ELSEVIER

European Journal of Pharmacology 402 (2000) 297–306

ejp

www.elsevier.nl/locate/ejphar

The lack of compound 48/80-induced contraction in isolated trachea of mast cell-deficient Ws/Ws rats in vitro: the role of connective tissue mast cells

Zullies Ikawati [a], Mototaka Hayashi [a], Masato Nose [b], Kazutaka Maeyama [a,*]

[a] *Department of Pharmacology, Ehime University School of Medicine, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan*
[b] *Department of Pathology II, Ehime University School of Medicine, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan*

Received 12 May 2000; received in revised form 30 June 2000; accepted 7 July 2000

Abstract

In the rat trachea, two types of mast cells have been identified, connective tissue mast cells and mucosal mast cells. Their different characteristics may account for their different biological functions. The role of connective tissue mast cells in tracheal contraction as one feature of the immediate reaction of asthma was studied in vitro in isolated trachea, using tissue derived from mast cell-deficient (Ws/Ws) rats, heterozygous (Ws/+) rats and control (+/+) rats, and compound 48/80 as a potent inducer of mast cell degranulation. The contractile response of tracheas from the three types of rats was also studied upon exposure to the following spasmogens: histamine, 5-hydroxytryptamine (5-HT), and carbachol. Histamine content in tissues reflected the differing mast cell numbers in strips from the three rat types. It was found that carbachol and 5-HT elicited tracheal contraction in a similar manner in strips from the three types of rats. Histamine had no contractile effect. Compound 48/80, at a dose of 25 μg/ml, elicited contraction in tracheas from both control (+/+) and heterozygous (Ws/+), but not in trachea from Ws/Ws rats. Compound 48/80-induced contractions in tracheas from +/+ rats were inhibited by 0.1 μM ketanserin and 0.1 μM nedocromil, but not by 0.1 μM mepyramine. Enzyme histochemistry confirmed that the degranulation occurred in connective tissue mast cells, but not in mucosal mast cells. We concluded that connective tissue mast cells play an important role in rat tracheal contraction via 5-HT release induced by compound 48/80. In addition, the specific mast cell-deficient (Ws/Ws) rats provide a good tool for studying the roles of mast cells in airway system.

Keywords: Compound 48/80; Connective tissue mast cell; Mast cell-deficient (Ws/Ws) rat; Tracheal contraction; 5-HT (5-hydroxytryptamine, serotonin); Histamine

1. Introduction

Mast cells have long been considered to be involved in the pathogenesis of a number of inflammatory diseases of the airways in humans and animal models (Galli, 1993). They play a pivotal role, especially in the immediate bronchoconstriction after antigen exposure, in attracting other cells responsible for the late reaction of asthma (Postma et al., 1989). Activated through the interaction of specific antigen and surface-bound antigen-specific immunoglobulin E, mast cells release and generate mediators to cause inflammation. Many of the mediators, such as histamine, leukotrienes, and prostaglandin D_2, exert direct spasmogenic activity on the muscle cells of the airways (Wasserman, 1997). This type of immediate reaction plays a prominent role in the genesis of asthma. Mast cells may also be activated by nonimmunologic stimuli induced by various substances such as neuropeptides, basic compounds, complement components, and certain drugs, e.g. opiates (Metcalfe et al., 1997).

In rodents, two major populations of mast cells have been identified, connective tissue mast cells and mucosal mast cells (Enerback, 1987; Gibson and Miller, 1986). In the case of rat trachea, mucosal mast cells exist as globule

* Corresponding author. Tel.: +81-89-960-5258; fax: +81-89-960-5263.
E-mail address: kazu@m.ehime-u.ac.jp (K. Maeyama).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00482-9

leukocytes, which are similar in their morphology and pharmacological responses to mucosal mast cells located in other organs of rats, and display a different distribution from connective tissue mast cells (Tam et al., 1988). There are, however, only a limited number of studies focusing on the distinct roles of the different types of mast cells in the airways. The individual characteristics of these cell types may account for their different functions, and may provide information useful for the development of selective anti-asthmatic drugs. Connective tissue mast cells are of interest to us because they can be stimulated both by immunologic and nonimmunologic stimuli.

Rats are increasingly used as models to study airway inflammation and bronchial responsiveness, but they have shown strain differences in terms of responsiveness both in vivo and in vitro. Mast cell-deficient rats, which were discovered by Niwa et al., 1991, are completely devoid of mast cells due to the deletion of 12-nucleotide bases in the tyrosine kinase domain of the c-*kit* receptor gene (Tsujimura et al., 1991). There are, however, very few investigators using these rats to study the airways. Nishida et al. (1998) reported the lack of active lung anaphylaxis in Ws/Ws rats sensitized with the nematode *Nippostrongylus brasiliensis*, suggesting the contribution of mast cells, but they did not take into account the different type of mast cells.

We, therefore, focused our attention on confirming the potential role of connective tissue mast cells in the airways. We also studied the tracheal contractile response to other spasmogens such as carbamylcholine chloride (carbachol), 5-hydroxytryptamine (5-HT) and histamine, and compared the results obtained with tissue from Ws/Ws rats with those obtained with tissue from heterozygous (Ws/+) and congenic (+/+) rats. We also determined the mechanism by which mast cells influence airway contraction by measuring the release of mediators into organ bath during contraction and by using specific antagonists. Comparison of the results from the three types of rats would provide us with important information concerning the role of connective tissue mast cells in the tracheal contractile response. To support the pharmacological data, a histological assessment was also performed.

2. Materials and methods

2.1. Preparation and protocols

2.1.1. Animals

The animal experiments performed in the present study were conducted according to the guidelines of the Animal Care Committee of Ehime University School of Medicine, and the experimental protocols were approved by this Committee.

Male and female Ws/+ rats, both of the Donryu strain, were crossed to obtain male Ws/Ws, heterozygous Ws/+, and their wild type, +/+ rats, using the procedure described by Niwa et al. (1991). All the Ws/Ws, Ws/+ and +/+ rats were used, with all groups weighing between 250–300 g and aged between 3–4 months. The animals were housed at a constant temperature of 22 ± 2°C with a humidity of 55 ± 10% on an automatically controlled 12:12-h light–dark cycle (lights on at 7:00 am), and had free access to food and water.

2.1.2. Measurement of histamine and 5-HT content in tissues and bath solution

The concentrations of histamine and 5-HT in trachea tissues and bath solution were determined by high-performance liquid chromatography (HPLC)–fluorometry (Yamatodani et al., 1985) and HPLC–electrochemical detection (Inoue et al., 1982), respectively. The lower limit for detection of histamine was 50 fmol, and that for 5-HT was 100 fmol. Briefly, rat tracheas were removed, weighed, and homogenized in 1 ml of 0.46 M perchloric acid containing 5 mM Na_2 EDTA and 1 mM sodium metabisulfite with a Polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenate was centrifuged at $10{,}000 \times g$ for 15 min at 4°C, and the supernatant was obtained. For histamine measurement, 50 μl of the supernatant was injected directly into a column packed with TSKgel SP-2SW cation exchanger (150×6 mm i.d.) (Tosoh, Tokyo, Japan). Histamine was eluted with 0.25 M potassium phosphate at a flow rate of 0.6 ml/min. The histamine was post-labeled with *o*-phthalaldehyde in alkaline conditions, and detected fluorometrically using an F1080 Fluorometer (Hitachi, Tokyo, Japan), with the excitation and emission wavelengths being 360 and 450 nm, respectively. For 5-HT measurement, 20 μl of the supernatant was injected directly into a column packed with TSK gel SP-2SW (150×4 mm i.d.) and eluted with 0.22 M Li propionate buffer (pH 6.0 adjusted with LiOH) containing 3.4 mM EDTA at a flow rate of 0.8 ml/min. Eluted 5-HT was determined through the use of an electrochemical detector (IRICA E-502, Kyoto, Japan) at 0.7 V of the working electrode potential.

To assay the amount of histamine in the 20 ml of organ bath solution, 400 μl of the sample was taken at different time points (0, 1, 5, 10, 15, and 20 min). Each sample was mixed with 200 μl of 1.15 M perchloric acid in 5 mM Na_2 EDTA and subjected to HPLC as previously described. Histamine and 5-HT contents are expressed as nanomole per gram of wet weight tissue.

2.1.3. Tissue preparation and measurement of tracheal contraction

The rats were anesthetized with diethyl ether vaporized in a glass jar and then exsanguinated. After exsanguination, the trachea was quickly removed. The lower part of the trachea was carefully stripped of connective tissue, then prepared as tracheal strips by opening it longitudi-

nally by cutting through the cartilage and dividing it into segments containing two to three cartilage rings.

The strips were mounted in 20 ml of organ bath solution containing Krebs–Henseleit buffer (in mmol/l: NaCl 118, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.5, glucose 5.6) containing 10^{-6} M indomethacin in order to avoid a possible modulation of contractile responses by prostaglandins derived from the epithelium (Holtzman, 1992). The solution was maintained at 37°C and bubbled with 95% O_2/5% CO_2. The tracheal strips were then allowed to stabilize for 60–90 min, during which time the bath solution was changed every 15 min at a resting tension of 1 g, which was found to be optimal for measuring changes in tension. Contraction was measured isometrically with TB-611T transducers (Nihon Kohden, Tokyo, Japan), with the signal being amplified by an AP-601G amplifier (Nihon Kohden). After the equilibration period of 60–90 min, a steady baseline was established.

Carbachol 100 μM was added to the bath solution to check the viability of preparations. This procedure was repeated once or twice at an interval of 30 min until the contraction was stable, i.e. less than 10% variation. The same was also done at the end of each experiment.

2.1.4. Contractile responses of rat trachea to various stimulants

In a series of experiments, concentration–response curves for carbachol, 5-HT and histamine were made in a cumulative way, using a range of 10^{-8}–10^{-4} M on the same preparation from each type of rat. After measurement of the contractions induced by these agents, all preparations were evaluated in terms of contractile response to compound 48/80. As the compound 48/80-induced contraction was not sustained, the contraction study was done with the optimal concentration that gave maximal contraction, based on a preliminary study. One tracheal strip was treated with only one concentration of compound 48/80.

In another series of experiments, the contraction elicited by compound 48/80 was also evaluated in the presence of ketanserin, a specific 5-HT_{2A} receptor antagonist, mepyramine, a specific histamine H_1 receptor antagonist and nedocromil, a mast cell stabilizer, which were added to the tracheal preparations for 20 min before administration of compound 48/80.

2.1.5. Enzyme histochemical study

Rat tracheas of +/+, Ws/+, and Ws/Ws rats were removed and fixed using 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 12 h at 4°C. The fixed specimens were washed with 0.1 M phosphate buffer, immersed in 20% sucrose/0.1 M phosphate buffer overnight at 4°C, and then frozen until studied. Sections were cut 4–5 μm thick using a cryotome before study.

Chloroacetate esterase activity of mast cells was visualized by enzyme histochemistry as described by Beckstead et al. (1981). Briefly, the sections were incubated for 60 min at 30°C with chloroacetate substrate solution, consisting of 0.5% naphthol AS-D chloroacetate and 0.02% hexazotized pararosaniline as chromogen, and were then counterstained with hematoxylin. This procedure was also applied for the trachea of control (+/+) rats after compound 48/80 administration. Four adjacent sections of each trachea were viewed at 400× magnification, and the number of mast cells in each section was counted. The average of four sections of each trachea was used for semi-quantitative analysis.

2.2. Drug used

Carbachol, compound 48/80, indomethacin, mepyramine, naphthol AS-D chloroacetate and pararosaniline were obtained from Sigma (St. Louis, MO, U.S.A.). Histamine diphosphate, 5-HT creatinine sulfate and ketanserin were obtained from Wako (Osaka, Japan). Nedocromil sodium, the disodium salt of pyranoquinoline dicarboxylic acid, was kindly supplied by Fujisawa–Fisons Pharmaceuticals (Osaka, Japan). All other chemicals were of the highest grade commercially available.

2.3. Statistical analysis

The results are expressed as the means ± S.E.M.. Statistical analysis was carried out by using a one-way analysis of variance followed by Student's paired *t*-test. *P* values

Table 1
Differences of histamine and 5-HT contents in rat tracheas between +/+, Ws/+, and Ws/Ws rats

	Histamine contents			5-HT contents		
	+/+	Ws/+	Ws/Ws	+/+	Ws/+	Ws/Ws
Whole tissue	86.06 ± 6.30 (n = 16)	30.71 ± 12.66 (n = 16)	0.71 ± 0.21 (n = 16)	4.92 ± 0.23 (n = 10)	1.53 ± 0.26 (n = 10)	ND
Percentage of +/+ rats trachea	100	35.68 ± 3.68[a]	0.83 ± 0.05[a]	100	31.10 ± 0.21[a]	ND

Data are means ± S.E.M. in nanomole per gram of tissue. ND, not detected.
[a] Data are means ± S.E.M. in percent.

less than 0.05 were considered to indicate significant differences.

3. Results

3.1. Histamine and 5-HT content in tracheal tissue of +/+, Ws/+ and Ws/Ws rats

Since histamine is the major mediator of mast cells and it is almost exclusively produced in and secreted from mast cells, the difference in tracheal histamine content was representative of the variable mast cell number in tracheal tissue between the three types of rats. We also measured the 5-HT content in rat trachea as a mediator released from rodent mast cells during degranulation. From the measurements, it was found that the histamine content of tracheal strips from Ws/Ws and Ws/+ rats was $0.83 \pm 0.05\%$ and $35.68 \pm 3.68\%$ ($n = 16$) of that in tracheal strips from control (+/+) rats, respectively (Table 1). The 5-HT content of tracheal strips from Ws/+ rats, this being almost similar in proportion to the ratio of histamine content, was $31.10 \pm 0.21\%$ ($n = 10$) of that found in tracheal strips from control rats, whereas 5-HT could not be detected in tracheal strips from Ws/Ws rats due to its very low concentration. This data indicated the difference in mast cell number between the three types of rats.

We also took notice of the difference in histamine and 5-HT content between upper and lower parts of the trachea in +/+ and Ws/+ rats. The content of histamine and 5-HT in the upper part of the trachea was approximately 77–79% of that in the lower part (Table 2).

3.2. Contractile response of tracheal strips from +/+, Ws/+ and Ws/Ws rats to carbachol, histamine, and 5-HT

Fig. 1 shows the contractile response of isolated tracheal strips to carbachol and 5-HT. The tracheal strips from +/+ rats showed contractile responses to both carbachol and 5-HT, but not to histamine with the dose range used in these experiments. Carbachol was also used to test the viability of tracheal preparations. The contraction elicited with 100 μM carbachol at the start of the experiment was not significantly different from that at the end of the experiments, indicating that the contractility of the preparation was well preserved during experiments.

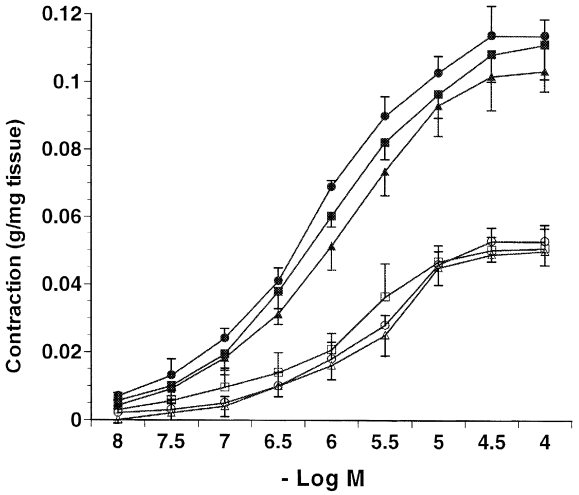

Fig. 1. Cumulative concentration–response curves for the contractile effect of carbachol (solid symbols) and 5-HT (open symbols) on isolated tracheal strips from +/+ (▲), Ws/+ (■) and Ws/Ws (●) rats. Each point represents the mean ± S.E.M. for five rats (ANOVA carbachol vs. 5-HT: $P \leq 0.01$ for +/+, Ws/+, and Ws/Ws rats; ANOVA for carbachol between +/+, Ws/+, and Ws/Ws rats: NS; ANOVA for 5-HT between +/+, Ws/+, and Ws/Ws rats: NS).

The contractile effect of carbachol was not significantly different between the three types of rats, with the average maximal tension being 0.110 ± 0.003 g/mg tissue ($n = 15$). The same was observed concerning the contractile effects mediated by 5-HT, with the average maximal tension being 0.051 ± 0.001 g/mg ($n = 15$). The effect of 5-HT on tracheal contraction was $46.81 \pm 0.76\%$ ($n = 15$) of that induced by carbachol.

In a preliminary study, it was found that the sensitivity of the lower and upper parts of the trachea to carbachol and 5-HT was significantly different (data not shown). Indeed, the lower part was more sensitive than the upper part. For this study, we used the lower part of the trachea. Histamine did not contract trachea with the dose range used in the experiments.

Table 2
Differences of histamine and 5-HT contents between upper and lower parts trachea of +/+, Ws/+, and Ws/Ws rats

Part of tracheas	Histamine contents			5-HT contents		
	+/+	Ws/+	Ws/Ws	+/+	Ws/+	Ws/Ws
Upper	75.19 ± 5.70 ($n = 8$)	26.55 ± 0.04 ($n = 8$)	0.70 ± 0.20 ($n = 8$)	4.33 ± 0.48 ($n = 5$)	1.35 ± 0.09 ($n = 5$)	ND
Lower	96.93 ± 8.75 ($n = 8$)[a]	34.88 ± 3.39 ($n = 8$)[a]	0.72 ± 0.20 ($n = 8$)	5.50 ± 0.79 ($n = 5$)[a]	1.71 ± 0.49 ($n = 5$)[a]	ND
Percentage of upper vs. lower	77.57 ± 5.21[b]	76.12 ± 3.74[b]	ND	78.73 ± 0.32[b]	78.95 ± 0.24[b]	ND

Data are means ± S.E.M. in nanomole per gram of tissue. ND, not detected.
[a] $P < 0.05$, compared to the upper part.
[b] Data are means ± S.E.M. in percent.

3.3. Effect of compound 48/80 on tracheal contraction

A final dose of 25 μg/ml compound 48/80 was used to elicit contraction. In tracheal strips from control (+/+) rats, compound 48/80 was found to elicit a contraction that was 63.24 ± 2.44% ($n = 5$) of the contraction induced by 5-HT (Fig. 2). The contraction pattern, as shown in the inset of Fig. 2, was different from that of the carbachol and exogenous 5-HT responses. Contraction occurred in 1 min, reached a maximum within 2–4 min, then gradually decreased over a period of 9–10 min, and disappeared by 15–20 min. The second administration of compound 48/80 to the organ bath elicited a very much smaller of contraction; indeed, in some preparations it did not give any effect.

With strips from Ws/+ rats, the maximum contraction elicited by compound 48/80 was 24.60 ± 3.70% ($n = 5$) of the 5-HT-induced contraction, as well as 38.90% of that observed with the +/+ rats. The second administration also did not give any effect. Regarding tracheal strips from mast cell-deficient (Ws/Ws) rats, no contraction occurred after compound 48/80 administration.

3.4. Effects of mepyramine, ketanserin and nedocromil on the contractile response induced by compound 48/80

Fig. 3 shows that preincubation with 0.1 μM mepyramine for 20 min before compound 48/80 administration did not alter the contraction ($n = 5$), indicating that the contraction did not involve the histamine H_1 receptor. Ketanserin (0.1 μM), a 5-HT_{2A} receptor antagonist, completely prevented the contractile effect of compound 48/80, indicating that the contraction is most likely due to 5-HT ($n = 5$). Nedocromil (0.1 μM), a mast cell stabilizer, was also found to prevent the contractile effect of compound 48/80 ($n = 5$).

3.5. Release of mast cell-derived mediators

From the results presented above, we found that the tracheal contraction in these rats was mostly due to 5-HT, not to histamine. In this experiment, we focused on histamine and 5-HT as major mast cell-derived mediators released from rat trachea. We, however, failed to obtain a time–course curve of 5-HT release into the organ bath due to the presence of very low concentrations. Therefore, instead of 5-HT, we measured histamine release into the organ bath solution. Histamine did not affect the tracheal contraction, but it was released from mast cells after compound 48/80 administration. Since histamine and 5-HT release from mast cells show a linear and positive correlation with each other (Pihel et al., 1998), we may assume that the pattern of histamine release from mast cells represents 5-HT release. The time course of histamine

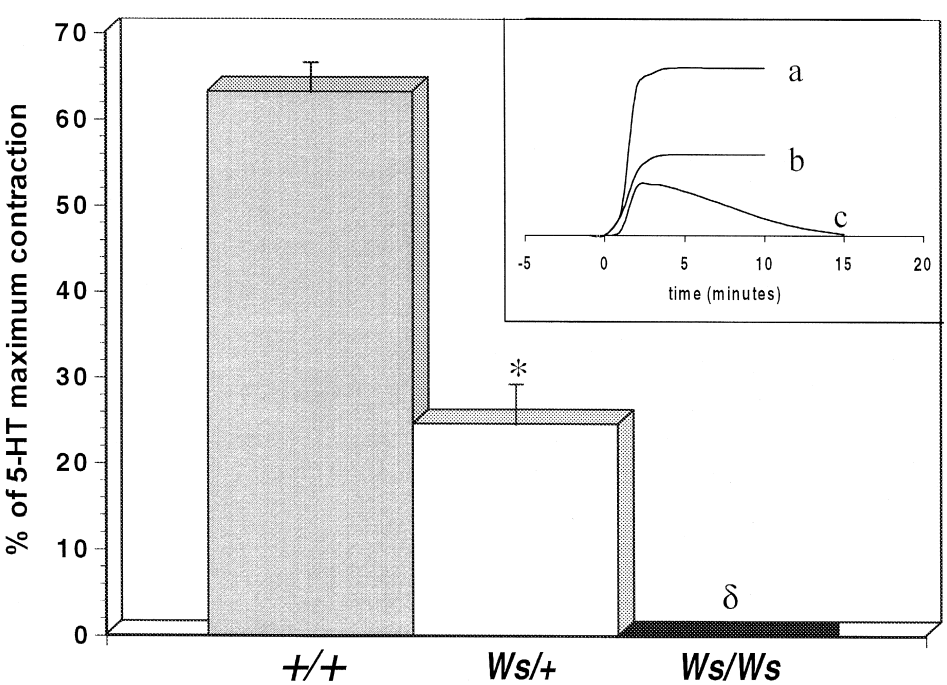

Fig. 2. Tracheal contraction induced by compound 48/80 in strips from +/+, Ws/+ and Ws/Ws rats. Inset shows the profile of time-dependent contraction induced by carbachol (a), 5-HT (b), and compound 48/80 (c) in +/+ rats, in proportional manner. Data represent the means ± S.E.M. for five rats. $^{*}P < 0.05$ compared to the control group, $^{\delta}P < 0.05$ compared to the Ws/+ group.

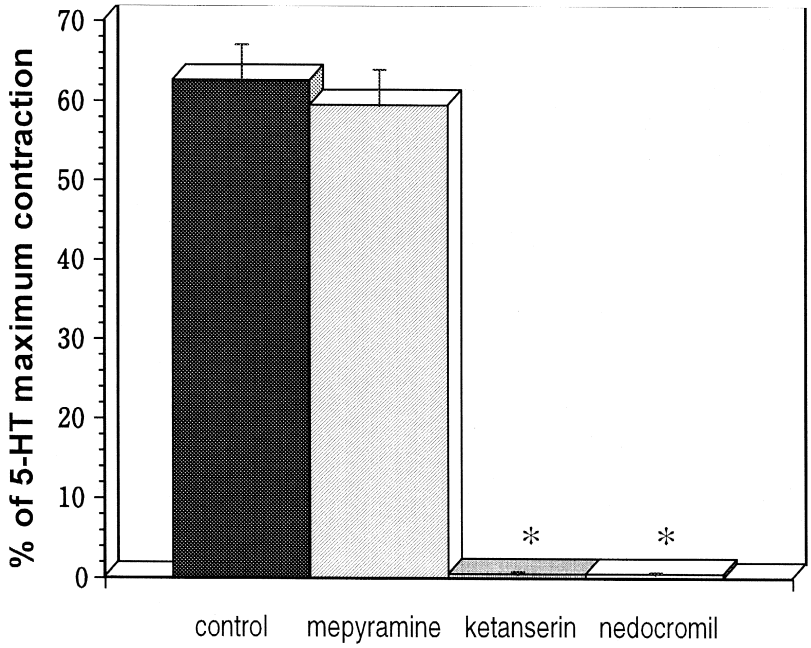

Fig. 3. Compound 48/80-induced contraction in tracheal strips from +/+ rats, preincubated for 20 min with 0.1 μM mepyramine ($n = 6$), 0.1 μM ketanserin ($n = 5$) and 0.1 μM nedocromil ($n = 5$), respectively. Each data point represents the mean ± S.E.M. for n rats. $^{*}P < 0.05$ compared to the control group.

release from tracheal strips from the three types of rats during compound 48/80 treatment is shown in Fig. 4. Concerning 5-HT, we only measured the remaining 5-HT content in tracheal tissue after compound 48/80 treatment, then compared this result with untreated tissue. We found that the 5-HT content in control tissue from +/+ rats was 4.94 ± 0.41 nmol/g ($n = 5$), whereas that in compound 48/80-treated tissue was 3.31 ± 0.36 nmol/g ($n = 5$), which means approximately 32.99% of 5-HT was released from mast cells after a single treatment with compound 48/80.

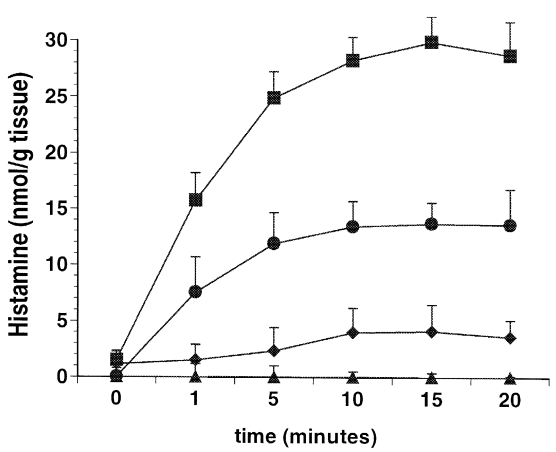

Fig. 4. Time course of histamine release from mast cells in tracheal strips from +/+ (■), Ws/+ (●) and Ws/Ws (▲) rats after compound 48/80 administration. Note the spontaneous release of histamine from mast cells of +/+ (◆) rat trachea.

3.6. Enzyme histochemical study of rat tracheal mast cells

From the enzyme histochemical study, we found esterase-positive cells stained red in the tissue from +/+ (control) and Ws/+, but not in tissue from Ws/Ws, rats. Both connective tissue mast cells and mucosal mast cells/globule leukocytes are known to contain a chymotrypsin-like serine protease that can be detected by enzyme histochemistry, using chloroacetate as substrate (Tam et al., 1988). We found that the mast cells population was greatest in tissue from +/+ rats, intermediate in tissue from Ws/+ rats, and absent in tissue from Ws/Ws rats (Fig. 5a–c).

Mast cell numbers in tissue from +/+, Ws/+ and Ws/Ws rats were 32 ± 6 cells/section ($n = 8$, four sections each of two tracheas), 11 ± 4 cells/section ($n = 8$, four sections each of two tracheas), and 0 ± 0 cell/section ($n = 8$, four sections each of two tracheas), respectively. After compound 48/80 treatment, mast cell numbers in tissue from +/+ rats decreased to be 7 ± 4 cells/section ($n = 8$, four sections each of two tracheas), indicating that degranulation had occurred.

The connective tissue mast cells were more abundant in the submucosal layer and on the abluminal surface, espe-

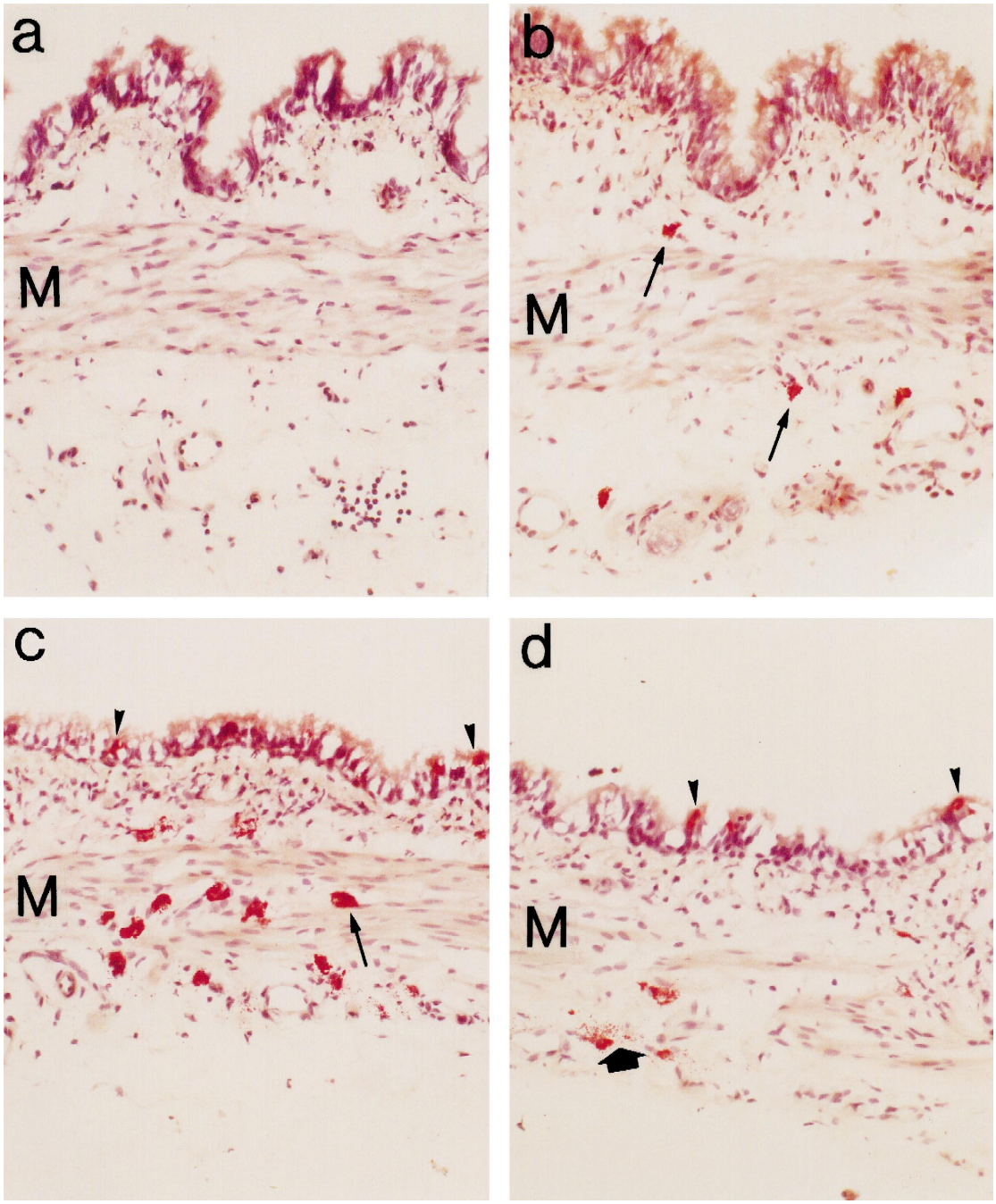

Fig. 5. Light micrographs of serial sections of the membranous part of trachea revealed by enzyme histochemistry for chloroacetate esterase activity.(a) Trachea from Ws/Ws rat. No esterase-positive cells were found, either in the epithelium or submucosa layer. M = muscle cells region (×100). (b) Trachea from Ws/+ rat. Significantly fewer esterase-positive cells, presumably connective tissue mast cells (arrow), were present around muscle cells (M) than in trachea from +/+ (control) rat (×100). (c) Trachea from +/+ (control) rat. Esterase positive-cells, presumably connective tissue mast cells (arrow), were present abundantly in submucosa layer around muscle cells (M) and presumably mucosal mast cells (arrowhead) are found in epithelial layer (×100). (d) Trachea from +/+ (control) rat after treatment with compound 48/80. Note that the number of connective tissue mast cells decreased significantly and some connective tissue mast cells showed evidence of the degranulation process (bold arrow). The mucosal mast cells (arrowhead) still remained (×100).

cially in the region close to smooth muscle cells and in the muscle itself, whereas the mucosal mast cells were found only in the epithelium. After administration of compound 48/80, connective tissue mast cells seemed to be degranulated, as indicated by a decrease in mast cell number and/or the degranulation process (Fig. 5d), whereas mucosal mast cells/globule leukocytes were not degranulated.

4. Discussion

The Ws/Ws rats discovered by Tsujimura et al., 1991 are devoid of connective tissue mast cells because of the deletion of 12-nucleotide bases in the tyrosine kinase domain of the c-*kit* receptor gene. As shown in Table 1, the histamine content of tracheal tissue from Ws/Ws and Ws/+ rats was approximately 0.83% and 35.68% of that in tissue from control (+/+) rats, respectively, this being representative of the different number of mast cells in tissue from the three types of rats. Comparison of the results obtained for the three types of rats, therefore, would be very useful to study more directly the role of mast cells in certain functions (Sugimoto et al., 1995; Guo et al., 1997; Huang et al., 1998; Andoh et.al, 1999).

In the present study, we observed the tracheal contractile response to various spasmogens, with a focus on compound 48/80 as a potent inducer of mast cell degranulation, and compared the results obtained for the three types of rats. Carbachol was used to test the viability and the contractility of tracheal smooth muscle, whereas histamine and 5-HT are compounds known to be released from mast cells in rodents after stimulation.

The finding of the present study demonstrates that the contractile response to compound 48/80 in tracheal strips from +/+, Ws/+ and Ws/Ws rats is significantly different, whereas the contractile responses to carbachol and 5-HT showed relatively similar patterns for tracheal strips from the three types of rats. As shown in Fig. 1, 5-HT elicited $46.81 \pm 0.76\%$ ($n = 15$) of the maximal contraction induced by carbachol, which is a similar result to that found with Fisher 344 rats by Joos et al. (1997). However, in a preliminary study, we found that the lower/distal part of the trachea was more sensitive to these spasmogens than the upper/proximal part. Carbachol-induced contraction is mediated by the muscarinic receptor. Vornanen and Tirri (1981) found a difference in terms of cholinergic responses along different sections of rat airways, where the bronchus was found to be more sensitive than the trachea. They suggested that the difference in sensitivity is probably due to altered activity of cholinesterase or to a difference in the level of muscarinic receptors along the various sections of the rat airways. Since, however, carbachol is extremely resistant to hydrolysis by cholinesterase, our results are perhaps more likely explained by a different density of the muscarinic receptor within the smooth muscle between the lower and upper parts of the trachea; the greatest density of muscarinic receptor is in the lower part of the airways (Allison and Jacoby, 1998).

5-HT can induce tracheal contraction by direct activation of 5-HT receptors present on the airway smooth muscle, as well as through an indirect mechanism such as facilitation of cholinergic neurotransmission (Dixon et al., 1980), cyclooxygenase product formation (Baklhe and Smith, 1977), β-adrenoceptor activation (Pluchino, 1972), release of endogenous peptide (Buckner et al., 1991), and microvascular leakage (O'Donnell et al., 1987). Pauwels et al. (1985) demonstrated that, in rat airways, 5-HT may predominantly act directly on smooth muscle because the 5-HT response was not antagonized by either atropine or bilateral vagotomy. Therefore, the most possible explanation is a difference in the number of 5-HT receptors within the smooth muscle of the upper and lower parts of the rat trachea.

Histamine did not give any effect regarding tracheal contractility in the three types of rats. This result is in accordance with previous findings obtained with Wistar rats (Church, 1975; Chand and Eyre, 1978) and with Fisher 344 rats (Joos et al., 1997; Van de Voorde and Joos, 1998). It possibly means that histamine receptors are not expressed in the muscle cells of rat trachea. Thus, concerning both the immediate mediators released from the rat mast cells, histamine and 5-HT, only the latter seems to elicit a substantial contraction.

We, however, failed to measure 5-HT release into the organ bath solution due to its very low concentration and limitations in terms of instrument sensitivity. It is probable that, after release, a high concentration of 5-HT directly acts at receptors in muscle cells to elicit contraction, with the remaining material being quickly degraded. This is supported by our finding of the presence of mast cells in the immediate vicinity of muscle cells, even muscle itself. However, 5-HT is known to be rapidly degraded. Holgate et al. (1993) reported that once released, 5-HT is rapidly degraded to 5-hydroxyindoleacetaldehyde followed by oxidation to 5-hydroxyindoleacetic acid. We, however, assumed that the pattern of 5-HT release would be similar to that we obtained for histamine.

Administration of 25 μg/ml compound 48/80 into the organ bath elicited contraction in the trachea from +/+ and Ws/+ rats, but not in trachea from Ws/Ws rats (Fig. 2). The contraction of Ws/+ rat trachea was also only 38.90% of that seen in +/+ rat trachea, this being proportional to the number of mast cells, supporting the idea of the involvement of mast cells in the tracheal contraction. The second administration of compound 48/80 to the organ bath elicited a much smaller contraction, and in some preparations it did not give any effect. The evidence indicates that mast cells had already degranulated and released the maximum amount of mediators (histamine and 5-HT) into the organ bath solution. It is also likely that tachyphylaxis occurred, which would decrease the effect of compound 48/80. We found that approximately 33% of 5-HT and 30% of histamine were released from mast cells in trachea after 25 μg/ml compound 48/80 treatment. This finding is in line with that of Cabado et al. (1999), who reported the release of around 20% of histamine from mast cells after stimulation with 2 μg/ml compound 48/80. We assumed that the remaining mediators were contained in the granules around the center of the cells and which may undergo degranulation less readily after stimu-

lation with compound 48/80. This could account for the occurrence of tachyphylaxis.

The contraction induced by compound 48/80 in trachea from +/+ and Ws/+ rats was completely inhibited by the 5-HT_{2A} receptor antagonist, ketanserin and by a mast cell stabilizer, nedocromil, but was not influenced by the histamine H_1 receptor antagonist, mepyramine. Even though Mapp et al. (1993) demonstrated that compound 48/80 might activate sensory nerves in guinea pig, Joos et al. (1997) reported that that such an effect was not found in rats. Moreover, we found that compound 48/80 did not induce contraction in Ws/Ws rat trachea. This evidence, consequently, allows us to exclude the effect of compound 48/80 on sensory nerves of rat trachea and, furthermore, an effect of nedocromil on sensory nerves in the rat trachea during contraction. We, therefore, may conclude that mast cells are involved in the compound 48/80-induced contraction, and the contraction was mostly due to the 5-HT, not histamine, released from mast cells as a result of degranulation.

A histological study was carried out to confirm the existence of mast cells in the three types of rats and their reaction before and after compound 48/80 administration. We applied enzyme histochemistry to detect the chloroacetate esterase activity of mast cells based on their chymotrypsin-like serine protease content. This method allowed us to see both types of mast cells, because each type of mast cell stains well. We found differences in mast cell number in Ws/Ws, Ws/+, and +/+ rat trachea (shown in Fig. 5a–c). The results obtained with trachea from control (+/+) rats were similar to those obtained by Tam et al. (1988), who demonstrated the existence of connective tissue mast cells and globule leukocytes/mucosal mast cells in Sprague–Dawley rat trachea. They reported that mucosal mast cells/globule leukocytes reside in and beneath the epithelium and are most abundant in the upper part of the trachea, whereas connective tissue mast cells can be found on the abluminal surface of the posterior membrane and in the lamina propria of the mucosa in the intercartilaginous spaces of the lower part of the trachea. We further found that the connective tissue mast cells were densely located in the vicinity of muscle cells and within the muscle itself. The distribution of subtypes of mast cells on rat trachea supports the previous finding of the different sensitivity in upper and lower parts of the trachea to compound 48/80. We also found that degranulation of connective tissue mast cells occurred after compound 48/80, but not in the case of mucosal mast cells. This confirmed the role of connective tissue mast cells in the contractile response of rat trachea.

The finding of vascular endothelial growth factor in the mucosal mast cells of rat trachea, but not in connective tissue mast cells, by Fan and Iseki (1999) supports the possibility of a different biological function for these mast cells. Vascular endothelial growth factor is a potent angiogenic mitogen that also increases vascular permeability. It works independently from histamine and is 50,000 times more potent than histamine in increasing vascular permeability. Woodbury et al. (1984) demonstrated that mucosal mast cells are functionally active during intestinal nematode infections. It is of primary interest that vascular endothelial growth factor has been shown to occur in globule leukocytes/mucosal mast cells, but not in connective tissue mast cells. In contrast, connective tissue mast cells contain much more histamine (and 5-HT) than globule leukocytes/mucosal mast cells, which is considered to play a central role in the early phase of the immunoglobulin E-dependent allergic response. We speculate that the inverse relationship between the occurrence of vascular endothelial growth factor in globule leukocytes/mucosal mast cells and that of histamine in connective tissue mast cells may represent a functional compensation for histamine by vascular endothelial growth factor in globule leukocytes/mucosal mast cells. A recent study proposed another role of mucosal mast cells, namely, that they contribute to enhancement of antigen transport across the rat tracheal epithelium (Yang et al., 1999). However, the precise function of mucosal mast cells in rat trachea still needs to be elucidated.

Finally, we conclude that connective tissue mast cells contribute to the early phase of the asthmatic condition evidenced by tracheal contraction, and that mucosal mast cells may have another role specifically in the late reaction of asthma. A study concerning the function of globule leukocytes/mucosal mast cells in airway disease is now being carried out in our laboratory. In addition, we suggest that Ws/Ws rats provide a good tool to study the role of mast cells in the airway system.

Acknowledgements

This study was financially supported by the foundation settled in Ehime University School of Medicine.

References

Allison, D.F., Jacoby, D.B., 1998. Muscarinic receptors and control of airway smooth muscle. Am. J. Respir. Crit. Care Med. 158, S154–S160.

Andoh, A., Kimura, T., Fukuda, M., Araki, Y., Fujiyama, Y., Bamba, T., 1999. Rapid intestinal ischaemia–reperfusion injury is supressed in genetically mast cell-deficient Ws/Ws rats. Clin. Exp. Immunol. 116, 90–93.

Baklhe, Y.S., Smith, T.W., 1977. Release of spasmogens from rat isolated lungs by tryptamines. Eur. J. Pharmacol. 46, 31–39.

Beckstead, J.H., Halverson, P.S., Ries, C.A., Bainton, D.F., 1981. Enzyme histochemistry and immunohistochemistry on biopsy specimens of pathologic human bone marrow. Blood 57, 1088–1098.

Buckner, C.K., Dea, D., Liberati, N., Krell, R.D., 1991. A pharmacologic examination of receptors mediating serotonin-induced bronchoconstriction in the anesthetized guinea pig. J. Pharmacol. Exp. Ther. 257, 26–34.

Cabado, A.G., Despa, M., Botana, M.A., Vieytes, M.R., Gonzalez, M., Botana, L.M., 1999. Membrane potential changes associated with calcium signals in human lymphocytes and rat mast cells. Life Sci. 64, 681–696.

Chand, N., Eyre, P., 1978. Histamine receptors of airway smooth muscle of ferret and rat. Res. Commun. Chem. Pathol. Pharmacol. 21, 55–65.

Church, M.K., 1975. Response of rat lung to humoral mediators of anaphylaxis and its modification by drugs and sensitization. Br. J. Pharmacol. 55, 423–430.

Dixon, M.D., Jackson, M., Richards, I.M., 1980. The effects of 5-hydroxytryptamine, histamine and acetylcholine on the reactivity of the lung of the anaesthetized dog. J. Physiol. (London) 307, 85–96.

Enerback, L., 1987. Mucosal mast cells in the rat and in man. Int. Arch. Allergy Appl. Immunol. 82, 249.

Fan, L., Iseki, S., 1999. Immunohistochemical localization of vascular endothelial growth factor in the globule leukocyte/mucosal mast cell of the rat respiratory and digestive tract. Histochem. Cell Biol. 111, 13–21.

Galli, S.J., 1993. New concepts about the mast cells. N. Engl. J. Med. 328, 257–265.

Gibson, S., Miller, H.R.P., 1986. Mast cells subsets in the rat distinguished immonohistochemically by their content of serine proteinase. Immunology 58, 181.

Guo, Y., Mochizuki, T., Morii, E., Kitamura, Y., Maeyama, K., 1997. Role of mast cells histamine in the formation of rat paw edema: a microdialysis study. Eur. J. Pharmacol. 331, 237–243.

Holgate, S.T., Robinson, C., Church, M.K., 1993. Mediators of immediate hypersensitivity. 4th edn. In: Middleton, E. Jr., Reed, C.E., Ellis, E.F., Adkinson, N.F. Jr., Yunginger, J.W., Busse, W.W. (Eds.), Allergy, Principles and Practice vol. 1 Mosby, Missouri, p. 272.

Holtzman, M.J., 1992. Arachidonic acid metabolism in airway epithelial cells. Annu. Rev. Physiol. 54, 303–329.

Huang, Z.L., Mochizuki, T., Watanabe, H., Kagoshima, M., Maeyama, K., 1998. Biphasic elevation of plasma histamine induced by water immersion stress, and their sources in rats. Eur. J. Pharmacol. 360, 139–146.

Inoue, M., Ueda, G., Yamasaki, M., Hiramatsu, K., Tanaka, Y., Kurachi, K., Maeyama, K., Yamatodani, A., 1982. Endometrial argyrophil cell adenocarcinoma with indole- or catecholamine precursor uptake and decarboxylation. Int. J. Gynecol. Pathol. 1, 47–58.

Joos, G.F., Levebvre, R.A., Bullock, G.R., Pauwels, R.A., 1997. Role of 5-hydroxytryptamine and mast cells in the tachykinin-induced contraction of rat trachea in vitro. Eur. J. Pharmacol. 338, 259–268.

Mapp, C.E., Boniotti, A., Papi, A., Chitano, P., Coser, E., Stefano, A.D., Saetta, M., Ciaccia, A., Fabbri, L.M., 1993. The effect of compound 48/80 on contractions induced by toluene diisocyanate in isolated guinea-pig bronchus. Eur. J. Pharmacol. 248, 67–73.

Metcalfe, D.D., Baram, D., Mekori, Y.A., 1997. Mast cells. Physiol. Rev. 77 (4), 1033–1064.

Nishida, M., Uchikawa, R., Tegoshi, T., Yamada, M., Matsuda, S., Sasabe, M., Arizono, N., 1998. Lack of active lung anaphylaxis in congenitally mast cell-deficient Ws/Ws rats sensitized with the nematode *Nippostrongylus brasiliensis*. APMIS 106, 709–716.

Niwa, Y., Kasugai, T., Ohno, K., Morimoto, M., Yamazaki, M., Dohmae, K., Nishimune, Y., Kondo, K., Kitamura, Y., 1991. Anemia and mast cell depletion in mutant rats that are homozygous at 'white spotting (Ws)' locus. Blood 78, 1936–1941.

O'Donnell, S.R., Braun, R.A., Reid, J.J., Wholohan, T., 1987. A histological method for studying the effects of drug on mediator-induced airway microvascular leakage in rodents. J. Pharmacol. Methods 17, 205–217.

Pauwels, R.M., Straeten, M.V.D., Wyne, J., Bazin, H., 1985. Genetic factors in non-specific bronchial reactivity in rats. Eur. J. Respir. Dis. 66, 98–104, Suppl.

Pihel, K., Hsieh, S., Jorgenson, J.W., Wightman, R.M., 1998. Quantal corelease of histamine and 5-hydroxytryptamine from mast cells and the effects of prior incubation. Biochemistry 37 (4), 1046–1052.

Pluchino, S., 1972. Direst and indirect effects of 5-hydroxytryptamine and tyramine on cat smooth muscle. Naunyn-Scmiedebergs Arch. Pharmakol. 272, 189–224.

Postma, D.S., Kotter, G.H., Sluiter, H.J., 1989. Pathophysiology of airway hyperresponsiveness. In: Weiss, S.T., Sparrow, D. (Eds.), Airway responsiveness and atopy in the development of chronic lung disease. Raven Press, New York, pp. 107–118.

Sugimoto, K., Maeyama, K., Alam, M.K., Sakurai, E., Onoue, H., Kasugai, T., Kitamura, Y., Watanabe, T., 1995. Brain histaminergic system in mast cells-deficient (Ws/Ws) rats: histamine content, histamine decarboxylase activity, and effects of (*S*)α-fluoromethylhistidine. J. Neurochem. 65, 791.

Tam, E.K., Calonico, L.D., Nadel, J.A., Mc.Donald, D.M., 1988. Globule leukocytes and mast cells in the rat trachea: their number, distribution, and response to compound 48/80 and dexamethasone. Anat. Embryol. 178, 107–118.

Tsujimura, T., Hirota, S., Nomura, S., Niwa, Y., Yamasaki, M., Tono, T., Morii, E., Kim, H.M., Kondo, K., Nishimune, Y., Kitamura, Y., 1991. Characterization of Ws mutant allele of rats: 12-base deletion in tyrosine kinase domain of c-*kit* gene. Blood 78, 1942.

Van de Voorde, J.V., Joos, G., 1998. Regionally different influence of contractile agonists on isolated rat airway segments. Respir. Physiol. 112, 185–194.

Vornanen, M., Tirri, R., 1981. Cholinergic responses in different sections of rat airways. Acta Physiol. Scand. 113, 177–182.

Wasserman, S.I., 1997. Biochemical mediators of allergic reactions. In: Grammer, R., Greenberger, L.C. (Eds.), Allergic Disease: Diagnosis and Management. 5th edn. JB Lippincott, New York, pp. 49–56.

Woodbury, R.G., Miller, H.R., Huntley, J.F., Newlands, G.F, Palliser, A.C., Wakelin, D., 1984. Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode infections in rat. Nature 312, 450–452.

Yamatodani, A., Fukuda, H., Wada, H., Iwaeda, T., Watanabe, T., 1985. High-performance liquid chromatographic determination of plasma and brain histamine without previous purification of biological samples: cation-exchange chromatography coupled with post-column derivatization fluorometry. J. Chromatogr. 344, 115–123.

Yang, P., Berin, M.C., Perdue, M.H., 1999. Enhanced antigen transport across rat tracheal epithelium induced by sensitization and mast cell activation. J. Immunol. 163, 2769–2776.