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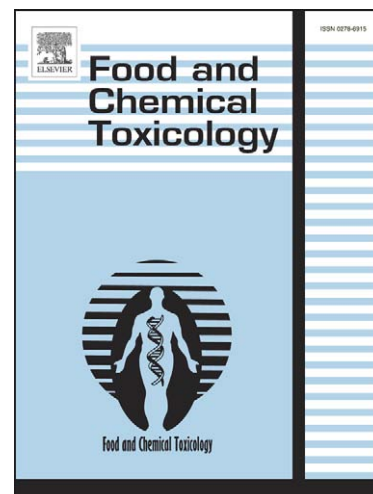
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Anti-allergic effects of *Teucrium japonicum* on mast cell-mediated allergy model

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Abbreviations: DNP-HSA, dinitrophenyl-human serum albumin; HMC-1, human mast cells; PCA, passive cutaneous anaphylaxis; PMACI, phorbol 12-myristate 13-acetate and calcium ionophore; RPMC, rat peritoneal mast cells

Abstract

The mast cell-mediated immediate-type allergic reaction is involved in many allergic diseases such as asthma, allergic rhinitis, and sinusitis. Stimulation of mast cells starts the process of degranulation resulting in release of mediators such as histamine and an array of inflammatory cytokines. In this report, we investigated the effect of aqueous extract of *Teucrium japonicum* Houttuyn (Labiatae) (AXTJ) on the mast cell-mediated allergy model and studied its possible mechanisms of action. AXTJ inhibited compound 48/80-induced systemic reactions and serum histamine release in mice. AXTJ decreased immunoglobulin E-mediated passive cutaneous anaphylaxis reaction. AXTJ reduced histamine release and intracellular calcium from rat peritoneal mast cells activated by compound 48/80. In addition, AXTJ attenuated activation of nuclear factor (NF)- κ B, and downstream tumor necrosis factor (TNF)- α expression in phorbol 12-myristate 13-acetate and calcium ionophore A23187-stimulated human mast cells. Our findings provide evidence that AXTJ inhibits mast cell-derived allergic reactions and involvement of intracellular calcium, TNF- α , and NF- κ B in these effects.

1. Introduction

The *Teucrium japonicum* Houttuyn (Labiatae) is a perennial herb that is widely distributed throughout Korea and Japan. The aqueous extract of *T. japonicum* (AXTJ) is used as a traditional oriental medicine for the treatment of aromatic stomach and digestive disorders. The extract is comprised of clerodane diterpene such as teucjaponin, tueponin, acacetin, and cirsimaritin (Min, 1991; Miyase, 1981).

Mast cells are important producers of mediators of inflammatory responses such as allergy and immediate-type allergic reaction. Immediate-type allergic reaction is mediated by histamine released in response to the antigen cross-linking of immunoglobulin E (IgE) bound to FcεRI on the mast cells. After activation via the FcεRI, the mast cells start the process of degranulation which results in the releasing of mediators, such as products of arachidonic acid metabolism and an array of inflammatory cytokines (Church and Levi-Schaffer, 1997; Galli et al., 2005; Miyajima et al., 1997).

Among the inflammatory substances released from mast cells, histamine is one of the best-characterized and most potent vasoactive mediators implicated in the acute phase of immediate hypersensitivity (Cook et al., 2002). Mast cell activation is initiated upon interaction of multivalent antigen with its specific IgE antibody attached to the cell membrane via FcεRI (Alber et al., 1991; Metzger et al., 1986). Anti-dinitrophenyl (DNP) IgE antibody and antigen have been established to induces passive cutaneous anaphylaxis (PCA) reactions

as a typical *in vivo* model for immediate hypersensitivity. Mast cell degranulation also can be elicited by nonimmunologic stimulators such as neuropeptides, basic compounds, complement components, and certain drugs (Oskeritzian et al., 2005). Compound 48/80 and polymers of basic amino acids, such as substance P, are some of the most potent stimulators of mast cells (Ennis et al., 1980). Thus, an appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylactic allergic reaction.

The signaling pathway leading to degranulation of mast cells after engagement of the FcεRI receptor has been extensively characterized (Beaven et al., 1984; Metcalfe et al., 1981). Activation of mast cells leads to phosphorylation of tyrosine kinase and mobilization of internal calcium. This is followed by activation of protein kinase C, nuclear factor (NF)-κB, and releasing of inflammatory cytokines. Although mast cells also store small amounts of cytokines in their granules, these cells dramatically increase the production of tumor necrosis factor-α (TNF-α) and other cytokines after their surface FcεRI are cross-linked with specific antigen (Bradding et al., 1993; Burd et al., 1989; Galli et al., 1991; Plaut et al., 1989).

In this study, we evaluated the effect of AXTJ on the systemic and local allergic reaction, and histamine release from mast cells. The intracellular calcium content was investigated to clarify the mechanism by which AXTJ inhibited histamine release from mast cells. In addition, the effect of AXTJ on phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (PMACI)-induced gene expression and secretion of TNF-α, and role of

- 1 NF- κ B in this effect were investigated using human mast cells (HMC-1).

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2. Materials and methods

2.1. Animals

The original stock of male ICR mice (6 weeks) and male Sprague-Dawley rats (8 weeks) were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea). The animals were housed 5 per cage in a laminar air flow room maintained under a temperature of $22\pm 2^{\circ}\text{C}$ and relative humidity of $55\pm 5\%$ throughout the study. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Reagents and cell culture

Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), o-phthaldialdehyde, PMA, calcium ionophore A23187, and metrizamide were purchased from Sigma (St. Louis, MO). SB 203580 was purchased from Calbiochem (La Jolla, CA). The human mast cell line (HMC-1) (Moller et al., 1998) was grown in Iscove's media (Life Technologies, Grand Island, NY) supplemented with 10% FBS and 2 mM glutamine at 37°C in 5% CO_2 . The passage ranging 4-8 of HMC-1 cells was used throughout the study.

2.3. Preparation of AXTJ

The *T. japonicum* was purchased from the Bohwa Dang (Jeonbuk, Korea). A voucher specimen (number WSP-07-08) was deposited at the Herbarium of the College of Pharmacy, Woosuk University. *T. japonicum* was grinded (1000 rpm, 30 sec) at room temperature using Micro Hammer-Cutter Mill (Culatti Co., Zurich, Swiss). The particle size was 0.5-2 mm after grinding. The plant sample (30 g) was extracted twice with purified water (500 ml) at 70°C for 5 h in water bath. The extract was filtered through Whatman No. 1 filter paper and the filtrate was lyophilized using 0.45 µm syringe filter. The yield of dried extract from starting crude materials was about 9.8 %. The dried extract was dissolved in saline or Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% bovine serum albumin) before use.

2.4. Compound 48/80-induced systemic anaphylaxis

Mice were given an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator, compound 48/80. AXTJ was dissolved in saline and intraperitoneally administered 1 h before the injection of compound 48/80 ($n=10/\text{group}$). In time dependent experiment, AXTJ (1000 mg/kg, BW) was injected at 5, 10, 15 and 20 min after compound 48/80 injection ($n=10/\text{group}$). Mortality was monitored for 1 h after induction of anaphylactic shock. After the mortality test, blood was obtained from the heart of each mouse to measure serum histamine content.

2.5. *Passive cutaneous anaphylaxis (PCA)*

An IgE-dependent cutaneous reaction was examined as previously described (Kim et al., 2005). PCA reaction was generated by sensitizing skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mouse tail vein. The mice were injected intradermally with 0.5 µg of anti-DNP IgE. After 48 h, each mouse was received an injection of 1 µg of DNP-HSA containing 4% Evans blue (1:4) via the tail vein. Thirty minutes after the challenge, the mice were killed and the dorsal skin (diameter, 1 cm) was removed for measurement of the pigment area. The amount of dye was determined colorimetrically after extraction with 1 ml of 1 M KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13) based on the method of Katayama *et al* (Katayama et al., 1978). The intensity of absorbance was measured at 620 nm in a spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan).

2.6. *Preparation of serum and histamine determination*

Preparation of serum and determination of histamine contents were examined as previously described (Kim et al., 2007a). Briefly, serum was withdrawn and the histamine content was measured by the o-phthaldialdehyde spectrofluorometric procedure. The fluorescent intensity was measured at emission 438 nm and excitation 353 nm using

spectrofluorometer (Shimadzu, RF-5301 PC, Kyoto, Japan).

2.7. Preparation of rat peritoneal mast cells (RPMC)

RPMC were isolated as previously described (Kim et al., 2006). In brief, rats were anesthetized with carbon dioxide and injected with 20 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄ and 0.1% gelatin), into the peritoneal cavity and the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells were aspirated by Pasteur pipette. The peritoneal cells were sedimented at 150 g for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells, i.e. macrophages and small lymphocytes. In brief, peritoneal cells were suspended in 1 ml of Tyrode buffer B, layered on 2 ml of metrizamide (22.5 w/v%), and centrifuged at 400 g for 15 min at 4°C. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue exclusion.

2.8. Inhibition of histamine release

Purified RPMC were resuspended in Tyrode buffer A for the treatment with

compound 48/80. RPMC were incubated with AXTJ (0.001 to 1 mg/ml) or media alone for 10 min at 37°C before the addition of compound 48/80 (5 µg/ml) and incubated for an additional 10 min. The cells were separated from the released histamine by centrifugation at 400 g for 5 min at 4°C. The cells were separated from the released histamine by centrifugation at 400 g for 5 min at 37°C.

2.9. Determination of intracellular Ca^{2+}

Morphological evaluation of cells was conducted with an Olympus IX71 inverted microscope (Olympus America, Melville, NY). Digital images were acquired using the Magnafire SP (Olympus) digital camera. Fluo-3/AM (2 µM, Molecular Probes, Eugene, OR) was used to determine intracellular Ca^{2+} following the manufacturer's protocol briefly described as following. RPMC were preincubated with Fluo-3/AM for 60 min. After washing the dye from the cell surface, cells were pretreated with AXTJ for 10 min before adding compound 48/80. The fluorescent intensity was recorded using fluorescent plate reader (Molecular Devices, Sunnyvale, CA) at an excitation of 485 nm and an emission of 520 nm and digitized.

2.10. Western blot analysis

HMC-1 cell (2×10^6 /ml) extracts were prepared by detergent lysis procedure. Samples

of protein (50 μ g) were electrophoresed using 12% SDS-PAGE, as described elsewhere (Lee et al., 2008) and then transferred to nitrocellulose membrane. The p65 NF- κ B was assayed using anti-NF- κ B (p65) antibodies (Santa Cruz Biotech, Santa Cruz, CA). Immunodetection was done using enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ).

2.11. Transient transfection and luciferase activity assay

For transient transfections, HMC-1 cells were seeded at 2×10^6 in a 6-well plate 1 day before transient transfection. The expression vectors containing the NF- κ B luciferase reporter construct (pNF- κ B-LUC, plasmid containing NF- κ B binding site; STANTAGEN, Grand Island, NY) were transfected with serum- and antibiotics-free Iscove's medium containing 8 μ l of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 5 h of incubation, medium was replaced with Iscove's medium containing 10% FBS and antibiotics. Cells were allowed to recover at 37°C for 20 h and subsequently were stimulated as indicated. Cell lysates were prepared and assay for luciferase activity using Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

2.12. RNA extraction and mRNA detection

The total cellular RNA was isolated from the RPMCs (1×10^6 /well in 24-well plate) after stimulation of PMA and A23187 with or without AXTJ using a TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. The first strand complementary DNA (cDNA) was synthesized using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). A reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNF- α and β -actin (internal control). The conditions for the reverse transcription and PCR steps were similar to those previously described (Kim and Sharma, 2004). The primer sets were chosen by the Primer 3 program (Whithead Institute, Cambridge, MA). The cycle number was optimized in order to ensure product accumulation in the exponential range. The amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide, documented using a Kodak DC 290 digital camera, and digitized with UN-SCAN-IT software (Silk Scientific, Orem, UT). The band intensity was normalized to that of β -actin in the same sample.

2.13. Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed by coating 96-well plates with 6.25 ng/well of monoclonal antibody with specificity for TNF- α . Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 and twice with

1 PBS alone. For the standard curve, rTNF- α was added to the serum, which was previously
2 determined to be negative to endogenous TNF- α . After exposure to the medium, the assay
3 plates were exposed sequentially to biotinylated anti-human TNF- α and 2,2'-azino-bis(3-
4 ethylbenzthiazoline-6-sulfonic acid) tablet substrates. Optical density was read within 10 min
5 of the addition of the substrate using a 405 nm filter.

7 2.14. Statistical analysis

8 Statistical analyses were performed using SAS statistical software (SAS Institute,
9 Cary, NC). Treatment effects were analyzed using one way analysis of variance followed by
10 Duncan's Multiple Range test. A value of $p < 0.05$ was used to indicate significant
11 differences.

3. Results

3.1. AXTJ attenuates allergic responses *in vivo*

The systemic allergic model (anaphylaxis) was used in order to assess the contribution of AXTJ in anaphylactic reaction. Compound 48/80 was used as a model of induction of systemic fatal allergic reaction. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. Compound 48/80 (8 mg/kg) induced fatal shock in 100% of animals (Table 1). When AXTJ was intraperitoneally administered at concentrations ranging from 10 - 1000 mg/kg for 1 h before the compound 48/80 injection, the mortality with compound 48/80 was dose-dependently reduced. AXTJ completely inhibited the compound 48/80-induced fatal shock at 500 mg/kg. In addition, the mortality of mice administered intraperitoneally with AXTJ (1000 mg/kg) 5, 10, 15 and 20 min after compound 48/80 injection increased time-dependently (Table 2). The effect of AXTJ on the compound 48/80-induced serum histamine release was investigated. AXTJ was administered at concentrations ranging from 10 - 1000 mg/kg 1 h before the compound 48/80 injection. Injection of compound 48/80 caused marked increase of serum histamine release, and this induction was inhibited by AXTJ treatment in a dose-dependent manner (Fig. 1). To confirm the anti-allergic effects of AXTJ in *in vivo* model, we used PCA model. PCA is one of the most important *in vivo* models of local allergic reaction (Wershil et al., 1987). Local extravasation is induced by a local injection of anti-DNP IgE followed by an

intravenous antigenic challenge. Anti-DNP IgE was injected into the right dorsal skin sites. As a control, the left dorsal skin site of these mice was injected with saline alone. AXTJ was administered intraperitoneally 1 h prior to the challenge with antigen. The administration of AXTJ (1 - 1000 mg/kg) showed a dose-dependent inhibition in the PCA reaction (Fig. 2).

3.2. AXTJ decreases histamine release and intracellular calcium levels

We evaluated the ability of AXTJ on compound 48/80-induced histamine release from RPMC. The level of released histamine was hardly detectable in unstimulated cells; however, RPMC released a high level of histamine when stimulated with compound 48/80 (5 μ g/ml) (Fig. 3A). AXTJ dose-dependently inhibited compound 48/80-induced histamine release. The concentration and duration of AXTJ treatment used in these studies had no significant effect on the viability of RPMC (data not shown).

Calcium movements across membranes of mast cells are critical to histamine release (Beaven and Metzger, 1993). To investigate the possible mechanism of AXTJ on the reduction of histamine release, we assayed the level of intracellular calcium. Figure 3B shows the stimulation of intracellular calcium when the RPMC was treated with compound 48/80 (5 μ g/ml). Preincubation of AXTJ (1 mg/ml) with RPMC decreased the compound 48/80-induced intracellular calcium level.

3.3 AXTJ inhibits activation of NF- κ B and expression of TNF- α

To evaluate the impact of AXTJ on downstream mast cell signaling, we examined the effect of AXTJ on the activation of a transcription factor, NF- κ B. Assessment of the activation of NF- κ B was determined by Western blot and NF- κ B-dependent gene reporter assay in HMC-1 cells. We used PDTC as a positive control. Stimulation of HMC-1 cells with PMA (20 nM) and A23187 (1 μ M) induced the nuclear translocation of p65 NF- κ B after 1 h of incubation (Fig. 4A). AXTJ inhibited the PMACI-induced nuclear translocation of p65 NF- κ B. To confirm the inhibitory effect of AXTJ on NF- κ B activation, we examined the effect of AXTJ on the NF- κ B-dependent gene reporter assay. HMC-1 cells were transiently transfected with a NF- κ B-luciferase reporter construct or an empty vector. Exposure of cells to PMACI increased the luciferase activity in the cells transfected with the NF- κ B-luciferase reporter construct (Fig. 4B). AXTJ significantly reduced the PMACI-induced luciferase activity.

Next, we examined whether AXTJ could regulate pro-inflammatory cytokines especially TNF- α in HMC-1 cells. The expression TNF- α is regulated by NF- κ B (Azzolina et al., 2003; Kim et al., 2007b). Stimulation of HMC-1 cells with PMACI during 4 h induced the gene expression of TNF- α . Treatment of AXTJ inhibited PMACI-induced TNF- α gene expression (Fig. 5A). Treatment of AXTJ also decreased PMACI-induced secretion of TNF- α in HMC-1 at 16 h (Fig. 5B). A specific inhibitor for NF- κ B, PDTC (10 μ M), abolished PMACI-induced secretion of TNF- α .

4. Discussion

Using *in vivo* and *in vitro* model, we show that AXTJ inhibits mast cell-mediated allergic responses. In the present study, we clearly demonstrated that AXTJ decreased compound 48/80 or IgE-induced anaphylactic reactions and PMACI-mediated expression of TNF- α . AXTJ significantly inhibited systemic allergic reaction and histamine release. These results indicate that immediate-type allergic reactions are inhibited by AXTJ. In addition, AXTJ administered mice are protected from IgE-mediated PCA, one of the most important *in vivo* models of anaphylaxis in local allergic reaction. This finding suggests that AXTJ might be useful in allergic skin reactions.

Intracellular calcium is critical to the degranulation of mast cells. Calcium movements across membranes of mast cells represent a major target for effective anti-allergic drugs, as these are essential events linking stimulation to secretion (Beaven et al., 1984). The transduction pathways modulating intracellular calcium is modified by ADP-ribosylating G-protein binding protein (Alfonso et al., 2000). Our results showing an attenuation of intracellular calcium in mast cells with AXTJ treatment is consistent with other reports (Lee et al., 2006). According to these observations, we speculate that decreased intracellular calcium might be involved in the inhibitory effect of AXTJ on histamine release.

HMC-1 cell line is a useful cell for studying cytokine activation pathways (Moller et al., 1998). The spectrum of cytokines produced by HMC-1 cells with PMACI stimulation

1 supports the well-recognized role of mast cells in immediate-type hypersensitivity. TNF- α
 2 plays a major role in triggering and sustaining the allergic inflammatory responses in mast
 3 cells. TNF- α promotes inflammation, leukocyte infiltration, granuloma formation and tissue
 4 fibrosis and is thought to be an initiator of cytokine related inflammation states by stimulating
 5 cytokine production (Hide et al., 1997; Jeong et al., 2002). These reports indicate that
 6 reduction of TNF- α from mast cell is a one of the key indicator of reduced allergic symptom.
 7 In the present study, AXTJ inhibited the expression and secretion of TNF- α in PMACI-
 8 stimulated HMC-1 cells. These results suggest that AXTJ reduces the allergic responses
 9 through decreasing the expression of pro-inflammatory cytokine.

10 NF- κ B is a transcription factor that induces the transcription of a variety of genes.
 11 Many of these genes encode molecules important in inflammatory processes, such as
 12 cytokines and adhesion molecules (Sen and Baltimore, 1986; Siebenlist et al., 1994). The role
 13 of NF- κ B activation and its regulation of cytokine production in allergic inflammation has
 14 been characterized (Blackwell et al., 1997). Expression of TNF- α is dependent on the
 15 activation of transcription factor NF- κ B in mast cells. In PMACI-stimulated mast cells, AXTJ
 16 decreased the nuclear translocation of p65 NF- κ B. In addition, a specific NF- κ B inhibitor,
 17 PDTC, reduced PMACI-induced TNF- α secretion. These data suggest that AXTJ has the
 18 inhibitory activity on NF- κ B activation and downstream TNF- α expression.

19 Because we used the whole water extract of *T. japonicum* not a purified component,

the active components that are responsible for the biological effect are not clear at this time. The effort for identify active components from AXTJ in allergic symptom is ongoing in our laboratory. However, the results presented in this report give an insight into the mechanism responsible for anti-allergic activity of AXTJ and evidence that AXTJ could contribute to the prevention or treatment of mast cell-mediated allergic diseases.

Conflict of interest statement

There are no conflicts of interest

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ACCEPTED MANUSCRIPT

Legends for figures

Figure 1. Effect of AXTJ on serum histamine release. Groups of mice ($n=10/\text{group}$) were intraperitoneally pretreated with 200 μl of saline or AXTJ. AXTJ was given at various doses 1 h before the injection of compound 48/80. The compound 48/80 solution was given intraperitoneally to the group of mice. The blood was obtained from the heart of each mouse and histamine content was measured by spectrofluorometer. Each bar represents the mean $\pm$ SEM of three independent experiments. *Significant difference at $p<0.05$.

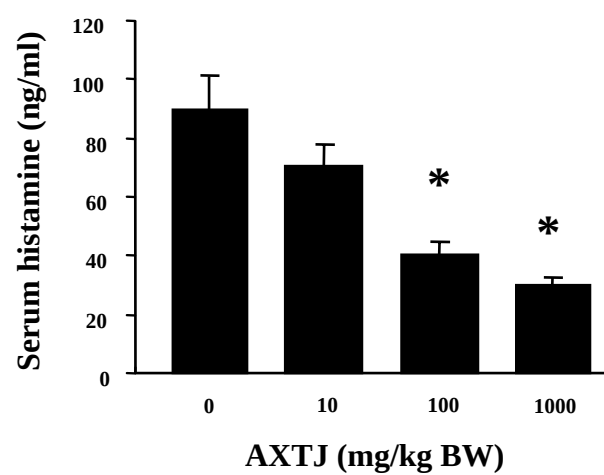
Figure 2. Effect of AXTJ on PCA reactions. AXTJ was intraperitoneally administered 1 h prior to the challenge with antigen. Each amount of dye was extracted as described in Materials and methods and measured by spectrophotometry. Each bar represents the mean $\pm$ SEM of three independent experiments.

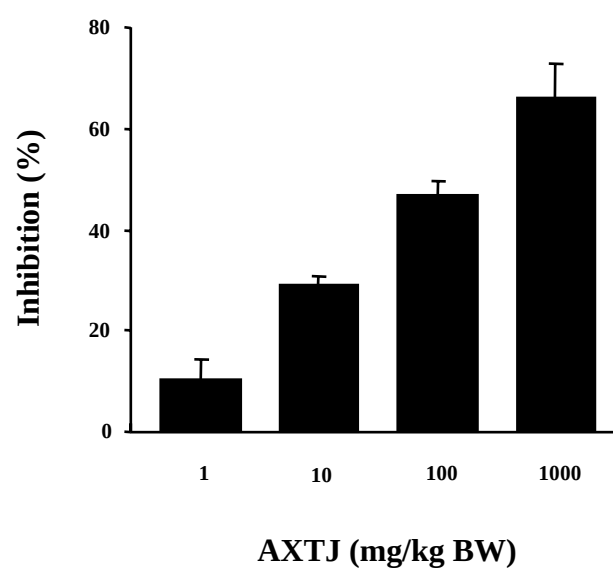
Figure 3. Effect of AXTJ on compound 48/80-induced histamine release and intracellular calcium in RPMC. A) RPMC (2×10^5 cells/ml) were preincubated with AXTJ for 10 min prior to incubation with compound 48/80 (5 $\mu\text{g}/\text{ml}$). Histamine content was measured by spectrofluorometer. B) Cells were preincubated 10 min with AXTJ (1 mg/ml) before adding compound 48/80 (5 $\mu\text{g}/\text{ml}$), and then another 10 min with compound 48/80. The level of

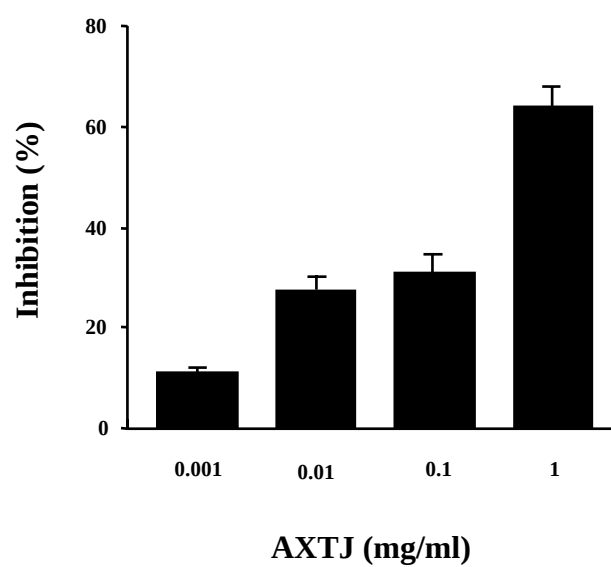
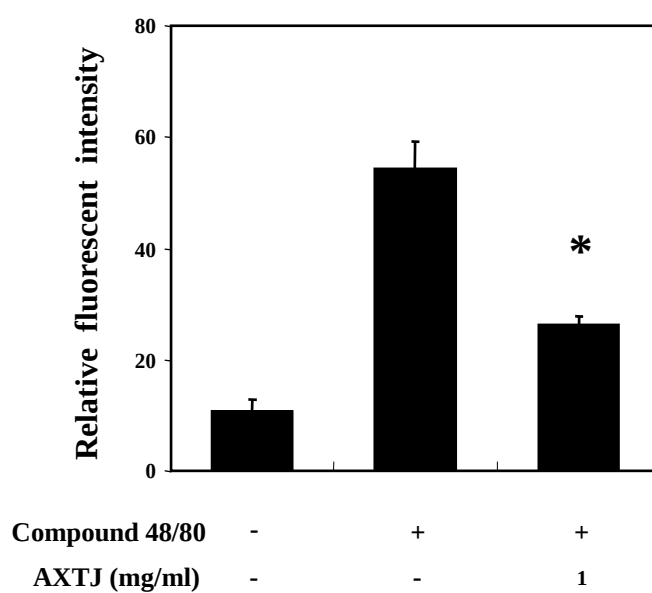
relative fluorescent intensity was recorded using fluorescent plate reader. Each bar represents the mean $\pm$ SEM of three independent experiments. *Significant difference at $p<0.05$.

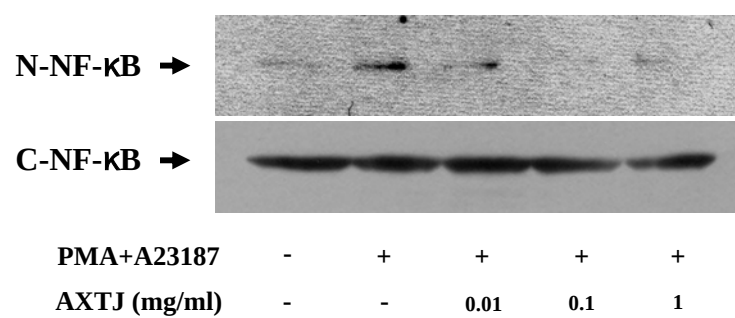
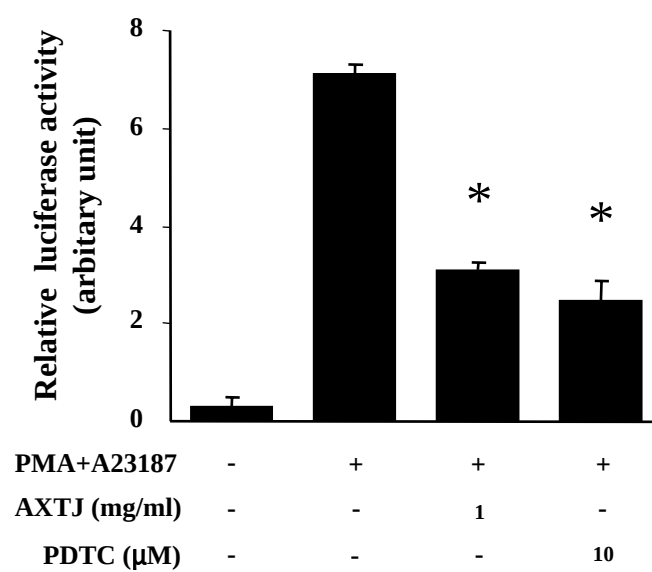
Figure 4. Effect of AXTJ on the activation of NF- κ B. HMC-1 cells were pretreated with AXTJ or PDTC (10 μ M) for 30 min prior to PMA (20 nM) and A23187 (1 μ M) stimulation. A) NF- κ B translocation were assayed by Western blot (N-NF- κ B, nucleus NF- κ B; C-NF- κ B, cytoplasmic NF- κ B). B) Cells were transiently transfected with the NF- κ B-luciferase reporter construct or empty vector. Then, the cells were incubated with PMA and A23187 with or without AXTJ. NF- κ B-dependent transcriptional activity was determined by luciferase activity assay. *Significant difference at $p<0.05$.

Figure 5. Effect of AXTJ on the gene expression and secretion of TNF- α . HMC-1 cells were pretreated with AXTJ or PDTC (10 μ M) for 30 min and stimulated by PMA (20 nM) and A23187 (1 μ M) for 4 h for mRNA and 16 h for protein. A) TNF- α expression was quantified by RT-PCR and the band intensity was normalized to that of β -actin in the same sample. B) The secretion of TNF- α was measured by ELISA as described in Materials and methods. Each data represents the mean $\pm$ SEM of three independent experiments. *Significant difference at $p<0.05$.

**Fig. 1**

**Fig. 2**

A**B****Fig. 3**

A**B****Fig. 4**

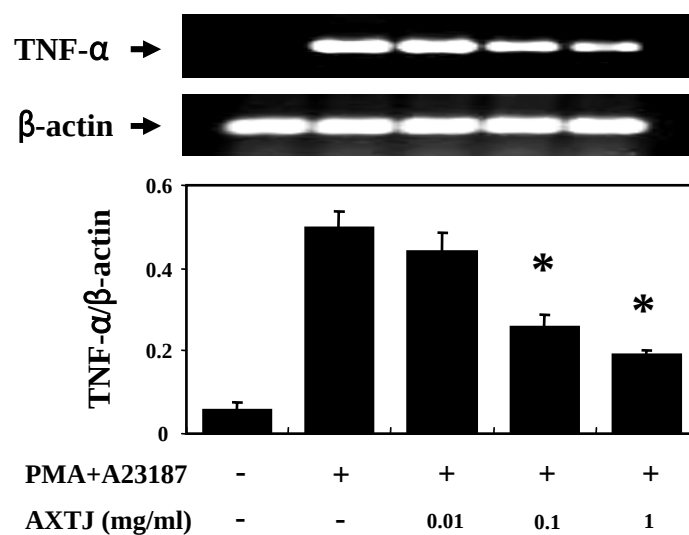
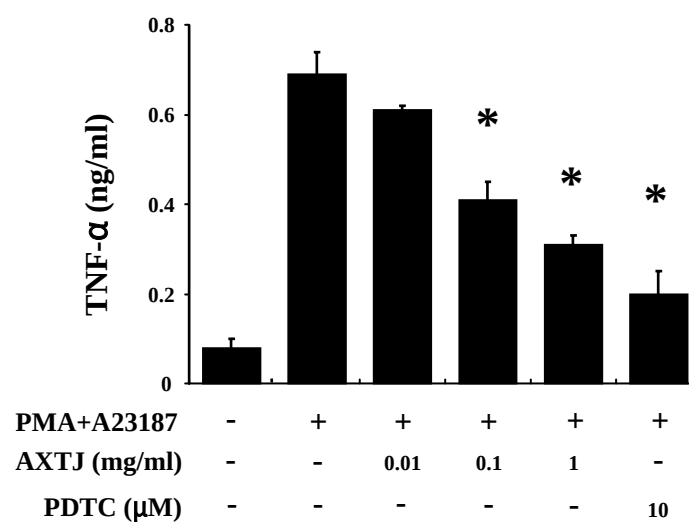
A**B****Fig. 5**

Table 1. Effect of AXTJ on compound 48/80-induced systemic anaphylaxis

AXTJ treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	+	100
10	+	100
50	+	90
100	+	50
500	+	0
1000	+	0
1000	-	0

Groups of mice ($n=10/\text{group}$) were intraperitoneally pretreated with 200 μl of saline or AXTJ at various doses 1 h before the intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice $\times 100/\text{total number of experimental mice}$.

Table 2. Time-dependent effect of AXTJ on compound 48/80-induced systemic anaphylaxis

AXTJ treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Time (min)	Mortality (%)
None (saline)	+		100
1000	+	0	0
	+	5	20
	+	10	50
	+	15	80
	+	20	100

Mice ($n=10/\text{group}$) were intraperitoneally pretreated with 200 μl of saline or AXTJ. AXTJ (1000 mg/kg) was given 5, 10, 15 and 20 min after the intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice $\times 100/\text{total number of experimental mice}$.