



Short communication

Adenine suppresses IgE-mediated mast cell activation



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ABSTRACT

Nucleobase adenine is produced by dividing human lymphoblasts mainly from polyamine synthesis and inhibits immunological functions of lymphocytes. We investigated the anti-allergic effect of adenine on IgE-mediated mast cell activation in vitro and passive cutaneous anaphylaxis (PCA) in mice. Intraperitoneal injection of adenine to IgE-sensitized mice attenuated IgE-mediated PCA reaction in a dose dependent manner, resulting in a median effective concentration of 4.21 mg/kg. In mast cell cultures, only adenine among cytosine, adenine, adenosine, ADP and ATP dose-dependently suppressed FcεRI (a high affinity receptor for IgE)-mediated degranulation with a median inhibitory concentration of 1.6 mM. It also blocked the production of LTB₄, an inflammatory lipid mediator, and inflammatory cytokines TNF-α and IL-4. In addition, adenine blocked thapsigargin-induced degranulation which is FcεRI-independent but shares FcεRI-dependent signaling events. Adenine inhibited the phosphorylation of signaling molecules important to FcεRI-mediated allergic reactions such as Syk, PLCγ2, Gab2, Akt, and mitogen activated protein kinases ERK and JNK. From this result, we report for the first time that adenine inhibits PCA in mice and allergic reaction by inhibiting FcεRI-mediated signaling events in mast cells. Therefore, adenine may be useful for the treatment of mast cell-mediated allergic diseases. Also, the upregulation of adenine production may provide another mechanism for suppressing mast cell activity especially at inflammatory sites.

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1. Introduction

Adenine is an ancestor molecule (Miller and Urey, 1959) and a component of biomolecules, such as nucleic acids and ATP. It can be produced from adenosine and 5'-methylthioadenosine (MTA) by purine nucleoside phosphorylase and MTA-phosphorylase, respectively (Avila et al., 2004). MTA is formed from decarboxylated S-adenosylmethionine in the synthesis of spermidine and spermine during cell proliferation and is metabolized to yield 5-methylthioribose-1-phosphate and adenine by MTA-phosphorylase. Therefore, adenine production from MTA

degradation contributes 85–97% of total adenine synthesis in dividing human lymphoblastoid cells (Kamatani and Carson, 1981).

Several biologic functions of adenine have been reported previously. Adenine inhibits human lymphoblast growth (Hershfield et al., 1977; Snyder et al., 1978), mitogen-stimulated transformation, cytoplasmic immunoglobulin production, and natural killer activity of human mononuclear leukocytes (Ito and Uchino, 1976; Kishi et al., 1985). In contrast to its growth inhibitory effects, adenine has protective role in survival of rat Purkinje cells (Watanabe et al., 2003) and erythrocytes in whole blood storage (Simon et al., 1962). It causes a biphasic effect on cAMP level in rat peritoneal mast cells (Sullivan et al., 1975a) and potentiates 48/80 induced degranulation (Sullivan et al., 1975b). Recently, adenine specific G protein-coupled receptor has been cloned and characterized in a mouse brain (Bender et al., 2002; von Kugelgen et al., 2008) and a pig kidney where it inhibits Na⁺-ATPase activity (Wengert et al., 2007).

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Mast cells are associated with various allergic diseases (Bischoff, 2007; Gregory and Brown, 2006). Mast cells express Fc ϵ RI, the high affinity receptor for IgE (Gilfillan and Tkaczyk, 2006). Administration of multivalent antigen induces cross-linking of Fc ϵ RI that are pre-occupied with IgEs specific to antigen, initiating signaling events resulting allergic reaction. The activated mast cells release intracellular granules that store preformed inflammatory mediators such as histamine, TNF- α and proteases by the degranulation process. Also, they synthesize and secrete inflammatory lipids and cytokines TNF- α and IL-4. A Src-family tyrosine kinase (SFK), Lyn, is associated with Fc ϵ RI (Kihara and Siraganian, 1994) and is activated in response to cross-linking of Fc ϵ RI caused by antigen treatment. Lyn phosphorylates tyrosine residues on immunoreceptor tyrosine-based activation motif (ITAM) in beta and gamma chain subunits of Fc ϵ RI complex. Phosphorylated ITAM of the gamma chain recruits Syk. This binding increases enzymatic activity of Syk. It phosphorylates tyrosine residues on the adaptor proteins linker of activated T cell (LAT) and Grb2-associated binder 2 (Gab2) to provide docking sites for signaling molecules to ensure the phosphorylation and activation of phospholipase C γ and the mobilization of calcium to generate critical signals for degranulation (Siraganian et al., 2010). Also activation of phosphatidylinositol 3-kinase and mitogen-activated protein (MAP) kinases promotes transcription factors for the production of cytokines and synthesis of eicosanoids, inflammatory lipids.

In this study we describe the anti-allergic effect of adenine and its action mechanism in mast cells. We show that adenine suppresses IgE-mediated passive cutaneous anaphylaxis (PCA) in mice and allergic reactions in mast cells such as Fc ϵ RI-mediated degranulation and secretion of an inflammatory lipid and cytokines by inhibiting Fc ϵ RI-mediated signaling events.

2. Materials and methods

2.1. Materials

The following materials were purchased from the indicated commercial sources: adenine, dinitrophenol (DNP)-specific monoclonal IgE, DNP-HSA, Evans blue dye, ADP, ATP, cytosine and thapsigargin from Sigma-Aldrich; PP2 from Calbiochem; fetal bovine serum from Gibco/Life Technology; Eagle's minimal essential medium and trypsin from Lonza; recombinant IL-3 from Peprotech; phospho-tyrosine mouse mAb (P-Tyr-100), anti-phospho-Syk (Tyr 525/526), anti-phospho-Src (Tyr 416), anti-Akt, anti-phospho-Akt (Ser473 and T308), anti-ERK and anti-phospho-ERK (Thr202/Tyr204), anti-phospho-p38 MAPK (Thr180/Tyr182) and anti-p38 MAPK from Cell Signaling Technology; anti-phospho-JNK (Thr183/Tyr185) and anti-JNK from Invitrogen; BD Calcium Assay Kit and ELISA sets for mouse TNF- α and IL-4 cytokine from BD Biosciences; TRIzol RNA extraction reagent from Invitrogen; Reverse Transcription Master Premix (5) from ELPIS Biotech; LTB $_4$ ELISA kit from Enzo Life sciences; ECL chemiluminescence kit from Millipore; RBL-2H3 cell line from American Type Culture Collection.

2.2. Induction of passive cutaneous anaphylaxis in mice

The BALB/c mice were purchased from DBL Korea and kept under specific pathogen-free conditions and maintained according to the guidelines of the Institutional Animal Care and Use Committee of Chungnam National University. PCA was performed as described with some modifications (Kim et al., 1999). Briefly, the mice were anaesthetized and injected intradermally with DNP-specific IgE (0.5 μ g each mouse) into the ear. After 24 h, DNP-HSA antigen (250 μ g) in 200 μ l PBS containing 1% Evans blue was injected

intravenously. PBS, adenine (2, 5 and 10 mg/kg) or ketotifen (10 mg/kg) was administered by intra-peritoneal injection 1 h before antigen treatment. The mice were sacrificed 1 h after antigen challenge, followed by the removal of ears for measurement of the amount of dye extravasated. The dye was extracted overnight from the ears in 500 μ l of formamide at 63 °C. The absorbance was measured in a microplate reader at 620 nm.

2.3. Preparation and culture of bone marrow-derived mast cells and RBL-2H3 cells

For the preparation of bone marrow-derived mast cells (BMMCs), femurs of 6 weeks old BALB/c mouse were taken and bone marrow was flushed with PBS and collected. The cells were suspended and cultured in RPMI 1640 medium containing 10% heat inactivated FBS, 1% penicillin and streptomycin, 2 mM glutamine, 50 μ M 2-mercaptoethanol, 25 mM HEPES [pH 7.4] supplemented with 10 ng/ml IL-3 in 5% CO $_2$ incubator at 37 °C. The non-adherent cells were reseeded to the fresh medium twice a week. By 4–5 weeks in culture, the cells were used for the further experiments after verifying that cells have granules stained with toluidine blue. RBL-2H3 cells were cultured in complete media (EMEM supplemented with 15% (v/v) FBS, 100 U/ml of penicillin and 100 μ g/ml of streptomycin) at 37 °C in 5% CO $_2$ incubator.

2.4. Sensitization and stimulation of BMMCs and RBL-2H3 mast cells

RBL-2H3 cells and BMMCs were sensitized with 100 ng/ml DNP-specific IgEs overnight in a complete growth medium. The cells were stimulated with 100 ng/ml DNP-HSA antigen after washing and preincubation for 30 min with Tyrode's buffer (10 mM HEPES [pH 7.4], 125 mM NaCl, 5 mM KCl, 1 mM MgCl $_2$, 2 mM CaCl $_2$, and 5.6 mM glucose) to measure degranulation, calcium ion mobilization, and LTB $_4$ secretion and to do immunoblots of signaling molecules. To measure cytokine mRNAs, cytokine secretion and cell viabilities, a complete growth medium was used to incubate the cells.

2.5. Measurement of degranulation in BMMCs and RBL-2H3 mast cells

The activity of a granule marker enzyme, β -hexosaminidase, in the medium and inside the cells after antigen stimulation was determined by a colorimetric assay (Ozawa et al., 1993) with a few modifications. Briefly, cells were transferred to 24-well cluster plates (1 $\times$ 10 ^{5} cells per 0.4 ml per well) and sensitized with IgE overnight. The cultures were washed twice and replaced with Tyrode's buffer (0.2 ml per well) for RBL-2H3 cells and BMMCs. The cultures were incubated for 30 min with adenine, adenosine, ADP, ATP, or cytosine before stimulation with antigen for 20 min. For thapsigargin-induced degranulation, RBL-2H3 cells prepared like above were stimulated with thapsigargin (5 μ M) for 20 min instead of antigen treatment. Production of *p*-nitrophenol from 5 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide was measured by a colorimetric assay. The values were expressed as percentages of β -hexosaminidase released into the medium.

2.6. Measurement of cytokine mRNA levels

RBL-2H3 cells were transferred to 6-well cluster plates (2 $\times$ 10 ^{5} cells per 2 ml per well), sensitized and stimulated as described in Section 2.4. After preincubation with adenine, cells were stimulated with antigen for 0.5 h or 2 h. The cells were washed with cold PBS. RNAs were extracted with TRIzol solution according to the manufacturer's protocol and 1 μ g of total RNA was used to synthesize

cDNA using Reverse Transcriptase Master Premix (5×) based on the manufacturer's protocol. The synthesized cDNA were used to determine mRNA levels by PCR using the following primers: rat TNF- α , CATCTTCTCAAACTCGAGTG, AGCAATGACTCCAAAGTAGAC; rat IL-4, CTGCTTCTCATATGTACCGGG, TTGAGTGTGTGAGCGTGG; rat β -actin, GATGAGATTGGCATGGCTTT, CACCTTCACCGTCCAGTTT. The PCR was performed at 94 °C for 40 s, at 60 °C for 40 s, and at 72 °C for 50 s for 30 cycles.

2.7. ELISA of TNF- α and IL-4

BMMCs were transferred to 12-well cluster plates (2×10^6 cells per ml per well), sensitized and stimulated as described in Section 2.4. The cells were stimulated with antigen for 6 h after pre-incubation with adenine for 30 min. Concentrations of TNF- α and IL-4 in the supernatants were measured using the ELISA kit according to the manufacturer's protocol.

2.8. Cell viability assay

Cell viability was measured using MTT assay. RBL-2H3 cells (1×10^5 cells per 100 μ l per well) were seeded in 96 well cluster plate. The cells were incubated in presence of different doses of adenine in Tyrode's buffer or complete culture medium for indicated times in figures and incubated with MTT solution (0.5 μ g/ml) for 2 h. After washing the cells, the formazan formed was dissolved in DMSO and measured at 575 nm using the microplate reader.

2.9. Measurement of LTB₄ release by ELISA

RBL-2H3 cells were transferred to 24-well cluster plates (1×10^5 cells per 0.4 ml per well), sensitized and stimulated for 20 min described in Section 2.4. The released LTB₄ into the medium after antigen stimulation for 30 min was measured by ELISA assay according to the protocol supplied by Enzo Life Sciences.

2.10. Immunoblotting analysis

RBL-2H3 cells (2×10^5 cells per 2 ml per well) were transferred to 6-well cluster plate, sensitized and stimulated as described in Section 2.4. Then cells were washed twice with ice-cold PBS, and then lysed in ice-cold lysis buffer for 30 min on ice (Park et al., 2009). Lysates were micro-centrifuged at 12,000 rpm at 4 °C for 20 min and were mixed with 4× sample buffer, boiled for 5 min, subjected to SDS-PAGE. Displayed proteins in the gel were transferred to the PVDF membrane. The membrane was blocked in 5% skim milk in Tris-buffered saline–Tween 20 (TBS–T) for 1 h and then incubated overnight with primary antibodies at 4 °C. After washing the membrane with TBS–T, immune-reactive proteins were detected with horseradish peroxidase-conjugated secondary antibody using chemiluminescence kit.

2.11. Calcium measurement

RBL-2H3 cells were transferred to 96-well cluster plates (5×10^4 cells per 100 μ l per well), sensitized and stimulated as described in Section 2.4. The calcium mobilization assay was performed according to the manufacturer's protocol. Briefly, the sensitized cells were loaded with calcium indicator dye and incubated for 1 h at 37 °C. Then cells were washed with Tyrode's buffer and treated with adenine in 100 μ l of Tyrode's buffer for 30 min. The calcium ion mobilization was measured at excitation 485 nm and emission 525 nm using the Twinkle Microplate Fluorometer (Berthold Technologies) after antigen stimulation. The data were recorded every 30 s in continuous mode and was calculated as ratio of fluorescent

reading of the antigen treated cells (F) and non-treated cells (F₀).

2.12. Statistical analysis

Data are presented as means $\pm$ SEM. The EC₅₀ and IC₅₀ calculations and statistical analysis were performed by using GraphPad Prism software. One way ANOVA followed by Dunnett's test was used to calculate the significant differences between treatment groups from in vivo experiments. In case of in vitro experiments, unpaired Student's *t*-test was performed. $P \leq 0.05$ was considered to indicate significance.

3. Results

3.1. Adenine suppresses IgE-mediated passive cutaneous anaphylaxis in mice

PCA in mice is a classical model mimicking type I allergic response in which mast cells are important (Kabu et al., 2006) although other factors besides mast cells can potentially modulate PCA. We examined the anti-allergic activity of adenine in IgE-mediated PCA in the mouse ear model (Kim et al., 1999). Intra-peritoneal administration of adenine (2, 5 and 10 mg/kg) 1 h before antigen injection suppressed the PCA reaction in a dose-dependent manner (Fig. 1). The median effective concentration (EC₅₀) was approximately 4.21 ± 0.117 mg/kg. The concentration that gave nearly complete suppression was 10 mg/kg. Adenine suppressed PCA more potently than ketotifen, an anti-allergy medicine, at the same dose of 10 mg/kg.

3.2. Adenine prevents Fc ϵ RI-mediated degranulation in mast cells

Mast cells degranulate intracellular granulates to the medium in response to antigen stimulation, releasing allergic mediators stored in them such as histamine, TNF- α and proteases. We investigated the effects of adenine on Fc ϵ RI-mediated degranulation of mast cells. It suppressed the degranulation in a dose-dependent manner in both RBL-2H3 cells (Fig. 2A) and BMMCs (Fig. 2B). The IC₅₀ values of adenine were 1.58 ± 0.094 mM for RBL-2H3 cells and 1.65 ± 0.093 mM for BMMCs. Nearly complete inhibition was achieved with 5 mM adenine. Cytosine did not give any effect on the degranulation, but adenosine and ADP significantly stimulated the degranulation at pharmaceutical concentrations (Fig. 2A). ATP stimulated the degranulation up to 2 mM but suppressed it at 5 mM by 40% (Fig. 2A). Pretreatment for 0 min or 30 min with adenine before antigen stimulation inhibited the mast cell degranulation with the same potency (Fig. 2C). Removal of adenine after 30 min preincubation completely abolished its inhibitory effect on the degranulation (Fig. 2C).

3.3. Adenine blocks thapsigargin-induced degranulation of mast cells

Besides Fc ϵ RI-mediated degranulation, mast cells degranulate in response to reagents that stimulate intracellular signaling molecules. Thapsigargin does not act on Fc ϵ RI but induced degranulation by stimulating signaling events such as intracellular Ca²⁺ mobilization, activation of phosphatidylinositol-3 kinase (Huber et al., 2000) and activation of Src tyrosine kinase activity (Chao et al., 1997) which are critical for Fc ϵ RI-mediated degranulation. Thapsigargin provoked degranulation and adenine prevented thapsigargin-induced degranulation in a dose-dependent manner, maximally at 5 mM concentration (Fig. 3).

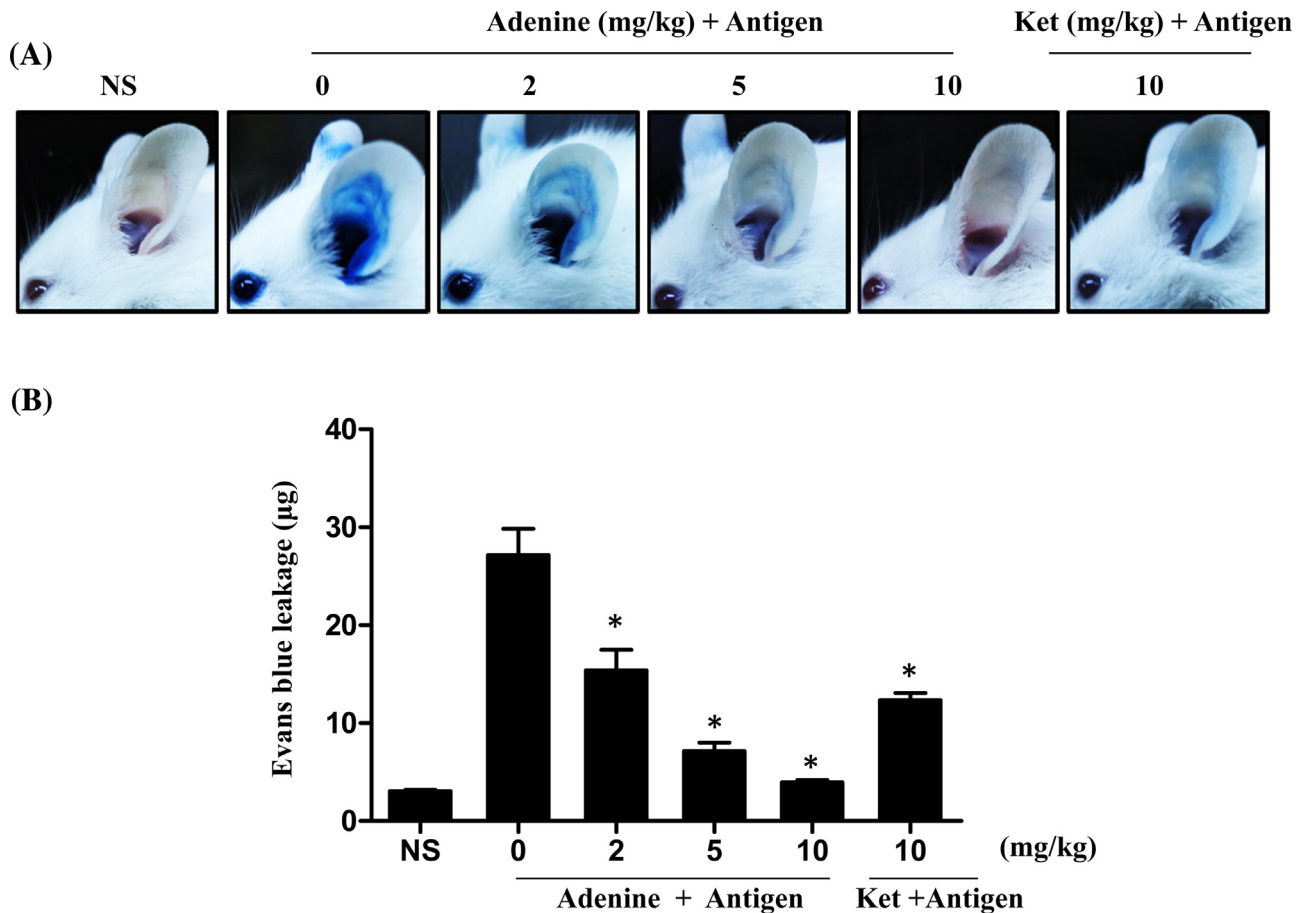


Fig. 1. Adenine suppresses IgE-mediated passive cutaneous anaphylaxis (PCA) in mice. (A) Each picture is a representative of three independent PCA experiments. (B) The means $\pm$ SEMs of values from three independent PCA experiments, each with 10 mice, are shown. Significant difference against a control group is indicated as * ($P < 0.05$). NS is no stimulation. Ket is ketotifen fumarate (10 mg/kg).

3.4. Adenine inhibits expression and secretion of cytokine genes in RBL-2H3 cells and BMMCs

The activated mast cells secrete various cytokines such as tumor necrosis factor (TNF)- α and IL-4 which are involved in allergic inflammation. Stimulation of mast cells with antigen for 6 h increased mRNA levels for both cytokines in RBL-2H3 cells and this increase was blocked by adenine in a dose-dependent manner, maximally at 5 mM concentration (Fig. 4A). Additionally, adenine inhibited secretion of these cytokines in BMMCs in a dose dependent manner (Fig. 4B). We examined the adenine effect on the cell viabilities in conditions used in these experiments. It was until 6 h that adenine did not diminish viabilities of RBL-2H3 cells up to 5 mM concentration when the cells were incubated in Tyrode's buffer or in complete culture media (Fig. 5A). After 6 h, adenine was cytotoxic even at 1 mM concentration (Fig. 5B), consisting with previous reports from lymphoblasts (Kishi et al., 1985; Snyder et al., 1978).

3.5. Adenine blocks LTB₄ production in antigen-stimulated mast cells

The activated mast cells synthesize and release inflammatory eicosanoids such as prostaglandins and leukotrienes that are mediating allergic responses (Boyce, 2007). Accordingly, we examined whether adenine inhibits the production of an eicosanoid LTB₄, which is one of the most potent chemoattractant, acting primarily on neutrophils, eosinophils, T cells, and mast cells. Adenine

suppressed secretion of LTB₄ in a dose-dependent manner in antigen-stimulated RBL-2H3 cells. The IC₅₀ value was approximately 0.91 ± 0.27 mM (Fig. 6). Nearly complete suppression of LTB₄ secretion was achieved with 1 mM adenine.

3.6. Adenine inhibits Fc ϵ RI-mediated intracellular signaling

Early Fc ϵ RI-mediated signaling events are initiated and relayed by tyrosine phosphorylation of proteins in mast cells (Siraganian et al., 2010). We investigated the possibility that adenine modulates Fc ϵ RI-mediated tyrosine phosphorylation of cellular proteins in RBL-2H3 cells. Western blot analysis with the phosphotyrosine specific antibody showed that antigen stimulation resulted in tyrosine phosphorylations of many proteins with a wide range of molecular weights (Fig. 7A). At the adenine concentration of 5 mM enough for complete inhibition to antigen-induced degranulation (Fig. 2), it attenuated the tyrosine phosphorylation of most proteins (Fig. 7A). The well-known Src inhibitor PP2 blocked the tyrosine phosphorylation more potently (Fig. 7A). Next, we examined the effect of adenine on tyrosine phosphorylation of individual proteins important to antigen-induced activation of mast cells. Adenine did not affect the phosphorylation of Lyn but substantially suppressed Syk phosphorylation critical for Syk activation in a dose-dependent manner (Zhang et al., 2000) (Fig. 7B). Subsequently, tyrosine phosphorylations of its target molecules LAT, PLC γ 2 and Gab2 were blocked in a dose-dependent manner (Fig. 7B). Also, in primary mast cells BMMC the phosphorylation of Syk and Gab2 was decreased (Fig. 7B).

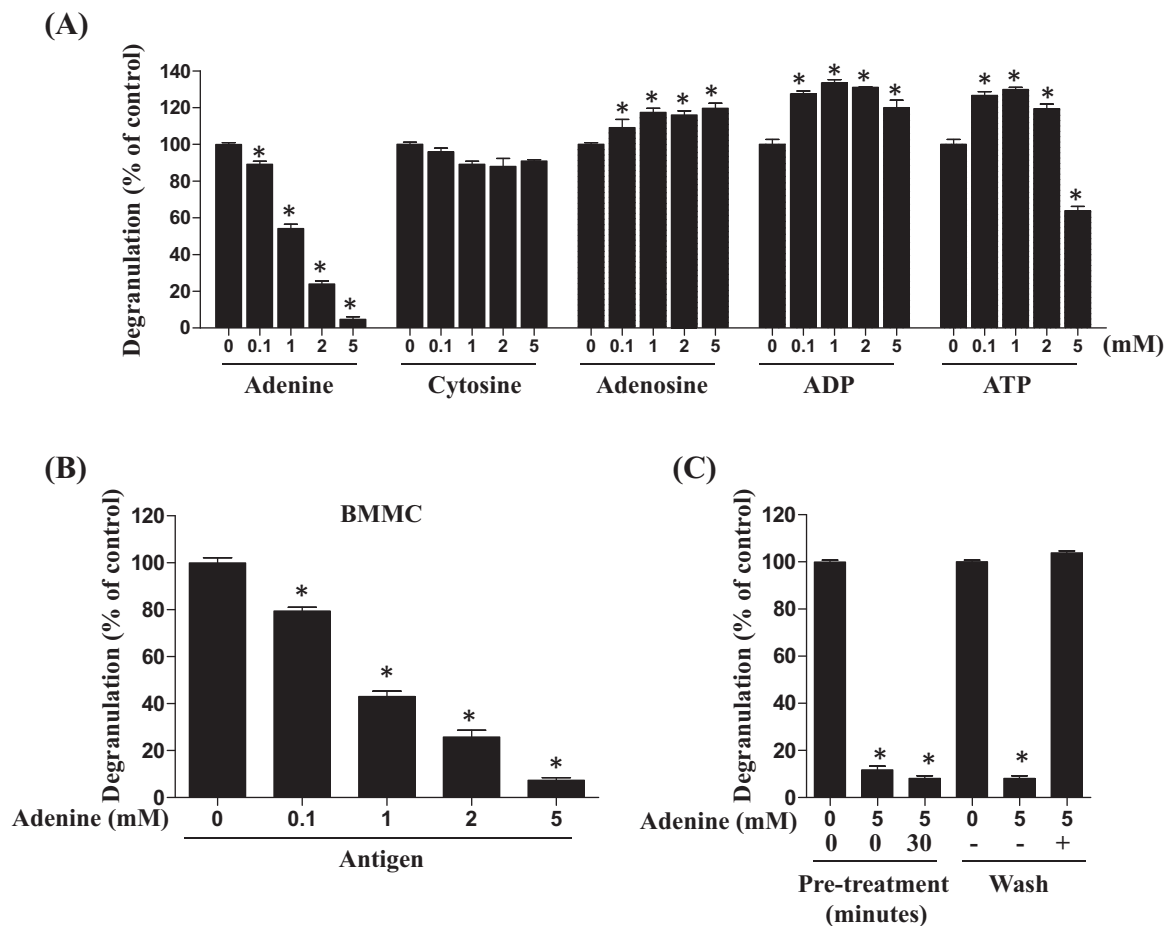


Fig. 2. Effects of adenine, ADP, ATP, and cytosine on FcεRI-mediated degranulation in mast cells. (A) Release of β-hexosaminidase was determined in antigen-stimulated RBL-2H3 cells after pre-treating indicated doses of adenine, cytosine, adenosine, ADP and ATP for 30 min in Tyrode's buffer. (B) Adenine inhibits antigen induced degranulation in BMMC. (C) Left half: concurrent treatment of adenine and antigen and preincubation of adenine for 30 min before antigen stimulation show the same potency in the antigen-induced degranulation in RBL-2H3 cells. Right half: the inhibitory effect of adenine on antigen-induced degranulation disappeared after washing out the adenine after 30 min of pre-treatment. The data are means ± SEM from three independent experiments. Significant difference against a control group is indicated as * ($P < 0.05$).

Next, we examined the impact of adenine on phosphatidylinositol 3-kinase and the MAP kinases which regulate the production of cytokines in antigen-stimulated RBL-2H3 cells. The phosphorylation of Akt, a surrogate for the activation of phosphatidylinositol 3-kinase, and MAP kinases (p38, ERK1/2, JNK) were also substantially inhibited by adenine in a dose-dependent manner (Fig. 8). Adenine suppressed the phosphorylation of Akt, ERK and JNK almost completely suppressed at

1 mM. However, it inhibited p38 phosphorylation minimally at 5 mM.

3.7. Adenine inhibits FcεRI-mediated Ca^{2+} mobilization in antigen-stimulated mast cells

Activation of PLCγ in FcεRI-mediated signaling elicits calcium mobilization which is critical for the degranulation in mast cells (Rivera and Gilfillan, 2006). Because adenine inhibited PLCγ activation (Fig. 7B), we investigated whether it inhibits the calcium mobilization in RBL-2H3 cells. Adenine attenuated the increase of cytosolic calcium concentration in a dose-dependent manner with the maximal inhibition at 1 mM (Fig. 9).

4. Discussion

We found that adenine suppresses IgE-mediated PCA in mice and activation of mast cells by inhibiting FcεRI-mediated signaling events leading to degranulation and secretion of inflammatory cytokines TNF-α and IL-4 and an inflammatory lipid LTB₄.

The PCA animal model used here is a well-established model for mast cell-mediated allergic reactions in vivo (Kabu et al., 2006). Intraperitoneal administration of adenine effectively suppressed the local allergic reaction in this model in a dose dependent manner (Fig. 1). The anti-PCA potency of adenine was better than ketotifen, a well-known mast cell stabilizer in the market; the EC₅₀ for adenine

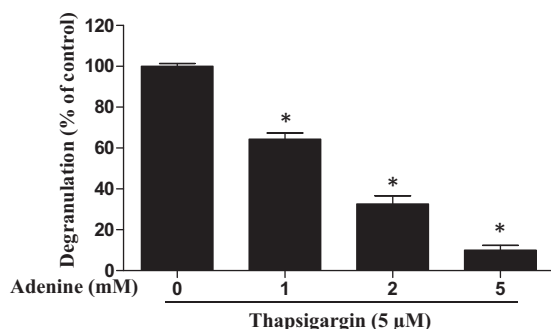


Fig. 3. Adenine suppresses thapsigargin induced mast cell degranulation. Release of β-hexosaminidase was determined in thapsigargin-stimulated RBL-2H3 cells after treating indicated doses of adenine for 30 min in Tyrode's buffer. The data are means ± SEM from three independent experiments. Significant difference against a control group is indicated as * ($P < 0.05$).

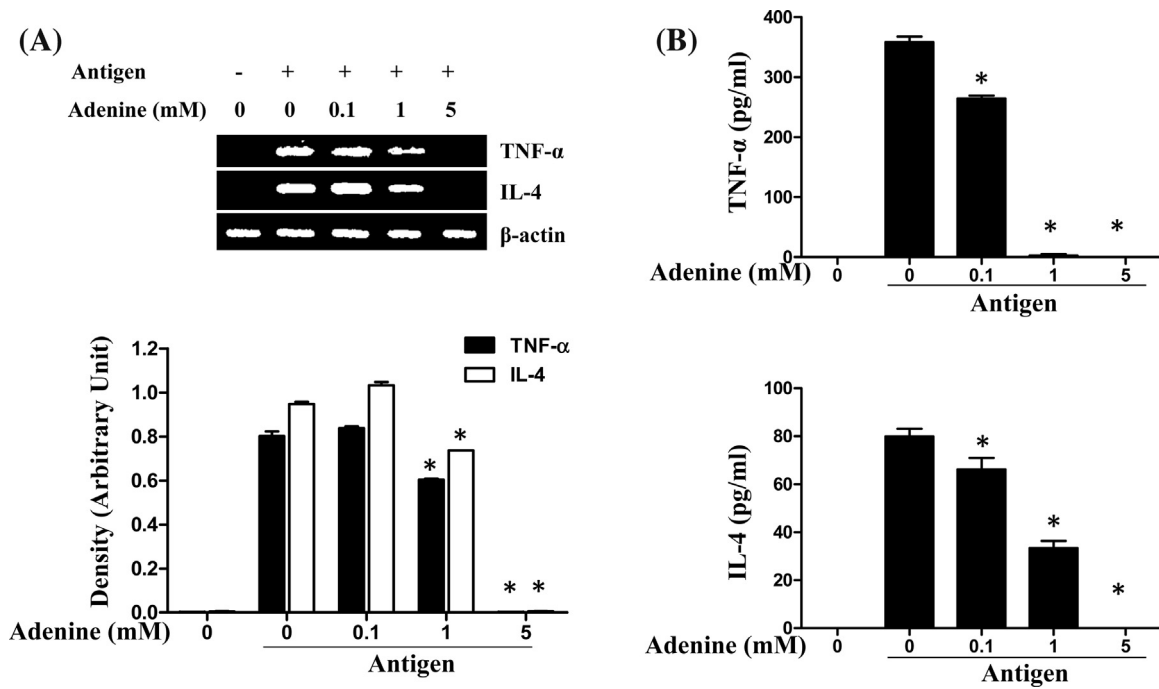


Fig. 4. Adenine attenuates the expression and secretion of TNF- α and IL-4 from mast cells. (A) IgE-sensitized RBL-2H3 cells were stimulated with antigen for RT-PCR. Cells were stimulated with 100 ng/ml of DNP-HSA for 0.5 h for TNF- α or 2 h for IL-4. Densitometric values from the PCR results are shown. (B) Secretion of TNF- α and IL-4 into the culture medium was determined 6 h after antigen stimulation from BMMCs. Significant difference against a control group is indicated as * ($P < 0.05$).

was 4.21 ± 0.117 mg/kg in this experiment and the EC_{50} of ketotifen is 10 mg/kg (Rossi et al., 2008). The adenine dose was in the range of 2–10 mg/kg (15–74 μ mol/kg) and was less than that required for inhibition of mast cell activation in vitro, which was over 100 μ M (Figs. 2 and 6). Adenine diffuses fast to the body and most of adenine is converted to adenine nucleotides, ADP and ATP through adenine salvage pathway (Bartlett, 1977; Roth et al., 1975). Therefore, we investigated the possibility that they inhibit Fc ϵ RI-mediated mast cell activation. Although adenine completely inhibited the activation at 5 mM concentration, ADP did not block but significantly stimulate the activation in a dose dependent manner in the same concentration range with adenine (Fig. 2A). ATP stimulated the degranulation in the concentration range from 0.1 mM to 2 mM as reported (Gao et al., 2013; Schulman et al., 1999) but suppressed the activation at 5 mM by 40% (Fig. 2A). Also, concurrent administration of adenine and antigen to diminish the conversion of adenine to its metabolites prevented the activation as much as antigen stimulation after 30 min preincubation of adenine (Fig. 2C). These

results suggest that adenine itself block Fc ϵ RI-mediated mast cell activation. However, we cannot completely exclude the possibility that adenine or its metabolites blocked PCA in mice by inhibiting not mast cells but other immune cells expressing IgE receptors because other factors besides mast cells can potentially modulate PCA although mast cells are important to a classical model mimicking type I allergic response (Kabu et al., 2006).

Adenine inhibited Fc ϵ RI-mediated activation of mast cells at pharmacological concentrations. This raised the question that adenine may interfere with the binding of IgE to the antigen or Fc ϵ RI. To resolve the notion, we examined whether adenine can modulated thapsigargin-independent degranulation which is not dependent on Fc ϵ RI. Thapsigargin mobilizes cytoplasmic calcium from ER and activates PI3K (Huber et al., 2000) and tyrosine kinase Src (Chao et al., 1997) which are critical for the mast cell activation. Adenine blocked degranulation induced by thapsigargin in a dose dependent manner (Fig. 3). These data reveal that the inhibition of Fc ϵ RI-mediated degranulation by adenine is specific to the

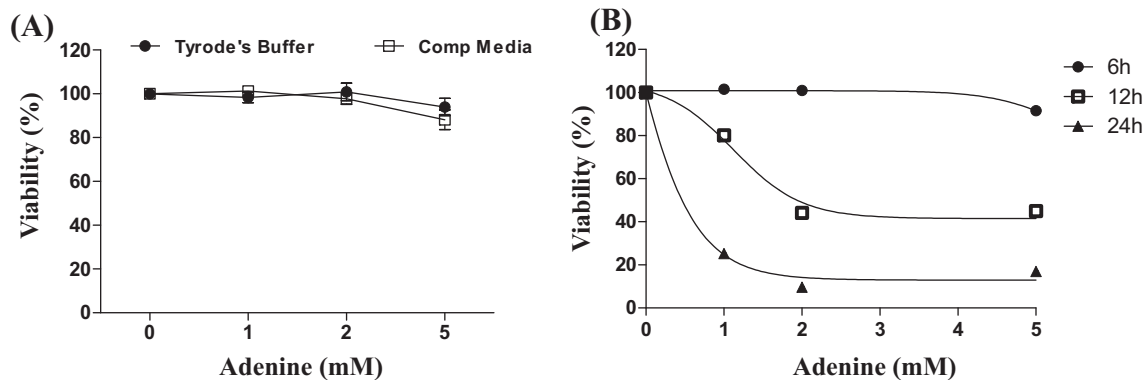


Fig. 5. Adenine is cytotoxic to mast cells after 6 h incubation. (A) RBL-2H3 cells were treated with indicated doses of adenine for 6 h in Tyrode's buffer or complete culture media to measure cell viability using MTT assay. (B) The indicated doses of adenine were treated for 6, 12 and 24 h in complete culture media to measure cell viability by using MTT assay. The data are presented as percentage of non-treated control group. Data are means $\pm$ SEM of three independent experiments.

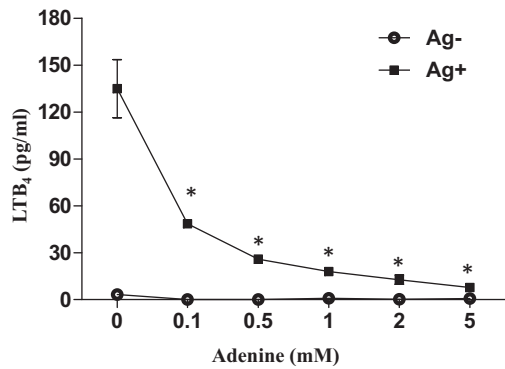


Fig. 6. Adenine inhibits LTB₄ secretion. The sensitized RBL-2H3 cells were stimulated with antigen for 30 min to measure released LTB₄ into the medium. The data are means ± SEM from three independent experiments. Significant difference against a control group is indicated as * ($P < 0.05$).

signaling events leading to mast cell activation. Also, pyrimidine base cytosine and adenine metabolites such as adenosine and ADP at pharmacological concentrations did not inhibit but enhanced the degranulation whereas ATP showed binary effects (Fig. 2A).

Antigen stimulation elicit phosphorylation of tyrosine residues in proteins which is essential for FcεRI-mediated early signaling events. Attenuation of antigen-induced tyrosine phosphorylation by adenine (Fig. 7A) suggests that adenine may inhibit tyrosine kinases. In support with this perception, adenine attenuated the tyrosine kinase Syk and subsequently tyrosine phosphorylation of PLCγ, Gab2 and LAT (Fig. 7B). However, adenine did not inhibit tyrosine phosphorylation of Lyn, an upstream kinase of Syk (Fig. 7B). These results are consistent with the observation that a selective inhibitor to SFKs, PP2, does not inhibit phosphorylation of SFKs, but inhibit phosphorylation of a target molecule of SFK (Wu et al., 2005). These results suggest that adenine attenuates antigen-induced tyrosine phosphorylation required for degranulation. How adenine inhibits antigen-induced tyrosine phosphorylation and which molecules are targets of adenine remain to be discovered.

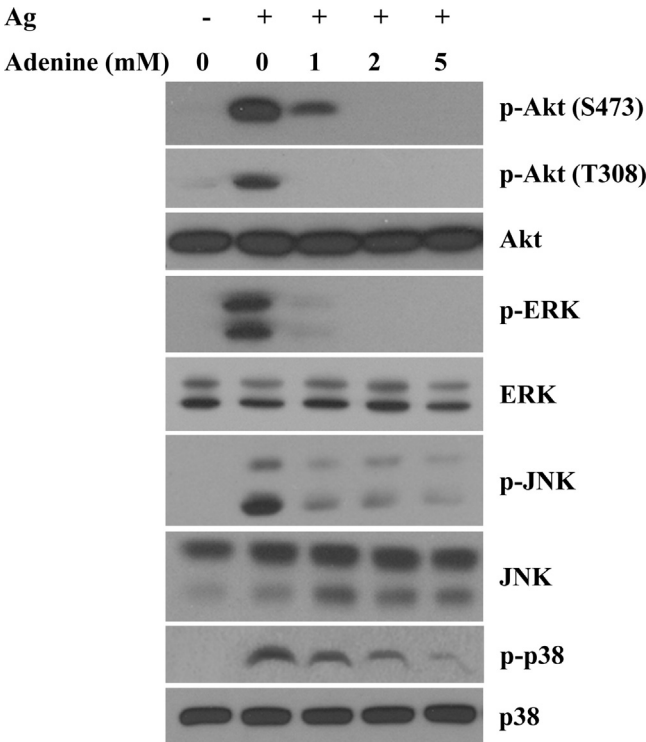


Fig. 8. Adenine suppresses phosphorylations of Akt and MAP kinases. The sensitized RBL-2H3 cells were stimulated with antigen for 20 min after pre-treating adenine for 30 min in Tyrode's buffer. Cell lysates were used for Western blot to detect the indicated proteins. Data shown are representatives of three independent experiments.

Adenine showed anti-allergic activity in this study and inhibits immunological functions of lymphocytes at pharmacological concentrations (Kishi et al., 1985). Its plasma concentration is 70 nM in healthy subjects and 1450 nM in patients with chronic renal failure (Slominska et al., 2002). Its local concentration in

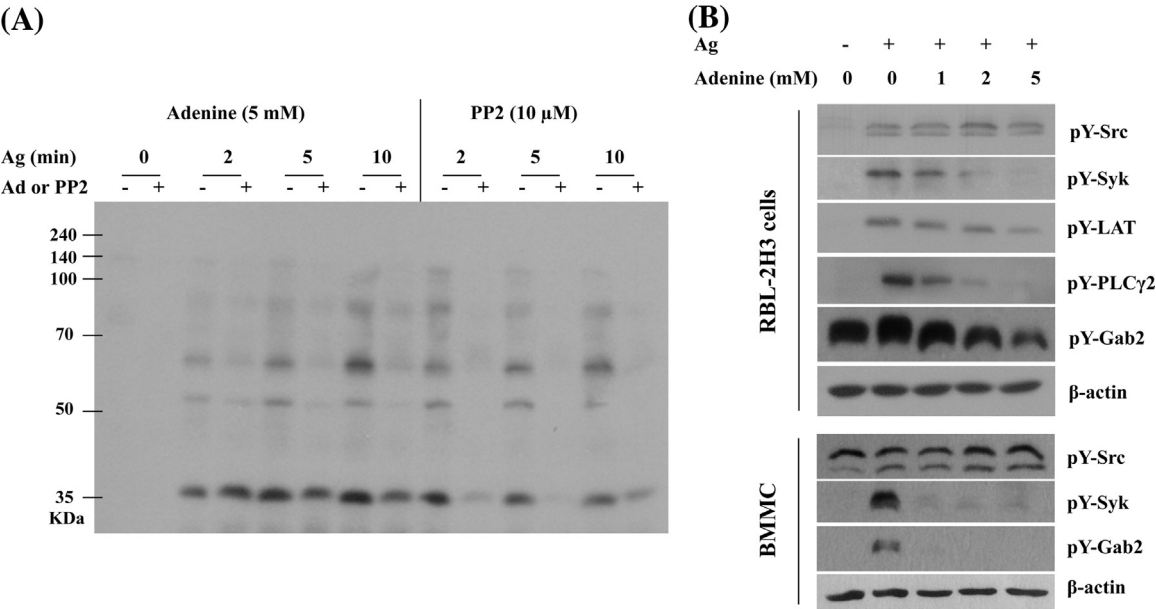


Fig. 7. Adenine attenuates antigen-induced tyrosine phosphorylation in mast cells. (A) The sensitized RBL-2H3 cells were pre-incubated with or without adenine (5 mM) or PP2 (10 μM) in Tyrode's buffer before stimulating with antigen for indicated time. The cell lysates were used for Western blotting to detect the tyrosine phosphorylation. (B) The sensitized mast cells (RBL-2H3 or BMMCs) were stimulated with antigen for 2 min after pre-treating various doses of adenine for 30 min in Tyrode's buffer. The mast cell lysates were used for Western blotting to detect phosphorylation of signaling molecules. Immunoblots shown are representatives of three independent experiments.

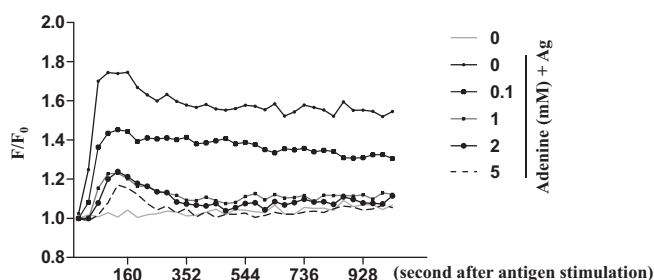


Fig. 9. Adenine inhibits antigen-induced increase of cytosolic Ca^{2+} concentration. The sensitized RBL-2H3 cells were stimulated by antigen to measure the cytosolic Ca^{2+} concentration. Data are presented as the ratio of fluorescence reading from antigen treated group (F) with the non-treated control group (F_0). A representative of three independent experiments is shown.

inflammatory sites may be much higher than the plasma concentration because proliferating lymphoblastoid cells make adenine substantially (Kamatani and Carson, 1981). Therefore it is possible that adenine in situ acts as an anti-mast cell agent in infection sites or inflaming tissues.

In conclusion, our data indicate that adenine suppresses PCA potently in mice and $\text{Fc}\epsilon\text{RI}$ -mediated signaling events in mast cell activation resulting in allergic responses in vitro such as degranulation and production of LTB_4 , $\text{TNF-}\alpha$ and IL-4 . The upregulation of adenine synthesis may provide another mechanism for suppressing mast cell activity at inflammatory sites.

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