluoxetine or fluvoxamine at various concentrations to examine their effects on 5-HT outflow. The resting outflow of 5-HT varied slightly from preparation to preparation. The resting outflow of 5-HT was 3.6 ± 0.6% (n = 4) in the experiments of PCA. PCA (1 µM – 1 mM) caused concentration-dependent increases in 5-HT outflow (Fig. 8A),

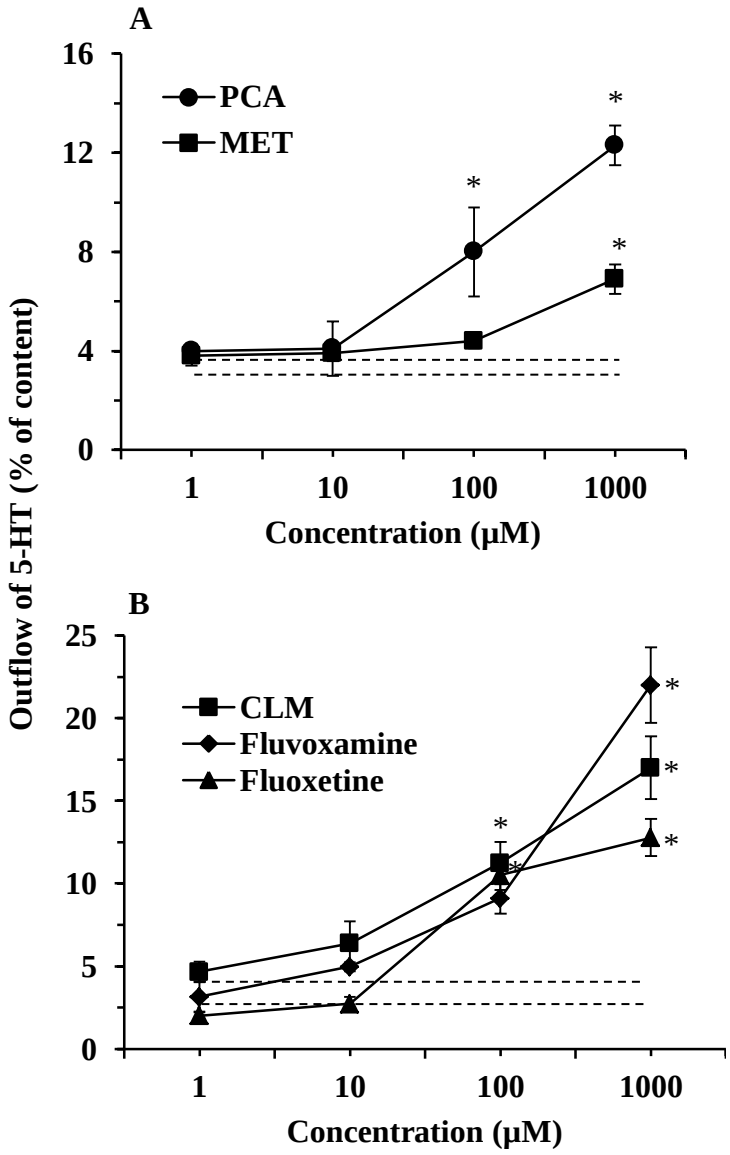

Fig. 8. Effects of amphetamine derivatives and 5-HT reuptake inhibitors on 5-HT outflow.

The concentration-dependent increases in 5-HT outflow in response to PCA (n = 4–6) and MET (n = 4) (A), and CLM (n = 8), fluvoxamine (n = 8) and fluoxetine (n = 5) for 10 min at 37°C (B). Data are means ± S.E.M. Dashed lines indicate upper and lower limits of S.E.M. of the resting outflow (n = 10). *P < 0.05 vs. the resting outflow (Dunnett's test).

and more than 100 μM of PCA caused a significant outflow of 5-HT. MET (1 μM – 1 mM) also caused concentration-dependent increases in 5-HT outflow (Fig. 8A), and the secretory response to high concentration of MET (1 mM) was significantly higher than the control. PCA was about ten times more effective in eliciting 5-HT outflow than MET. These results suggest that chemoreceptor cells in the chicken aorta have a potency of 5-HT secretory response to amphetamine derivatives like neurons.

The resting outflow of 5-HT was 4.0 ± 0.4% (n = 10) in the experiments of 5-HT uptake inhibitors. CLM, fluvoxamine and fluoxetine (1 μM - 1 mM) also increased 5-HT outflow in concentration-dependent manners (Fig. 8B). The significant secretory responses to CLM and fluoxetine appeared above 0.1 mM, and fluvoxamine at 1 mM. Fluvoxamine at 1 mM was more effective than fluoxetine or CLM at the same concentration. These results suggest that 5-HT-containing chemoreceptor cells in the chicken aorta respond to 5-HT uptake inhibitors similar to the neurons in the CNS.

3.4. Extracellular Ca^{2+}-independent outflow of 5-HT by amphetamine derivatives and 5-HT uptake inhibitors

The mechanism of 5-HT outflow by amphetamine derivatives is controversial. It has been reported that outflows of 5-HT and dopamine by amphetamine derivatives including PCA and MET are a Ca^{2+}-dependent (Crepsi et al., 1997) or an independent manner (Wichems et al., 1995; Crepsi et al., 1997). The effect of extracellular Ca^{2+} removal on 5-HT outflow in response to PCA and 5-HT uptake inhibitors was

examined (Fig. 9). The resting outflow of 5-HT was not affected by Ca^{2+} removal. The secretory responses to PCA (1 mM) and 5-HT uptake inhibitors (0.1 mM), CLM, fluoxetine and fluvoxamine, were also not significantly attenuated by the removal of extracellular Ca^{2+}.

Next, the effect of voltage-dependent Ca^{2+} channel blockers on 5-HT outflow induced by PCA was examined (Fig. 10). The response to PCA (1 mM) was not affected by ω-conotoxin GVIA (1 μM), an N-type Ca^{2+} channel blocker, and nifedipine (1 μM), an L-type Ca^{2+} channel blocker, the concentration of which effectively inhibited 5-HT outflow induced by excess KCl (Ito et al., 1999).

These results suggest that extracellular Ca^{2+}-independent mechanisms are involved in 5-HT outflow induced by PCA and 5-HT uptake inhibitors from chicken aortic chemoreceptor cells.

3.5. Extracellular NaCl-dependent outflow of 5-HT by CLM but not PCA

It is reported that 5-HT is taken up into the cells through 5-HT transporters in an extracellular NaCl-dependent manner (Rudnick and Wall, 1992; Seidel et al., 2005; Sitte et al., 2001). Therefore, the effects of changes in extracellular concentration of NaCl on 5-HT outflow in response to PCA and CLM were examined (Fig. 11). When NaCl was substituted by sucrose, the resting 5-HT outflow in the presence of NaCl at 10 mM or absence of NaCl appeared to be greater, but not significantly**,** than that in the presence of NaCl at 20 mM or 140 mM. PCA (1 mM) significantly elicited 5-HT outflow regardless of the presence or absence of extracellular NaCl. CLM (0.1 mM) failed to produce the outflow of 5-HT at NaCl concentrations less than 20 mM.

However, CLM induced a significant increase in 5-HT outflow in the presence of 20 mM NaCl to the same extent as that in the presence of 140 mM NaCl. These results suggest that extracellular NaCl-dependent mechanisms are involved in 5-HT outflow from chicken aortic chemoreceptor cells induced by CLM, while the effect of PCA on 5-HT outflow is independent of extracellular NaCl.

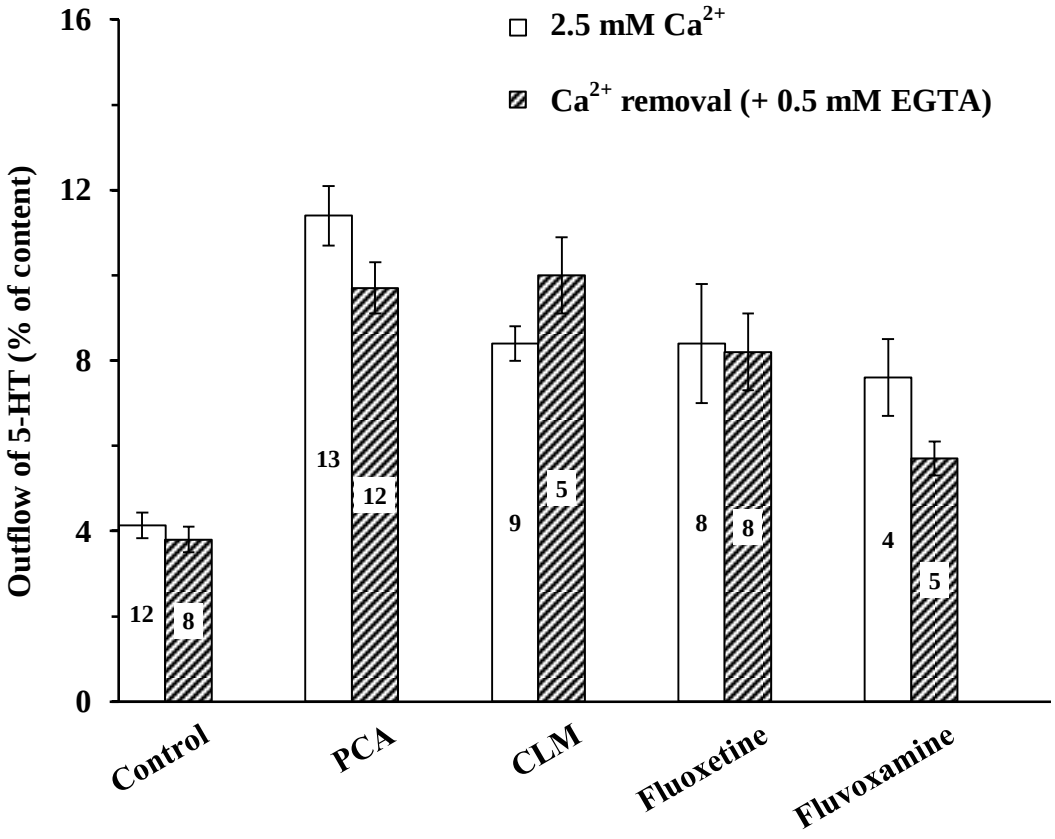

Fig. 9. Effects of extracellular Ca^{2+} removal on 5-HT outflow induced by PCA and 5-HT uptake inhibitors.

The resting 5-HT outflow (control, n = 4–5) and the outflow of 5-HT in response to PCA (1 mM), CLM (0.1 mM), fluvoxamine (0.1 mM) and fluoxetine (0.1 mM) for 10 min at 37°C in the presence (open columns) and absence (hatched columns) of extracellular Ca^{2+} were examined. Data are means ± S.E.M. and numbers in the column indicate numbers of experiments.

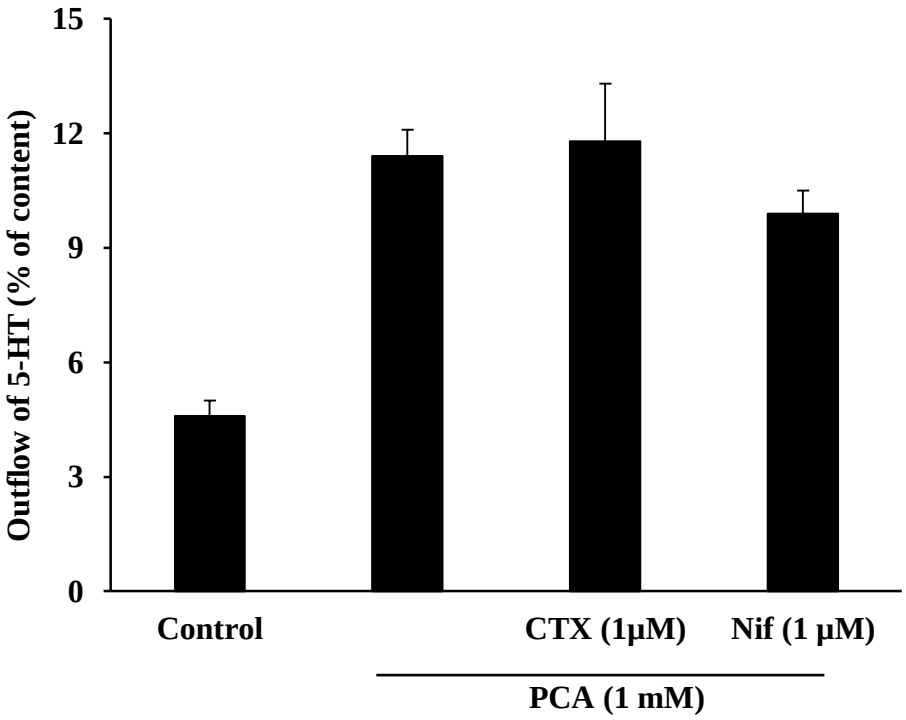

Fig. 10. Effect of L-and N-type Ca^{2+}channel blockers on PCA-induced 5-HT outflow.

The resting 5-HT outflow (control, n = 4–5) and the outflow of 5-HT induced by PCA (1 mM) in the presence of ω-conotoxin GVIA (CTX, 1 μM), or nifedipine (Nif, 1 μM). Data are means ± S.E.M.

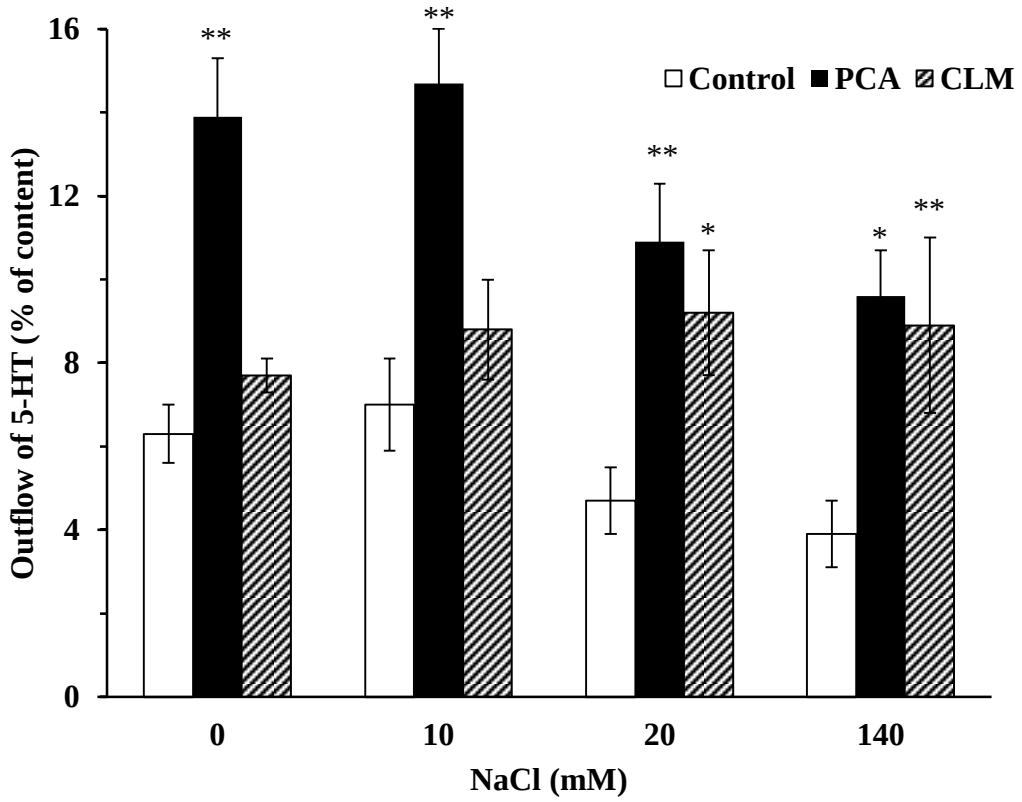

Fig. 11. Effects of extracellular NaCl on 5-HT outflow induced by PCA and CLM.

The resting 5-HT outflow (control, n = 4‑8, open columns) and the 5-HT outflow in response to PCA (1 mM, n = 4‑7, filled columns) and CLM (0.1 mM, n = 4, hatched columns) at 0, 10, 20 and 140 mM NaCl. Data are means ± S.E.M. *P < 0.05 and **P < 0.01 vs. control (Dunnett's test).

4. Discussion

The present results demonstrated that amphetamine derivatives, MET and PCA, and 5-HT uptake inhibitors, fluoxetine, fluvoxamine and CLM, concentration-dependently increased 5-HT outflow from chemoreceptor cells of chicken thoracic aorta. 5-HT outflows by these drugs were temperature-dependent and extracellular Ca^{2+}-independent responses. CLM but not PCA increased 5-HT outflow in an extracellular NaCl-dependent manner.

It has been shown that 5-HT is localized in the epithelioid cells in the wall of the chicken aorta, forming a band of ~ 1 mm in width (Miyoshi et al., 1995), indicating that 5-HT outflow from the chicken aorta arises from these epithelioid cells. Our group has reported that these cells are chemoreceptor cells and that the chicken aorta containing these cells (chemoreceptive ring) release 5-HT in response to nicotinic agonists, depolarization and hypoxia (Ito et al., 1997, 1999, 2001). In this study, amphetamine derivatives increased 5-HT outflow from chicken thoracic aorta. It has been well established that amphetamine derivatives enhance the outflow of monoamine neurotransmitters. MET and PCA are capable of effluxing ^{3}H-dopamine and ^{3}H-5-HT from rat brain synaptosomes (Berger et al., 1992; McKenna et al., 1991), and thus amphetamine derivatives decrease tissue content of these neurotransmitters in several brain regions (Murnane et al., 2012). It is reported that amphetamine derivatives are capable of enhancing reverse transport of monoamines resulting in their efflux from neurons (Robertson et al., 2009; Heal et al., 2013). The amphetamine derivatives inhibit VMAT and MAO in 5-HT-containing neurons (Sulzer et al., 2005; Fleckenstein et al., 2009) which increase cytosolic 5-HT concentration. Taken

together, it is suggested that PCA and MET promote 5-HT outflow by reversing the 5-HT transport across the cellular membrane in chicken chemoreceptor cells (Fig. 12A). In the present study, PCA was more effective in increasing 5-HT outflow than MET. It is also reported that PCA is about ten times more potent for 5-HT outflow than MET in the rat brain synaptosomes (Berger et al., 1992). From these results, it is suggested that chemoreceptor cells of chicken thoracic aorta have a similar sensitivity to amphetamine derivatives to 5-HT-containing neurons in the mammalian CNS.

5-HT uptake inhibitors such as CLM, fluoxetine and fluvoxamine are reported to elicit 5-HT outflow from various regions of the brain (Bel and Artigas, 1992; Fuller, 1994; Beyer and Cremers, 2008; Nagayasu et al., 2013). These drugs are well-known to inhibit the reuptake of 5-HT released from the neurons by blocking plasma membrane 5-HT transporters (Rothman and Baumann, 2003; Slattery et al., 2004; Kristensen et al., 2011). This was also the case in the chicken aortic chemoreceptor cells, in which these inhibitors were also effective in increasing 5-HT outflow. These results suggest that, like the 5-HT-containing neurons, these chemoreceptor cells express 5-HT transporters (serotonin transporters, SERT) on their plasma membrane (Fig. 12B). Potencies of 5-HT uptake inhibitors including fluoxetine on 5-HT outflow are different (Nagayasu et al., 2013). In this study, however, there were no differences in the potency among CLM, fluoxetine and fluvoxamine on 5-HT outflow, although they had different K_i values for binding to human 5-HT transporters (Apparsundaram et al., 2008). This discrepancy may be explained by (1) the short drug incubation period of 10 min and (2) the reduction of inward currents through basal K^+ channels by high concentrations of 5-HT uptake inhibitors (Kobayashi et al., 2004). Alternatively, (3) chicken 5-HT transporters might have different sensitivity to these

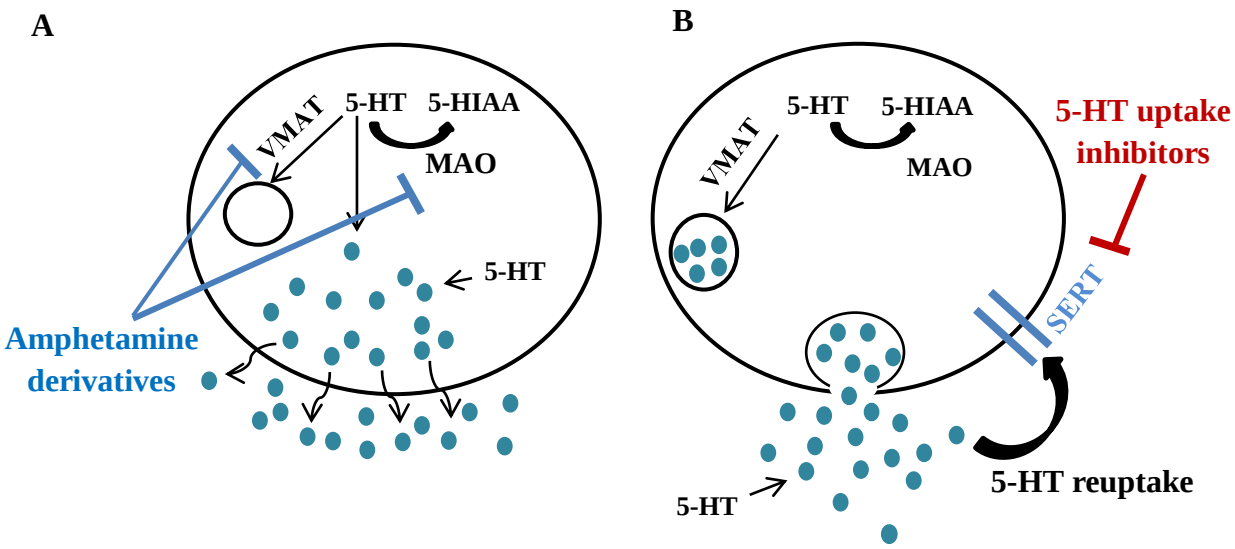

Fig. 12. 5-HT outflow from chemoreceptor cells in the chicken thoracic aorta by amphetamine derivatives and 5-HT uptake inhibitors

(A) 5-HT is transported by VMAT to secretory vesicles and then stored. Alternatively, 5-HT is metabolized to 5-HIAA by MAO. The amphetamine derivatives increase the cytosolic 5-HT concentration by inhibiting VMAT and MAO. The cytosolic 5-HT leaks out across the cellular membrane into the extracellular space. (B) 5-HT is spontaneously released from the chemoreceptor cells in the chicken thoracic aorta. 5-HT uptake inhibitors increase the extracellular 5-HT concentration by inhibiting NaCl-dependent serotonin transporter (SERT).

drugs from the mammalian neurons. Further investigation is needed to address this issue.

In this study, spontaneous 5-HT outflow was not affected by extracellular Ca^{2+} removal. 5-HT uptake inhibitors increased 5-HT outflow even in the absence of extracellular Ca^{2+}, indicating that spontaneous and the 5-HT uptake inhibitor-evoked 5-HT outflows are not due to Ca^{2+}-dependent exocytosis. Amphetamine derivatives are reported to promote 5-HT outflow in an extracellular Ca^{2+}-independent manner (Wichems et al., 1995) and in both Ca^{2+}-dependent and independent manners in synaptosomes, suggesting that the former is associated with Ca^{2+} influx through voltage-dependent Ca^{2+} channels and the latter with carrier-mediated release (Crepsi et al., 1997). In this study, however, we did not observe the Ca^{2+}-dependent 5-HT outflow in response to PCA. This difference may be explained by a difference in preparations, i.e., synaptosomes and chemoreceptor cells, which may have different compartmentalization of 5-HT (Gobbi et al., 2002).

In the 5-HT-containing chemoreceptor cells, the lowering of extracellular NaCl to 10 mM or below increased the spontaneous 5-HT outflow. Low NaCl also increases catecholamine outflow from the cat adrenal medulla (Teraoka et al., 1990). Activities of monoamine transporters including 5-HT transporters are coupled with extracellular NaCl (Sitte et al., 2001; Adams and DeFelice, 2002; Quick, 2003), with an EC_{50} of about 15 mM (Seidel et al., 2005) or less (Sitte et al., 2001). It is likely that the increase in spontaneous 5-HT outflow at low NaCl results from the inhibition of 5-HT transporters, because CLM failed to induce 5-HT outflow at low NaCl, under which 5-HT transporter activity has already been attenuated. This hypothesis was supported by the fact that CLM produced 5-HT outflow at more than 20 mM NaCl.

Unlike CLM, however, 5-HT outflow induced by PCA was not affected by the lowering extracellular NaCl concentration, indicating that the mechanisms producing 5-HT outflow by CLM are different from those by amphetamine derivatives.

Chapter II

Effects of H_2S on 5-HT-contaning chemoreceptor cells in chicken thoracic aorta

1. Introduction

5-HT-containing epithelioid cells in the chicken thoracic aorta are chemoreceptor cells because of their similar morphological and functional characteristics to mammalian carotid chemoreceptor cells. Recently, it has been reported that endogenous H_2S is a key mediator of the hypoxic response in a variety of O_2-sensitive tissues including carotid body (Olson et al., 2006, 2008, Olson and Whitfield, 2010; Dombkowski et al., 2011 Olson, 2015).

H_2S is considered to be one of the gasotransmitters. It has been report that H_2S is produced in the mammalian cells and plays important roles in various biological functions (Kimura, 2011). Several enzymes such as cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) produce H_2S (Olson, 2015). In rodent carotid glomus cells, CBS- and CSE-catalyzed H_2S is reported to mediate the carotid response to hypoxia (Li et al., 2010; Smith and Yuan, 2012; Prabhakar, 2012; Makarenko et al., 2012). Exogenous H_2S also activates carotid body type I cells in rat, cat and rabbit (Buckler, 2012; Jiao et al., 2015).

H_2S affects a variety of ion channels and receptors (Tang et al., 2010) including transient receptor potential (TRP) A1 and V1 channels and voltage-dependent Ca^{2+} channels. TRPA1 and TRPV1 are members of the TRP channel family, which serve to increase membrane cation permeability in both excitable and non-excitable cells (Venkatachalam and Montell, 2007; Gees et al.,

2010). H_2S activates TRPA1 and TRPV1 in the smooth muscles of the urinary bladder, airways, and gastrointestinal tract (Trevisani et al., 2005; Streng et al., 2008; Tang et al., 2010). Our group has previously shown that H_2S activates TRPA1 and evokes Ca^{2+} signals in rat sensory neurons (Miyamoto et al., 2011) and RIN14B cells, which induces 5-HT release (Ujike et al., 2015). H_2S is oxidized to polysulfides (H_2S_n), which activate ion channels with a greater potency than that of H_2S. It has recently been reported that sodium trisulfide (Na_2S_3) is found in the brain and also activates TRPA1 in the astrocytes (Kimura et al., 2013, 2015).

In cultured smooth muscle cells from gastric fundus of mice, H_2S reportedly elicits depolarization and then activates of L-type Ca^{2+} channels, resulting in an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Meng et al., 2015). In the rodent carotid glomus cells, H_2S increases the $[Ca^{2+}]_i$, which is inhibited by a L-type Ca^{2+} channel blocker (Makarenko et al., 2012) and removal of extracellular Ca^{2+} (Peng et al., 2010). Our group's previous study has indicated that 5-HT-containing chemoreceptor cells in the chicken aorta have voltage-dependent L- and N-type Ca^{2+} channels, and that 5-HT release by depolarization is abolished by nifedipine and ω-conotoxin GVIA, L- and N-type Ca^{2+} channel blockers, respectively (Ito et al., 1999).

In this Chapter, the aim of my study is to investigate the effect of NaHS, an H_2S donor, on the 5-HT release from chemoreceptor cells in the chicken thoracic aorta. To reveal the mechanism of action of H_2S, the effects of TRP and voltage-dependent Ca^{2+} channel blockers on 5-HT release were examined. In addition, the expression of TRPA1 in 5-HT-containing epithelioid cells of the chicken thoracic aorta was detected.

2. Materials and Methods

2.1. Animals

2.2. Preparation of aortic tissue

2.3. Measurement of 5-HT outflow

These are the same as those in Chapter I.

2.4. Immunohistochemistry

The aortic strips containing the chemoreceptive ring were cut longitudinally, stretched into a sheet and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at 4^{o}C overnight. PBS with the following composition was used (mM): NaCl 137, Na_2HPO_4 8.1, KCl 2.7, KH_2PO_4 1.5. For whole mount immunohistochemical labeling, the tissues were treated with 1% Triton X-100 in PBS for 30 min three times and incubated in blocking solution (1% normal horse serum with 1% Triton X-100 in PBS). The tissues were incubated with 6% H_2O_2 in methanol at room temperature for 1 h to block endogenous peroxidase. The tissues were then incubated with primary antibody, anti-5-HT (1:50, 5-HT-H209, Novus Biologicals, Littleton, CO, USA), and anti-TRPA1 antibody (1:1000, ab58844, Abcam, Cambridge, UK) in blocking solution at 4^{o}C for 2 days. Visualization of the primary antibodies was performed using commercially available kits (VECTASTAIN Universal ABC kit Elite, Peroxidase substrate kit, Vector laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The tissues were mounted on glass slides with glycerol and images were captured through an all-in-one fluorescence microscope (BZ-710; Keyence, Osaka, Japan). For double immunofluorescence,

paraffin-embedded sections (3 μm) of aortic strips were deparaffinized in xylene and rehydrated through a graded alcohol series. The tissues were treated with methanol containing 0.3% H_2O_2. After blocking with 5% normal donkey serum, the sections were incubated with anti-5-HT (1:50, 5-HT-H209, Novus Biologicals) and anti-TRPA1 antibodies (1:400, ab58844, Abcam) at 4°C overnight. Next, the sections were treated with Alexa Fluor 546 conjugate donkey anti-mouse IgG antibody (1:500, A10040, Thermo Fisher Scientific, Waltham, MA, USA) and Alexa Fluor 488 conjugate donkey anti-Rabbit IgG antibody (1:500, A-21206, Thermo Fisher Scientific) for 30 min at room temperature for 5-HT and TRPA1, respectively. Nuclei were labeled with Hoechst 33342 (1:5000, Dojindo, Kumamoto, Japan) for 5 min at room temperature. Digital images of sections were acquired through an all-in-one fluorescence microscope (BZX-710; Keyence). For checking of the antibody specificity, we confirmed that negative control staining without primary antibody incubation showed no positive reaction in immunofluorescence study.

2.5. Drugs

Following drugs were used. Sodium hydrosulfide (NaHS) (Strem Chemicals, Newburyport, MA, USA), nifedipine, ruthenium red (Wako Pure Chemical Industries, Osaka, Japan), ω-conotoxin GVIA (Peptide Institute, Osaka, Japan), HC030031, SB366791 (Tocris, Bristol, UK), and trans-cinnamaldehyde (Aldrich Chemistry, St Louis, USA) were prepared from stock solutions and dissolved in the Hepes-buffered saline solution. Sodium trisulfide (Na_2S_3) (Dojindo, Kumamoto, Japan) was prepared made just before use.

2.6. Data analysis

All data were expressed as means ± S.E.M. Statistical comparisons between two groups were performed by unpaired Student's *t*-test. For multiple comparisons, one-way ANOVA, followed by Dunnett's test or Williams' test, were used. A P value of less than 0.05 was considered significant.

3. Results

3.1. Concentration-dependent outflow of 5-HT by H_2S

The effect of NaHS, an H_2S donor, on 5-HT outflow from chicken thoracic aorta was examined. Chicken aorta with a 5-HT-containing chemoreceptor ring was incubated at 37°C for 10 min in the Hepes-buffered solution containing various concentrations of NaHS. In the absence of NaHS, 5-HT outflow was around 6% during 10 min-incubation time. This outflow was considered to be the resting release of 5-HT from 5-HT-containing cells. NaHS (30 μM-3 mM) caused a concentration-dependent increase in 5-HT outflow (Fig. 13). These results suggest that H_2S is capable of releasing 5-HT from chemoreceptor cells in the chicken aorta.

3.2. Extracellular Ca^{2+}-dependent outflow of 5-HT by H_2S

It has been previously shown that 5-HT release from chemoreceptor cells in the chicken aorta during hypoxia depends on Ca^{2+} influx through voltage-dependent L- and N-type Ca^{2+} channels (Ito et al., 1999). Therefore, next, the effect of extracellular Ca^{2+} on the 5-HT outflow induced by H_2S was examined (Fig. 14). 5-HT outflow in the presence of NaHS (0.3-3 mM) in the normal solution was statistically higher than the resting release (control). Although the resting 5-HT outflow was not affected by the removal of extracellular Ca^{2+}, 5-HT outflow in response to 0.3 mM NaHS was abolished by the removal of extracellular Ca^{2+}. On the other hand, 5-HT outflow evoked by 1 and 3 mM NaHS was significantly reduced but not abolished by the removal of extracellular Ca^{2+}. These results suggest that 5-HT outflow induced by

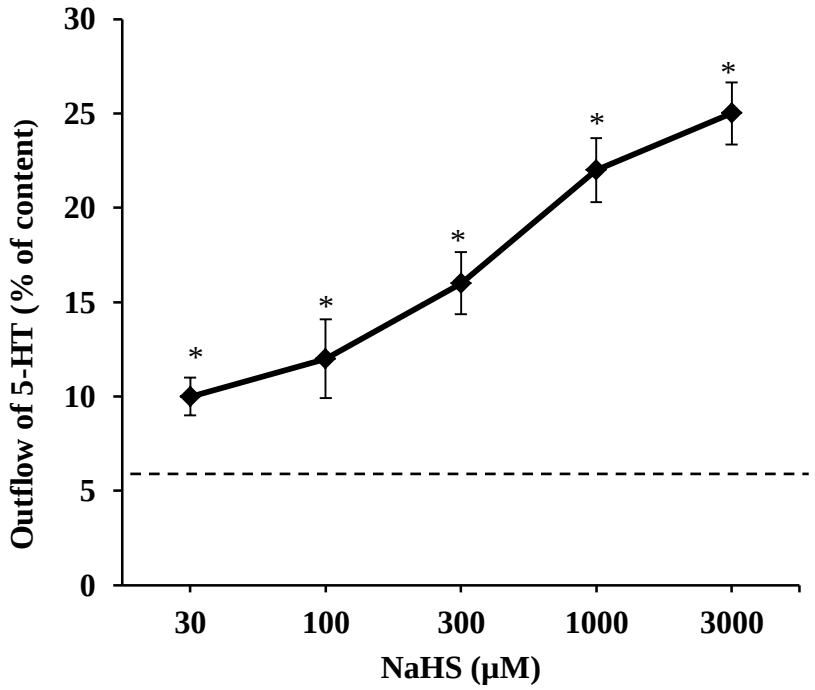

Fig. 13. Effect of H_2S on 5-HT outflow

Concentration-dependent increase in 5-HT outflow in response to NaHS (30 µM-3 mM) for 10 min at 37ºC (n = 5-14). Data are means ± S.E.M. Dashed line indicates the resting release of 5-HT. *P < 0.05 vs. resting release (Williams' test).

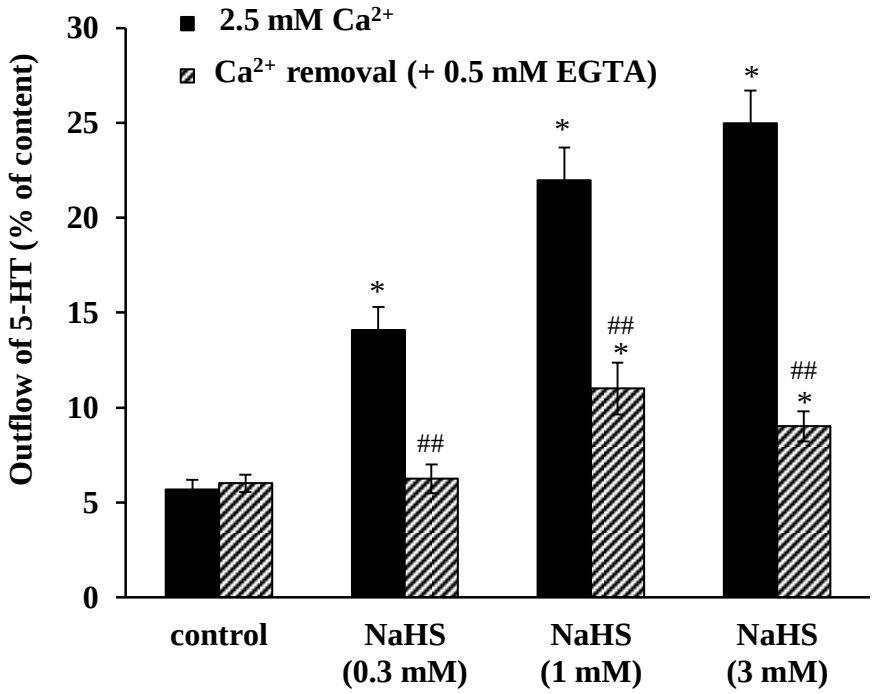

Fig. 14. Effects of extracellular Ca^{2+} removal on 5-HT outflow induced by H_2S.

The resting 5-HT outflow (control, n = 14) and the outflow of 5-HT in response to H_2S (0.3-3 mM, n = 5-8) for 10 min at 37ºC in the presence (black columns) and absence of (hatched columns) of extracellular Ca^{2+} (0.5 mM EGTA). The values in the black columns were taken from Fig. 13. Data are means ± S.E.M. *P < 0.05 vs. control (Williams' test), $^{\#\#}P < 0.01$ vs. NaHS/2.5 mM Ca^{2+} (Student's *t*-test).

H_2S is largely dependent on extracellular Ca^{2+}, whereas the resting release of 5-HT is not. A small amount of 5-HT appeared to be released even in the absence of extracellular Ca^{2+} in response to high concentrations of H_2S.

3.3. Contribution of voltage-dependent Ca^{2+} channels to outflow of 5-HT by H_2S

It has been reported that H_2S increases the $[Ca^{2+}]_i$ and induces Ca^{2+} waves in cultured astrocytes and hippocampal slices, which are blocked by inhibitors of various types of Ca^{2+} channel including L- and N-types (Nagai et al., 2004). Therefore, the effects of voltage-dependent Ca^{2+} channel blockers on the 5-HT outflow in response to H_2S were examined (Fig. 15). 5-HT outflow induced by 0.3 mM NaHS was significantly higher than the resting release. However, it was significantly inhibited by nifedipine (1 μM) or ω-conotoxin GVIA (1 μM), which are L- and N-type voltage-dependent Ca^{2+} channel blockers, respectively. These results suggest that voltage-dependent Ca^{2+} channels are involved in 5-HT release from the chemoreceptor cells in the chicken aorta.

3.4. Contribution of TRPA1 channels to outflow of 5-HT by H_2S

H_2S reportedly activates TRPV1 in the airway and the urinary bladder (Trevisani et al., 2005; Patacchini et al., 2005). In addition, TRPA1 is activated by H_2S in neuronal and secretory cells (Miyamoto et al., 2011; Ujike et al., 2015). Therefore, the effects of TRPA1 and TRPV1 channel blockers on 5-HT outflow evoked by H_2S were

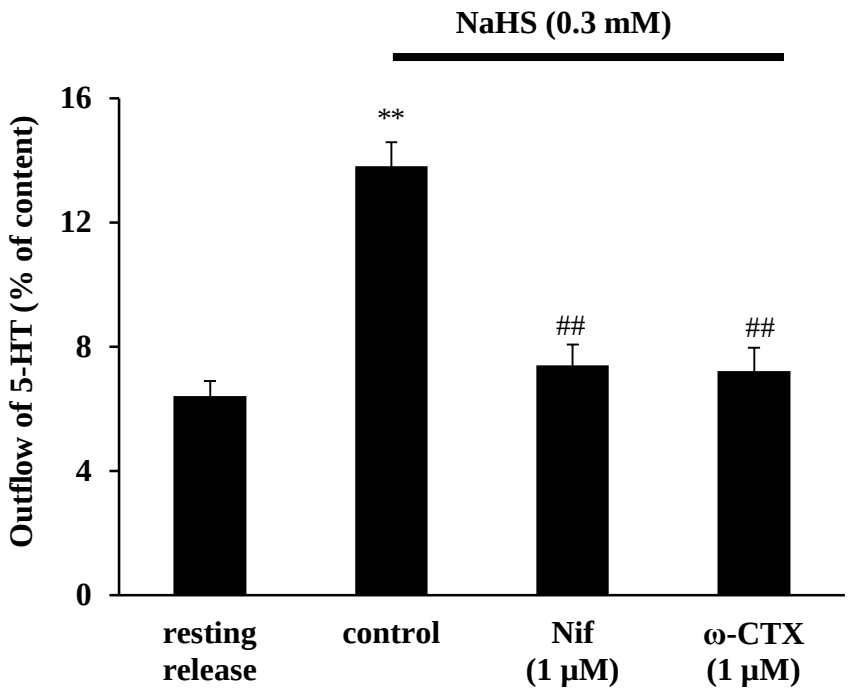

Fig. 15. Effects of L-and N-type Ca^{2+} channel blockers on 5-HT outflow by H_2S .

The resting 5-HT outflow (n=11, resting release) and the outflow of 5-HT induced by NaHS (0.3 mM, n=14) in the absence and presence of nifedipine (Nif, 1 µM, n = 11) or ω-conotoxin GVIA (CTX, 1 µM, n = 13). Data are means ± S.E.M. **P < 0.01 vs. basal release, $^{\#\#}P < 0.01$ vs. control (Dunnett's test).

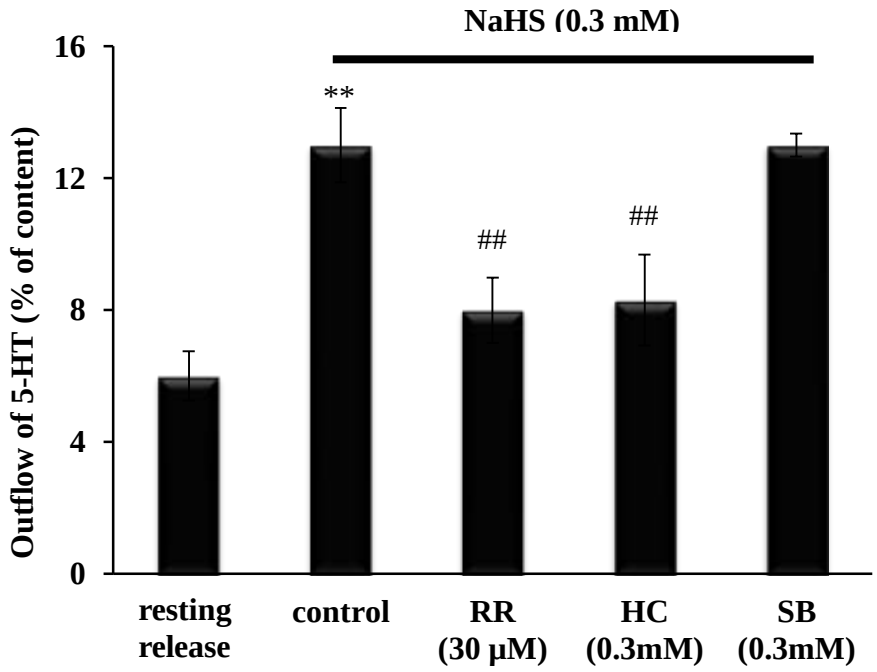

Fig. 16. Effects of TRP channel blockers on 5-HT outflow by H_2S

The resting 5-HT outflow (resting release, n = 5) and the outflow of 5-HT induced by NaHS (0.3 mM, n = 7) in the absence and presence of ruthenium red (RR, 30 µM, n=4), HC030031 (HC, 0.3 mM, n = 6) or SB366791 (SB, 0.3 mM, n = 6). Data are means ± S.E.M. **P < 0.01 vs. resting release, $^{\#\#}$P < 0.01 vs. control (Dunnett's test).

examined (Fig. 16). 5-HT outflow induced by 0.3 mM NaHS (control) was significantly higher than the resting release. The effect of NaHS (0.3 mM) on 5-HT outflow was significantly inhibited by ruthenium red (30 μM, a non-selective TRP channel blocker) and HC030031 (0.3 mM, a selective TRPA1 channel blocker) but not by SB366791 (0.3 mM, a selective TRPV1 channel blocker). These results suggest that TRPA1 channels are also involved in the H_2S-evoked 5-HT release from the chemoreceptor cells in the chicken aorta.

3.5. Concentration-dependent outflow of 5-HT by cinnamaldehyde

TRPA1 is activated by a variety of plant-derived and environmental irritants, such as cinnamaldehyde, isothiocyanate, allicin, and acrolein (Bandell et al., 2004; Bautista et al., 2006; Iwasaki et al., 2008), all of which interact with cysteine residues in the ion channel protein (Bautista et al., 2006; Macpherson et al., 2007). Therefore, the effect of cinnamaldehyde, a TRPA1 agonist, on 5-HT outflow was examined to confirm the contribution of TRPA1 to 5-HT outflow from chicken thoracic aorta with chemoreceptive ring.

Cinnamaldehyde (0.1‑3 mM) caused a concentration-dependent increase in 5-HT outflow (Fig. 17A), and more than 0.3 mM of cinnamaldehyde caused a significant outflow of 5-HT. Next, the effect of TRPA1 channel blocker on the 5-HT outflow evoked by cinnamaldehyde was examined. The cinnamaldehyde (0.3 mM)-evoked 5-HT outflow was significantly higher than the resting release. The cinnamaldehyde-induced 5-HT outflow was significantly inhibited by HC030031

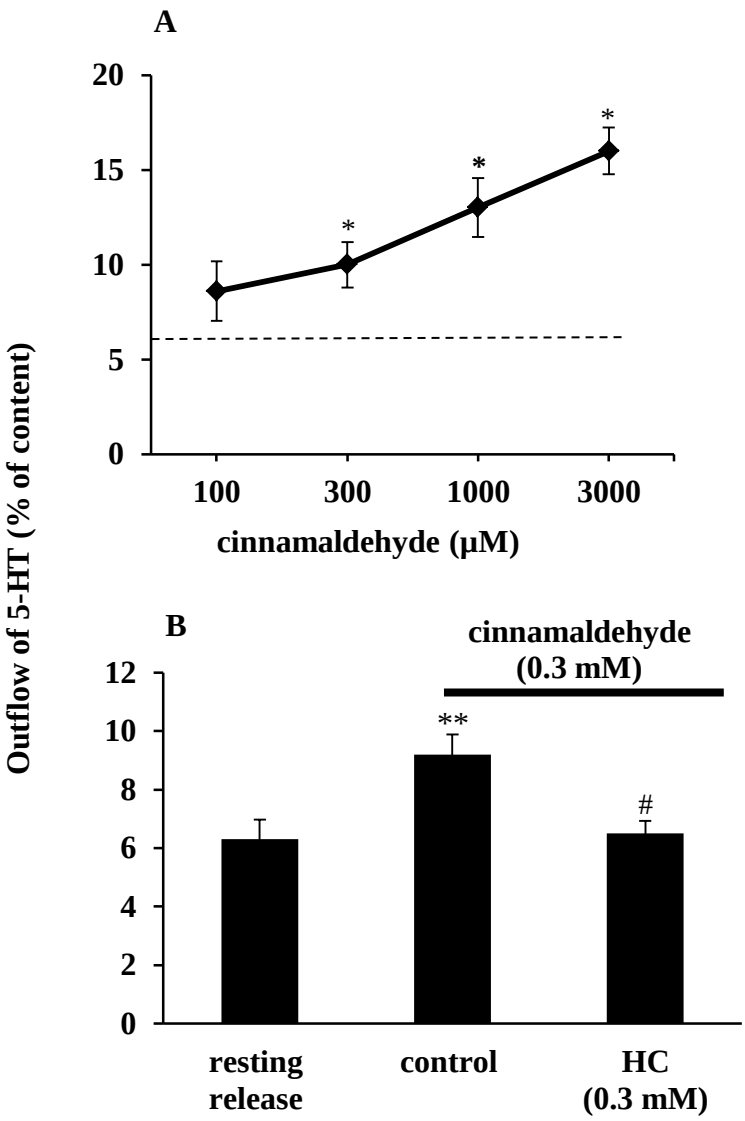

Fig. 17. Effect of cinnamaldehyde on 5-HT outflow

(A) Concentration-dependent increase in 5-HT outflow in response to cinnamaldehyde (0.1-3 mM) for 10 min at 37ºC (n = 4-10). Dashed line indicates the resting release of 5-HT. (B) The resting 5-HT outflow (resting release, n = 5) and the outflow of 5-HT induced by cinnamaldehyde (0.3 mM, n = 9) in the absence and presence of HC030031 (HC, 0.3 mM, n=8). Data are means ± S.E.M. **P < 0.01 vs. resting release, $^{\#}$P < 0.05 vs. control (Dunnett's test).

(0.3 mM) (Fig. 17B). These results confirm our idea that TRPA1 is involved in H_2S-evoked 5-HT release from the chemoreceptor cells in the chicken aorta.

3.6. Concentration-dependent outflow of 5-HT by Na_2S_3

Polysulfide, a mixture of substances with varying numbers of sulfurs (H_2S_n), is generated from H_2S by 3-mercaptopyruvate sulfurtransferase (3-MST) (Kimura et al., 2015). It has recently been reported that polysulfide such as Na_2S_3 excites mouse sensory neurons via the activation of TRPA1 and causes acute pain (Hatakeyama et al., 2015). Therefore, the effect of Na_2S_3 on 5-HT outflow from chicken thoracic aorta with a chemoreceptive ring was examined. As shown in Fig 18A, Na_2S_3 (3-30 μM) caused a concentration-dependent and significant increase in 5-HT outflow. Next, the effect of TRPA1 channel blocker on 5-HT outflow evoked by Na_2S_3 was examined. The Na_2S_3 (10 μM)-evoked 5-HT outflow was significantly higher than the resting release. The Na_2S_3 (10 μM)-induced 5-HT outflow was significantly inhibited by HC030031 (0.3 mM) (Fig. 18B). These results suggest that Na_2S_3 has an effect similar to H_2S to stimulate the chemoreceptor cells through TRPA1.

3.7. Expression of TRPA1 in 5-HT-containing cells

TRPA1 channels are expressed in numerous excitable and non-excitable cells and involved in manifold physiological and pathological functions in the body (Fernandes et al, 2012; Mori et al., 2016). In this study, the expression of TRPA1 protein in the chicken thoracic aorta was examined. The distribution of TRPA1 in the

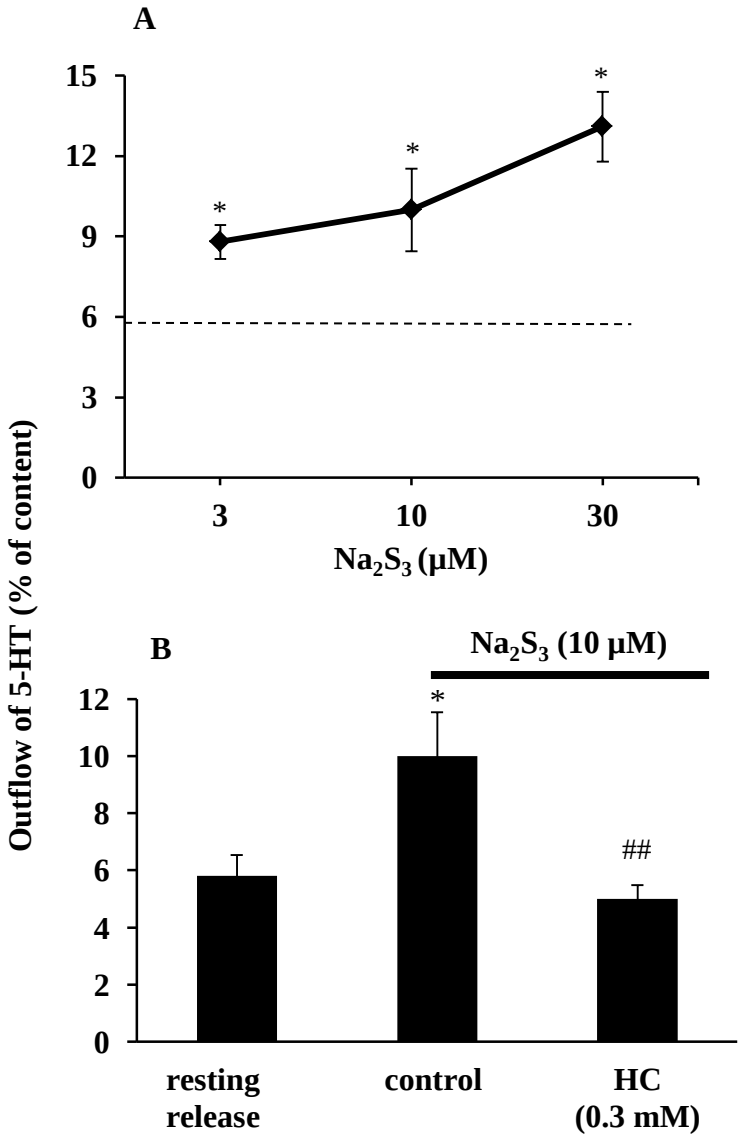

Fig. 18. Effect of Na_2S_3 on 5-HT outflow

(A) Concentration-dependent increase in 5-HT outflow in response to Na_2S_3 for 10 min at 37ºC (n = 6). Dashed line indicates the resting release of 5-HT. (B) The resting 5-HT outflow (resting release, n = 6) and outflow of 5-HT induced by Na_2S_3 (10 µM, n = 7) in the absence and presence of HC030031 (HC, 0.3 mM, n = 5). Data are means ± S.E.M. *P < 0.05 vs. basal release, $^{\#}$P < 0.05 vs. control (Dunnett's test).

tissue was first examined by immunohistochemical analysis using whole mount preparation. TRPA1 immunoreactivity was detected in the wall of the chicken aorta (Fig. 19). The area of TRPA1 expression was similar to that of 5-HT immunoreactive cells (i.e; the chemoreceptive ring). Next, double immunofluorescence analysis for TRPA1 and 5-HT was examined (Fig. 20). Both TRPA1 and 5-HT immunoreactivities were detected near the surface of the aortic wall. In addition, TRPA1 expression was localized to 5-HT-containing cells. These results suggest that TRPA1 is expressed in the 5-HT-containing chemoreceptor cells in the chicken thoracic aorta.

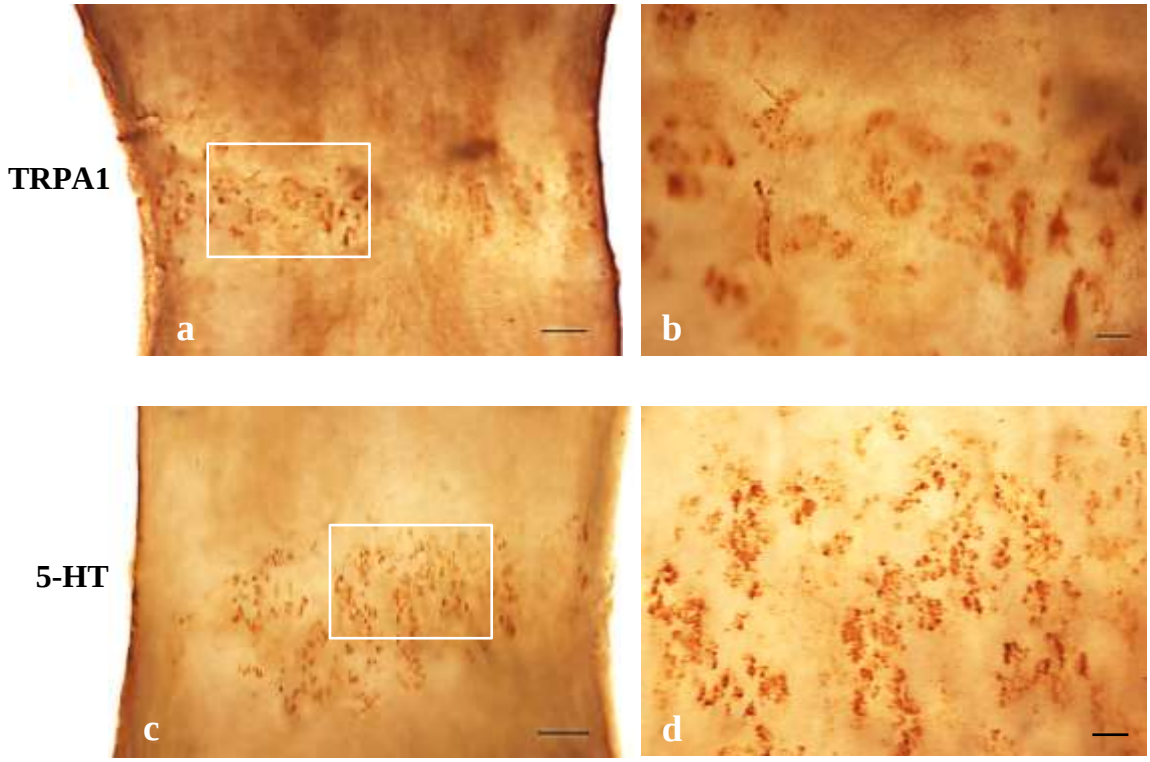

Fig. 19. Distribution of TRPA1 and 5-HT in the chicken thoracic aorta with chemoreceptive ring

Distribution of TRPA1 (a) and 5-HT immunoreactive cells (c) in the inner wall of the chicken thoracic aorta. High magnification images of TRPA1 (b) and 5-HT (d) immunoreactive clusters from the boxed regions of (a) and (c). Scale bars indicate 400 μm (a and c), 100 μm (b and d).

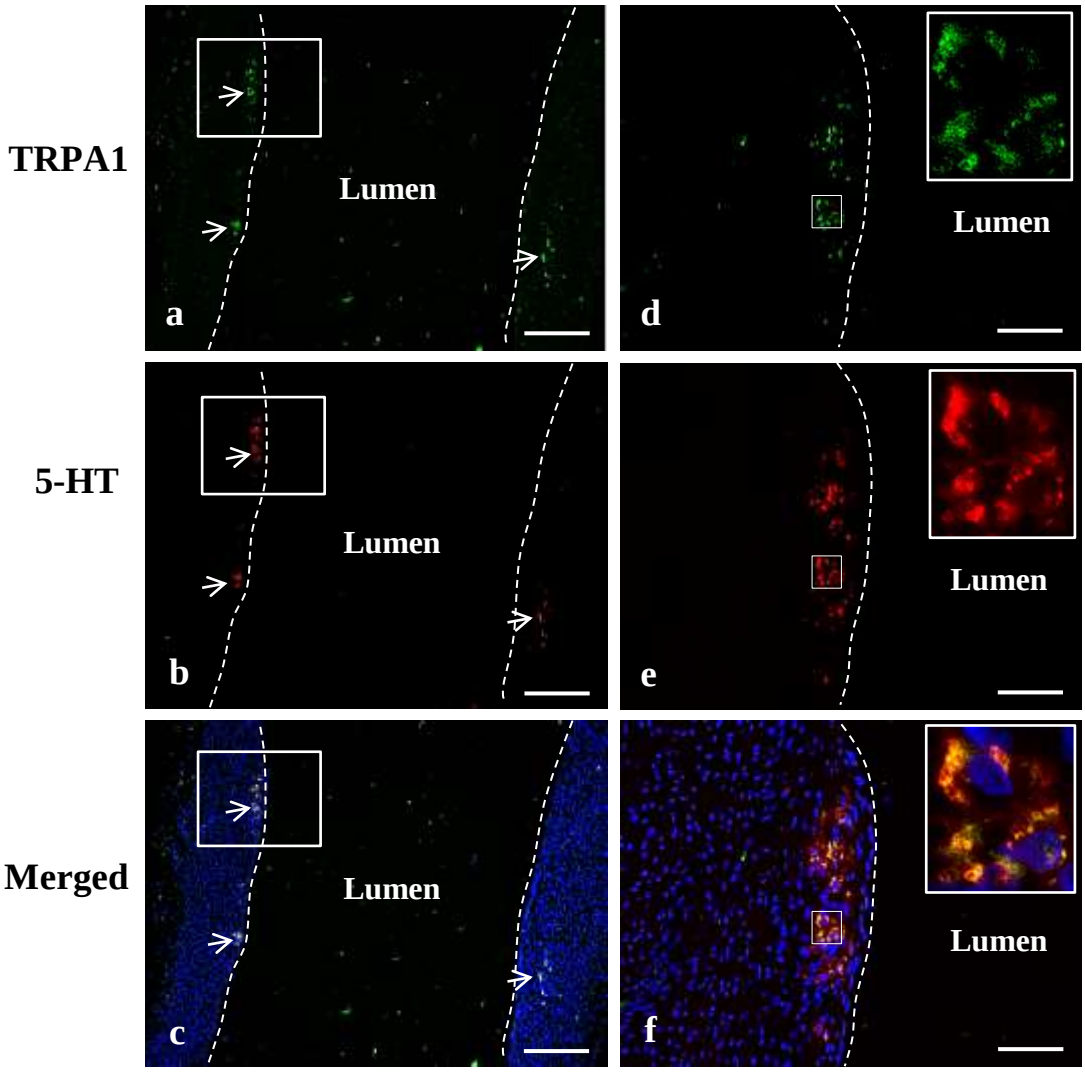

Fig. 20. Double immunofluorescence labeling for TRPA1 and 5-HT in the chicken thoracic aorta with chemoreceptive ring

Immunofluorescence labeling of TRPA1 (a) and 5-HT (b) in the chicken aorta. (c) TRPA1-expressing 5-HT-containing chemoreceptor cells are indicated by arrows. (d, e and f) High magnification images from boxed regions of (a), (b), and (c), respectively. Dotted lines are indicating the border between vascular endothelium and lumen on the images. Scale bars indicate 200 μm (a-c), and 50 μm (d-f).

4. Discussion

In this study, we found that H_2S caused a concentration-dependent increase in 5-HT release from chicken thoracic aorta with the chemoreceptive ring, which was inhibited by the removal of extracellular Ca^{2+}. The H_2S-evoked 5-HT outflow was also inhibited by voltage-dependent L- or N-type Ca^{2+} channel blocker and a selective TRPA1 channel blocker. Cinnamaldehyde, a TRPA1 agonist, and Na_2S_3, a polysulfide, mimicked the secretory response to H_2S. The expression of TRPA1 was localized to 5-HT-containing epithelioid cells in the aortic wall.

H_2S releases catecholamine from rat and trout adrenal chromaffin cells and 5-HT from RIN14B cells in a Ca^{2+}-dependent manner (Perry et al., 2009; Zhu et al., 2012; Ujike et al., 2015). In the present study, H_2S triggered 5-HT release from the chicken aortic preparation, and this release was markedly reduced by the removal of extracellular Ca^{2+}, suggesting that H_2S caused Ca^{2+}-dependent exocytosis of 5-HT. On the other hand, perceptible release of 5-HT in response to H_2S at high concentrations was observed even in the absence of extracellular Ca^{2+}. It is reported that some secretagogues cause catecholamine secretion from adrenal chromaffin cells by mediating intracellular Ca^{2+} mobilization even in the absence of extracellular Ca^{2+} (Asano et al., 1995). In this experiment, although we did not examine this phenomenon precisely, similar mechanisms might be involved in 5-HT release from chicken chemoreceptor cells in response to H_2S at high concentrations.

TRPA1 channels are non-selective cationic channels permeable to Na^+ and Ca^+ (Gees et al., 2010). Our group has previously shown that H_2S stimulates rat sensory

neurons and RIN14B cells via the activation of TRPA1 (Miyamoto et al., 2011; Ujike et al., 2015). It has been reported that H_2S evokes time- and concentration-dependent increases in the $[Ca^{2+}]_i$ in CHO cells expressing mouse or human TRPA1 (Streng et al., 2008). A TRPA1 agonist causes Ca^{2+} influx and 5-HT release from rat enterochromaffin cells (Nozowa et al., 2009). In this study, 5-HT release by H_2S was significantly inhibited by a non-selective TRP blocker (ruthenium red) and a selective TRPA1 blocker (HC030031). In addition, a TRPA1 agonist (cinnamaldehyde) mimicked the secretory response to H_2S. These results suggest that H_2S activates TRPA1 in the chicken thoracic chemoreceptor cells and releases 5-HT. TRPA1 is expressed in many kinds of neuronal and non-neuronal cells (Smith et al., 2004; Du et al., 2007; Atoyan et al., 2009; Lee et al., 2012; Cho et al., 2014). In this study, it was found that TRPA1 was localized to 5-HT-containing chemoreceptor cells in the chicken aorta. It is also reported that H_2S is capable of activating TRPV1 in sensory neurons (Trevisani et al., 2005; Patacchini et al., 2005). However, TRPV1 is unlikely to be involved in 5-HT release by H_2S in chicken aortic chemoreceptor cells because 5-HT release was not inhibited by a selective TRPV1 blocker (SB366791). In addition, it has recently been reported that polysulfide salts, i.e., sodium tri-and tetrasulfide (Na_2S_3 and Na_2S_4), activate TRPA1 channels in astrocytes much more potently than H_2S (Kimura et al., 2013, 2015). This was also the case in the present study that Na_2S_3 increased 5-HT release by the activation of TRPA1. Taken together, it is concluded that TRPA1 plays an essential role in the H_2S and Na_2S_3-induced 5-HT release from chemoreceptor cells in the chicken aorta.

The TRPA1 activation produces depolarization through the increase in membrane Na^+ permeability in addition to Ca^{2+} (Raisinghani et al., 2011), which in

turn elicits further Ca^{2+} entry through voltage-dependent Ca^{2+} channels. There are two possible pathways to induce Ca^{2+} entry into chemoreceptor cells in response to H_2S (Fig. 21); one could involve TRPA1 channels with its high Ca^{2+} permeability, while the other could involve voltage-dependent Ca^{2+} channels stimulated by the depolarization associated with TRPA1 activation. It has been reported that H_2S increases the $[Ca^{2+}]_i$ and induces Ca^{2+} waves in cultured astrocytes and hippocampal slices, which are also blocked by various types of Ca^{2+} channel inhibitors including L- and N-type ones (Nagai et al., 2004). In the present study, 5-HT release by H_2S was abolished by nifedipine and ω-conotoxin GVIA (respective L- and N-type voltage-dependent Ca^{2+} channel blockers). Thus, it is suggested that Ca^{2+} entry via L- and N-type Ca^{2+} channels, but not direct Ca^{2+} entry via TRPA1 channels, plays a major role in 5-HT release triggered by H_2S in the chicken aortic chemoreceptor cells.

In mammals, pyridoxal 5'-phosphate-dependent enzymes such as CBS and CSE produce H_2S either from cysteine or from the combination of cysteine and homocysteine. In addition, H_2S is also synthesized from cysteine by 3-MST in conjunction with cysteine aminotransferase (Kamoun, 2004; Kimura, 2011; Mikami et al., 2011; Olson, 2015; Kimura et al., 2013). Endogenous H_2S functions as an oxygen sensor in various tissues(Olson, 2015; Olson et al., 2008). Recently, H_2S generated by CSE and CBS was shown to be a physiological mediator of carotid body responses to hypoxia (Li et al., 2010; Makarenko et al., 2012; Prabhakar, 2012). The chemoreceptor cells in the chick aorta have similar morphological and functional characteristics to mammalian carotid chemoreceptor cells (Miyoshi et al., 1995; Ito et al., 2001). In addition, TRPA1 plays a critical role in the O_2 sensing of vagal and

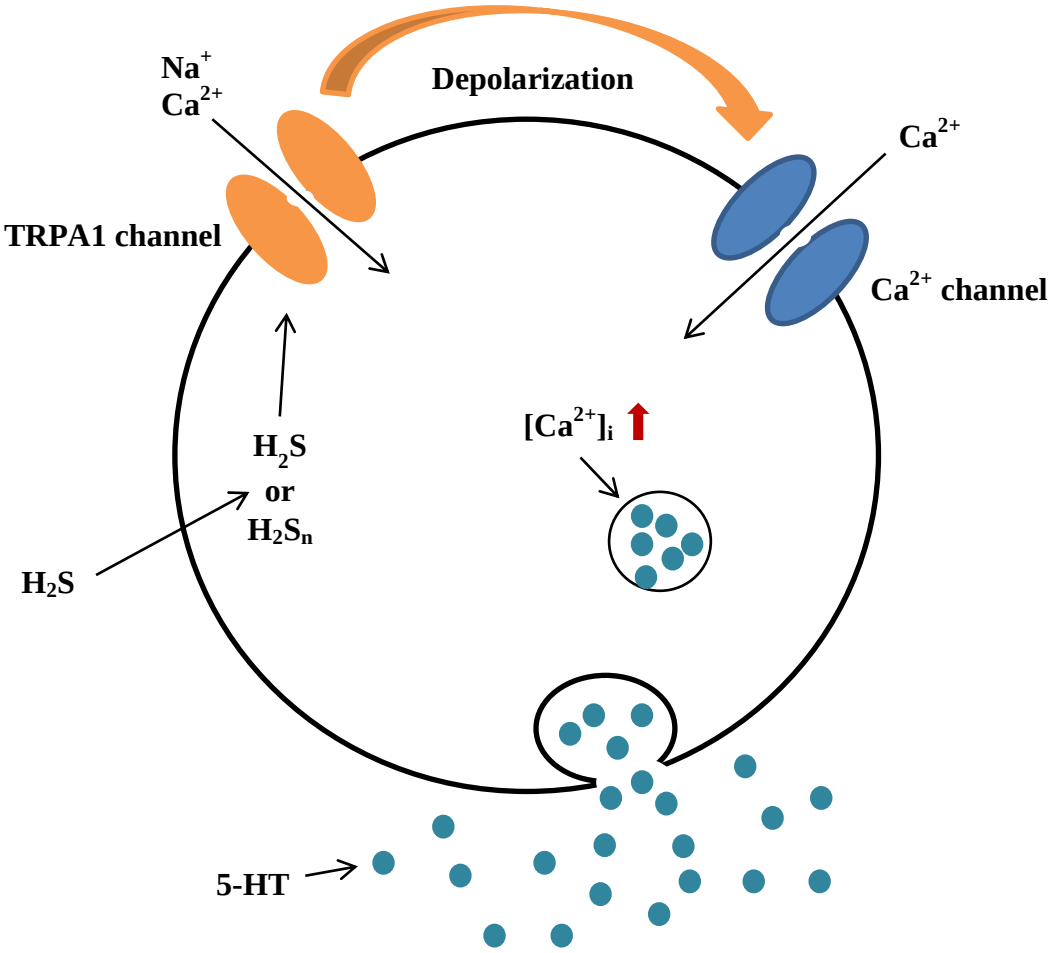

Fig. 21. 5-HT release from chemoreceptor cells in the chicken thoracic aorta by H_2S

H_2S and H_2S_n activate TRPA1 expressed in chemoreceptor cells, resulting in the membrane depolarization, and Ca^{2+} entry through voltage-dependent Ca^{2+} channels and then 5-HT release.

sensory neurons (Takahashi et al., 2011; Mori et al., 2016). A TRPA1 antagonist causes the dose-dependent attenuation of the hypoxic ventilator response, indicating that TRPA1 contributes to the hypoxic chemoreflex in mice (Pokorski et al., 2014). In addition, TRPA1 is a major oxidant sensor in sensory neurons that is activated by hypochlorite and hydrogen peroxide (Bessac et al., 2008). Present results suggest that endogenous H_2S also contributes to the O_2 sensing of chemoreceptor cells in the chicken aorta via TRPA1. Further investigation is needed to address this issue.

Conclusion

The present results indicate that amphetamine derivatives and 5-HT uptake inhibitors increase 5-HT outflow from chemoreceptor cells of chicken thoracic aorta. It is suggested that these cells have 5-HT transport, storage and uptake activities, which are similar to those of 5-HT-contaning neurons in the mammalian CNS.

H_2S triggered the release of 5-HT from the chicken thoracic aorta containing chemoreceptor cells, which was inhibited by TRPA1 and voltage-dependent Ca^{2+} channel blockers. The expression of TRPA1 was localized to 5-HT-containing chemoreceptor cells in the aortic wall. It is suggested that H_2S activates TRPA1 expressed in chemoreceptor cells, resulting in their depolarization, and Ca^{2+} entry through voltage-dependent Ca^{2+} channels and then 5-HT release. The generation of endogenous H_2S might be associated with O_2 sensitivity in chemoreceptor cells of chicken thoracic aorta via TRPA1.

The chicken thoracic aorta may become a useful model for examining the effects of drugs on 5-HT-containing neurons or chemoreceptor cells *in vitro*, because 5-HT outflow from aortic strips was easily detected with HPLC equipped with an electrochemical detector.

Summary

In the chicken thoracic aorta, 5-HT-containing cells aggregate and form a band (1 mm width). Because of their similarities in morphological and functional characteristics to mammalian carotid chemoreceptor cells, these cells are also considered as chemoreceptor cells. To reveal the mechanisms of 5-HT release from these cells, I investigated 5-HT outflow from the chemoreceptor cells in the chicken thoracic aorta.

1. Amphetamine derivatives, PCA and MET, caused concentration-dependent increases in 5-HT outflow. PCA was more effective in increasing 5-HT outflow than MET. In addition, PCA increased 5-HT outflow in an incubation time- and temperature-dependent manner.

2. 5-HT uptake inhibitors, CLM, fluoxetine and fluvoxamine, caused concentration-dependent increases in 5-HT outflow.

3. The outflows of 5-HT induced by PCA and 5-HT uptake inhibitors were not dependent on extracellular Ca^{2+} concentration. It is suggested that 5-HT uptake inhibitor- and PCA-evoked 5-HT outflows are not due to Ca^{2+}-dependent exocytosis.

4. 5-HT outflow induced by CLM was dependent on extracellular NaCl concentration. On the other hand, the 5-HT outflow by PCA was not affected by extracellular NaCl concentration.

5. It is suggested that CLM suppresses 5-HT uptake by inhibiting membrane 5-HT transporters, which results in the increase in extracellular 5-HT levels. On the other hand, PCA inhibiting VMAT and MAO increases cytosolic 5-HT levels, resulting in enhancing the efflux of 5-HT.

6. H_2S triggered 5-HT release from chemoreceptor cells in the chicken thoracic aorta in a concentration-dependent manner. The release of 5-HT in response to H_2S was reduced by extracellular Ca^{2+} removal. These results suggest that H_2S produces 5-HT release from these cells by Ca^{2+}-dependent exocytosis.

7. 5-HT release by H_2S was abolished by nifedipine and ω-conotoxin GVIA, L- and N-type voltage-dependent Ca^{2+} channel blockers, respectively.

8. 5-HT release by H_2S was significantly inhibited by ruthenium red, a non-selective TRP blocker and HC030031, a selective TRPA1 blocker. Cinnamaldehyde, a TRPA1 agonist and Na_2S_3, a polysulfide, mimicked the secretory response to H_2S.

9. The existence of TRPA1-positive cells was detected in the wall of chicken thoracic aorta, which was similar to that of 5-HT-positive cells. Double staining revealed that the expression of TRPA1 was localized in the 5-HT-containing cells.

10. The present results have demonstrated that 5-HT-containing chemoreceptor cells in the chicken thoracic aorta have 5-HT transport, storage and uptake activities, which are similar to those of 5-HT-containing neurons in the mammalian CNS, and that the TRPA1 activation by H_2S causes depolarization, which activates voltage-dependent Ca^{2+} channels. The Ca^{2+} entry through these Ca^{2+} channels is suggested to mainly evoke 5-HT release from the chemoreceptor cells in the chicken thoracic aorta.

11. It is suggested that endogenous H_2S plays a role in the O_2 sensing in chemoreceptor cells of chicken thoracic aorta via TRPA1. The chicken thoracic aorta may become a useful model for examining the effects of drugs on 5-HT-containing neurons or chemoreceptor cells *in vitro*, because 5-HT outflow from aortic strips was easily detected with HPLC equipped with an electrochemical detector.

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