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Differential regulation of IL-4 expression and degranulation by anti-allergic olopatadine in rat basophilic leukemia (RBL-2H3) cells

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Abstract

Olopatadine hydrochloride (olopatadine) is an anti-allergic drug that functions as a histamine H_1 antagonist and inhibits both mast cell degranulation and the release of arachidonic acid metabolites in various types of cells. In this study, we examined the ability of olopatadine to inhibit the expression of cytokine genes *in vitro* via high-affinity receptors for immunoglobulin E in mast cells, using a rat basophilic leukemia (RBL-2H3) cell line and an *in vivo* mouse model. Levels of gene expression in RBL-2H3 cells were determined by semi-quantitative RT–PCR, and serum interleukin-4 (IL-4) level in mice was quantified by ELISA. Olopatadine inhibited significantly the induction of IL-4 expression by mast cells both *in vivo* and *in vitro*. Olopatadine inhibited Ca^{2+} influx through receptor-operated channels (ROC) without affecting Ca^{2+} release from intracellular stores. Comparative analysis of olopatadine with other anti-allergic drugs and the ROC blocker SKF-96365 demonstrated that the potency of inhibition of Ca^{2+} influx correlated with the degree of suppression of degranulation and arachidonic acid release. Inhibition of Ca^{2+} influx decreased phosphorylation of p38 mitogen-activated protein kinase and c-Jun NH_2-terminal kinase, which participate in regulation of cytokine (e.g. IL-4) gene expression. However, the rank order of inhibition of Ca^{2+} influx did not correspond to reduction of IL-4 expression, suggesting that an unknown mechanism(s) of action, in addition to inhibition of Ca^{2+} influx, is involved in the expression of cytokines in mast cells.

Keywords: Olopatadine; RBL-2H3; Degranulation; IL-4; Ca^{2+} influx

1. Introduction

Olopatadine hydrochloride (11-[(*Z*)-3-(dimethylamino)-propylidene]-6,11-dihydrodibenz[*b*,*e*]-oxepin-2-acetic acid monohydrochloride) is a new anti-allergic drug that exhibits potent histamine H_1 receptor antagonism and additional anti-inflammatory activities [1]. Olopatadine has proven to be useful for treatment of allergic rhinitis, chronic urticaria, eczema dermatitis, purigo, cutaneous pruritus, psoriasis vulgaris, erythema exsudativum multiforme and allergic conjuctivitis in double-blind clinical trials [1]. It is available both orally (Allelock®) and as an ophthalmic solution (Patanol®). Olopatadine suppressed the late-phase reaction as well as early-phase reactions in experimental allergic animal models [2,3]. Suppression of the immediate response may be due to H_1 antagonism and inhibition of both degranulation and lipid mediator release from inflammatory cells. Olopatadine inhibits the release of histamine, tryptase, LTs and PGD_2 from human primary mast cells [4] and nasal mucosal mast cells [5]. The inhibition of lipid mediator release from inflammatory cells may contribute to suppression of the late-phase response by olopatadine in animal models [1], but the mechanism by which it does remains to be fully elucidated.

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Abbreviations: H_1, histamine receptor-1; RBL, rat basophilic leukemia; IgE, immunoglobulin E; FcεRI, high-affinity receptor for IgE; ELISA, enzyme-linked immunosorbent assay; LT, leukotriene; PG, prostaglandin; IL, interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; ROC, receptor-operated channel; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; DSCG, disodium chromoglycate; DNP, dinitrophenol; IBMX, isobutylmethylxanthine; fluo-3-AM, fluo-3-acetoxymethyl ester; FLIPR, fluorometric imaging plate reader; RT–PCR, reverse transcription–polymerase chain reaction; cAMP, adenosine 3′,5′-cyclic monophosphate; PDE, phosphodiesterase; TNP, trinitrophenyl; HAS, human serum albumin; BSA, bovine serum albumin; olopatadine, olopatadine hydrochloride.

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Mast cells play important roles in both immediate allergic reactions and late-phase reactions [6]. Mast cell activation is regulated by aggregation of FcεRI, leading to degranulation, and release of arachidonic acid metabolites such as LTs and PGD_2 in the early phase. Aggregation of FcεRI by antigens results in the recruitment of the tyrosine kinase Syk, leading activation of phospholipase C and the members of MAPK family [7,8]. Phospholipase C generates the second messenger inositol 1,4,5-trisphosphate, which induces the release of Ca^{2+} from intracellular stores [7,8]. The released Ca^{2+}, in combination with phosphatidylinositol 3-kinase, which is activated following aggregation of FcεRI, triggers Ca^{2+} influx through ROCs [9,10]. Elevation of intracellular Ca^{2+} level causes degranulation and arachidonic acid release from cellular membranes by Ca^{2+}-dependent phospholipase A_2 [7,11]. Furthermore, mast cells concomitantly synthesize and release a variety of cytokines, including IL-3, IL-4, IL-5, IL-13, GM-CSF and TNF-α during the late-phase reaction. These cytokines play important roles in the infiltration of inflammatory cells and induction of the late-phase reaction [6]. Members of MAPK family (ERKs, JNKs and p38 MAPK) are believed to mediate the expression of cytokines [12,13]. However, the mechanism regulating cytokine gene expression in mast cells is not fully understood.

In this study, we examined the effects of olopatadine on (i) the expression of several cytokine genes, as well as (ii) the degranulation and (iii) release of arachidonic acid via FcεRI signaling in RBL-2H3 cells, which are frequently used as mast cells. Since RBL-2H3 cells do not contain H_1 receptors, inhibition of mediator release in this cell line might involve mechanisms other than H_1 antagonism [14]. In addition, we investigated how this anti-allergic drug inhibits early- and late-phase reactions of mast cells.

2. Materials and methods

2.1. Materials

Olopatadine hydrochloride, DSCG, tranilast, amlexanox, loratadine and cetirizine hydrochloride were synthesized in our laboratories. Anti-TNP mouse IgE was produced in our laboratory with hybridoma (TIB142) purchased from American Type Culture Collection. The following materials were purchased from the indicated commercial sources: ketotifen fumarate (Sigma); astemizole (Sigma); SKF-96365 (Biomol Research Laboratories); fluo-3-AM (Molecular Probes); Pluonic F-127 (Sigma); DNP-specific-IgE (Sigma); DNP-HSA (Sigma); TNP-BSA (LSL); 4-methylumberlliferyl-*N*-acetyl-β-D-glucosaminide (Sigma); IBMX (Sigma); forskolin (Sigma); and PGE_2 (Sigma). All anti-allergic drugs were dissolved in dimethyl sulfoxide at a concentration of 0.1 mol/L and stored at −20° before use. The final concentration of dimethyl sulfoxide in the assay system described below was 0.1% (v/v).

2.2. Cell culture

RBL-2H3 cells were grown in Eagles' minimal essential medium (Nissui) containing 10% (v/v) heat-inactivated fetal bovine serum (JRH Biosciences), 2 mmol/L L-glutamate (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). Cultures were maintained under 5% CO_2 at 37° in tissue culture flasks. Cells were detached with trypsin–EDTA solution (Invtirogen). After the cells were washed, they were resuspended in medium and used for subsequent experiments.

2.3. Animals

BALB/c mice and BALB/c nude mice (6 weeks of age) were purchased from Charles River, Japan. WBB6F1-W/W^v mice and control mice (WBB6F1-+/+) were purchased from Japan SLC. The animals were housed under controlled temperature (19–25°) and humidity (30–70%) and given access to food and water *ad libitum*. All mice received humane care in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, formulated by the Japanese Pharmacological Society, and the study protocol was approved by the Bioethics Committee of Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd.

2.4. β-Hexosaminidase secretion assay

Degranulation was determined by measuring the release of a granule marker, β-hexosaminidase. RBL-2H3 cells were grown on 2% (w/v) BSA-coated 96-well plates (1×10^5 cells/well) and were sensitized overnight with 100 ng/mL DNP-specific IgE. The growth medium was replaced with modified-Tyrode's assay buffer (119 mmol/L NaCl, 4.74 mmol/L KCl, 2.54 mmol/L $CaCl_2$, 1.19 mmol/L $MgSO_4$, 1.19 mmol/L KH_2PO_4, 10 mmol/L HEPES, 5 mmol/L glucose, 0.1% (w/v) BSA, pH 7.3). The IgE-sensitized RBL-2H3 cells were preincubated for 15 min, and then exposed to the anti-allergic drugs for 15 min at the indicated concentration. After incubation, the cells were stimulated with 100 ng/mL DNP-HSA for 1 hr. At the end of the experiment, the supernatant was incubated with an equal volume of substrate solution (0.2 mol/L citrate, 1 mmol/L 4-methylumberlliferyl-*N*-acetyl-β-D-glucosaminide, pH 4.5). The enzyme reaction was terminated by the addition of 0.2 mol/L Tris–HCl buffer (pH 11). The fluorescence of the product (4-methylumbelliferone) was measured by spectrofluorometery on an automatic microtiter plate reader (ARVO SX 1420 Multilabel Counter, Wallac) at an excitation wavelength of 355 nm and emission wavelength of 450 nm. To determine the total amount of β-hexosaminidase release, the remaining cells

were lysed by treatment with assay buffer containing 1% (v/v) Triton X-100 prior to incubation with substrate using the same procedure as for determination of activity in the supernatant. Percentage β-hexosaminidase release was calculated by dividing the fluorescence of the supernatant by the sum of fluorescences of the supernatant and the cell lysate. The effects of various treatments on β-hexosaminidase release are reported as % of control.

2.5. Measurement of intracellular Ca^{2+}

The Ca^{2+} responses in RBL-2H3 cells were assessed using fluo-3-AM in conjunction with a FLIPR (Molecular Devices). RBL-2H3 cells were sensitized overnight with 100 ng/mL DNP-specific IgE on 2% (w/v) BSA-coated 96-well plates (1×10^5 cells/well). The growth medium was replaced with assay buffer. Fluo-3 loading was achieved by exposing the cells to 4 μmol/L fluo-3-AM in the presence of 1% (v/v) fetal bovine serum and 0.04% (w/v) Pluonic F-127 in the dark for 1 hr. The fluo-3-loaded RBL-2H3 cells were washed three times with assay buffer, and then treated with the anti-allergic drugs at the indicated concentration for 5 min. The cells were immediately placed on the FLIPR and stimulated with 100 ng/mL DNP-HSA at 1 min. The change in fluorescence of fluo-3 was monitored at an excitation wavelength of 488 nm and emission wavelength of 540 nm.

2.6. Arachidonic acid release assay

RBL-2H3 cells were sensitized overnight with 100 ng/mL DNP-specific IgE on 2% (w/v) BSA-coated 24-well plates (5×10^5 cells/well), and 0.25 μCi [^{3}H] arachidonic acid was added to the growth medium. Growth medium was replaced with assay buffer. The IgE-sensitized RBL-2H3 cells were treated with the anti-allergic drugs for 15 min and stimulated with 100 ng/mL DNP-HSA for 1 hr. At the end of the experiment, radioactivities in the supernatant and in the solubilized cells were measured using a liquid scintillation counter (TRI-CARB 2700TR, Packard). The percentage of arachidonic acid release was calculated by dividing the radioactivity in the supernatant by the sum of radioactivities in the supernatant and cell lysate. The effects of treatment on arachidonic acid release are reported as % of control.

2.7. Semi-quantification of cytokine mRNA by reverse transcription–polymerase chain reaction

RBL-2H3 cells were sensitized overnight with 100 ng/mL DNP-specific IgE on 2% (w/v) BSA-coated 6-well plates (2.5×10^6 cells/well). The IgE-sensitized RBL-2H3 cells were treated with the anti-allergic drugs for 15 min and stimulated with 100 ng/mL DNP-HSA. After incubation, the cells were washed twice with ice-cold PBS. Total RNA was isolated according to the manufacturer's instructions (RNeasy, Qiagen). First-strand cDNA synthesis was performed from 1 μg of total RNA in 20-μL volumes with oligo(dT) priming using the Superscript First-strand Synthesis System (Invitrogen). PCR was performed with 1 μL of the generated cDNA using 1 U of ExTaq polymerase (Takara). Based on previous reports [15,16], the following primer pairs (including PCR product sizes in parentheses) were synthesized by Invitrogen. IL-2, 5′-CTG TGT TGC ACT GAC GCT TGT C-3′ and 5′-CTG AGT CAT TGT TGA GAT GAT GC-3′ (444 bp); IL-3, 5′-GTA TGC TGC TCC CGC TCC TGA TG-3′ and 5′-CAT TCC ACG GTC ATA GGG CGA AAG-3′ (473 bp); IL-4, 5′-ACC TTG CTG TCA CCC TGT TC-3′ and 5′-TTG TGA GCG TGG ACT CAT TC-3′ (351 bp); IL-6, 5′-GAA ATG ATG GAT GCT TCC AAA CTG G-3′ and 5′-GGA TAT ATT TTC TGA CCA CAG TGA GG-3′ (414 bp); IL-13, 5′-GCT CTC GCT TGC CTT GGT GGT C-3′ and 5′-CAT CCG AGG CCT TTT GGT TAC AG-3′ (276 bp); TNF-α, 5′-CAA GGA GGA GAA GTT CCC AA-3′ and 5′-CGG ACT CCG TGA TGT CTA AG-3′ (501 bp); GM-CSF, 5′-GCA TGT AGA TGC CAT CAA AGA AGC-3′ and 5′-CAT TTC TGG ACC GGC TTC CAG C-3′ (342 bp); β-actin, 5′-TAA CCA ACT GGG ACG ATA TG-3′ and 5′-ATA CAG GGA CAG CAC AGC CT-3′ (202 bp). The denaturation, annealing, extension, and cycle conditions were as follows: IL-2: 94° for 60 s, 60° for 45 s, 72° for 45 s and 34 cycles; IL-3: 94° for 60 s, 60° for 45 s, 72° for 45 s and 24 cycles; IL-4: 94° for 60 s, 58° for 45 s, 72° for 45 s and 24 cycles; IL-6: 94° for 60 s, 63° for 45 s, 72° for 45 s and 34 cycles; IL-13: 94° for 60 s, 60° for 45 s, 72° for 45 s and 25 cycles; TNF-α: 94° for 60 s, 60° for 45 s, 72° for 45 s and 25 cycles; GM-CSF: 94° for 60 s, 63° for 45 s, 72° for 45 s and 34 cycles; β-actin: 94° for 60 s, 58° for 45 s, 72° for 45 s and 18 cycles. The PCR reaction was performed with a GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were electrophoresed in 2% (w/v) agarose gels and stained with SYBR Green I (Molecular Probes). The detection and densitometric analysis of bands were performed with a FluorImager SI System (Molecular Dynamics). The sizes of bands were confirmed with reference to molecular size markers (100 bp DNA Ladder Marker, Takara). The value of each cytokine mRNA was normalized to the amount of β-actin mRNA, which was utilized as a housekeeping gene for each experimental condition.

2.8. IL-4 production in vivo

Mice were passively sensitized by intravenous injection of 30 μg of anti-TNP mouse IgE antibody. Twenty-four hours after sensitization, mice were injected intravenously with 5 μg TNP-BSA. Olopatadine was orally administered 30 min before TNP-BSA injection. Serum from individual mice was obtained at 2 hr after stimulation, and IL-4 concentration was measured using an OptEIA ELISA kit (Pharmingen), according to the manufacturer's manual.

2.9. Western blot analysis

The IgE-sensitized RBL-2H3 cells (2.5×10^6 cells/well) were treated with the anti-allergic drugs for 15 min and stimulated with 100 ng/mL DNP-HSA. After incubation, the cells were washed twice with ice-cold PBS. The cells were lysed with ice-cold lysis buffer (150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% (v/v) Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, 10 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L Tris–HCl, pH 7.5) for 30 min on ice. Insoluble material was removed by centrifugation and the supernatant was boiled with Laemmli's buffer containing dithiotheritol for 3 min. Proteins were separated by 7.5% SDS–PAGE and blotted onto a PVDF membrane (Immobilon, Millipore). Proteins were detected using an immunoblotting technique with the following antibodies. Anti-phosphotyrosine monoclonal antibody PY-20 (Wako); anti-phosphotyrosine monoclonal antibody P-Tyr-102 (Cell Signaling Technology); anti-p38 MAP kinase antibody (Cell Signaling Technology), anti-phospho-p38 MAP kinase antibody (Cell Signaling Technology); anti-SAPK/JNK antibody (Cell Signaling Technology); and anti-phospho-SAPK/JNK antibody (Cell Signaling Technology). Horseradish peroxidase-linked whole antibody (Amersham Bioscience) was used as a secondary antibody. The antibodies were detected with Super Signal West Pico Chemiluminescent Substrate (Pierce) and analyzed using a Lumino Image Analyzer LAS-1000 Plus (Fujifilm).

2.10. Measurement of cAMP

The IgE-sensitized RBL-2H3 cells (5×10^5 cells/well) were preincubated for 15 min with 1 mmol/L of IBMX and exposed to the anti-allergic drugs in the presence of 1 mmol/L IBMX. Additionally, the cells were preincubated without IBMX and were exposed to drugs in the presence of 100 μmol/L of forskolin. The termination of reactions and cAMP measurements were carried out as described in the cAMP kit manual (CIS Bio International).

3. Results

3.1. Effects of olopatadine on degranulation, arachidonic acid release and Ca^{2+} entry in RBL-2H3 cells stimulated with antigen

Stimulation of β-hexosaminidase release and Ca^{2+} influx by the antigen DNP-HSA was confirmed to occur in IgE-sensitized RBL-2H3 cells in a concentration-dependent manner (Fig. 1). Antigen stimulation caused a sustained increase of Ca^{2+} that may be associated with influx of external Ca^{2+}. A concentration of 100 ng/mL of DNP-HSA was selected for optimal cell stimulation in subsequent experiments. Olopatadine concentration-dependently inhibited the release of β-hexosaminidase (Fig. 2A) and arachidonic acid (Fig. 2B) from antigen-stimulated RBL-2H3 cells. In agreement with a previous investigation using human mast cells [4], olopatadine inhibited release of the arachidonic acid metabolite LTB_4 in RBL-2H3 cells (data not shown). Since inhibition of degranulation by some anti-allergic drugs has been explained by the suppression of Ca^{2+} influx [14,17–19], the effect of olopatadine on the Ca^{2+} influx induced by antigen was examined in the present study using fluo-3-AM in conjunction with the FLIPR system. As shown in Fig. 2C, olopatadine inhibited Ca^{2+} influx.

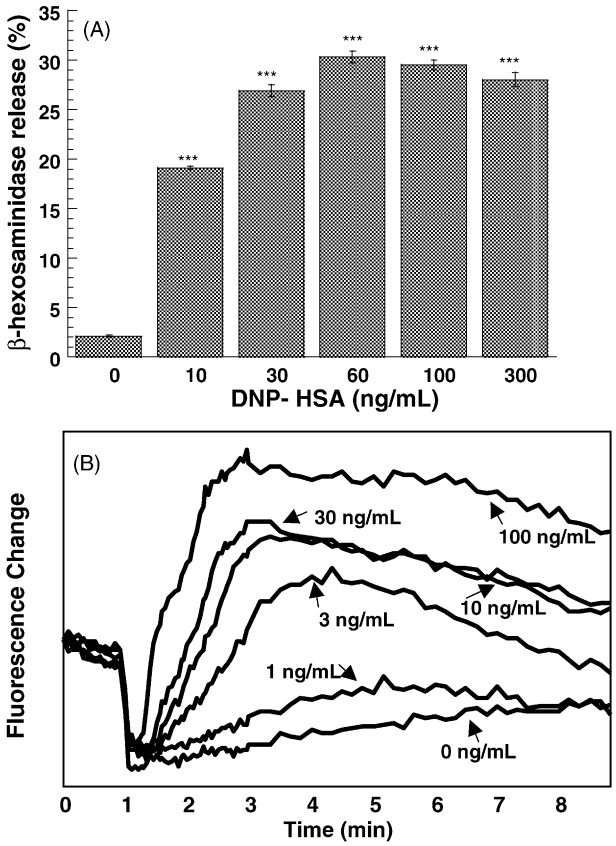

Fig. 1. Degranulation of β-hexosaminidase and Ca^{2+} response in antigen-stimulated RBL-2H3 cells. (A) RBL-2H3 cells were RBL-RB sensitized overnight with 100 ng/mL DNP-specific IgE, and stimulated with the indicated concentrations of DNP-HSA. The enzymatic activities of β-hexosaminidase in the supernatant and solubilized cells were measured with 4-methylumberlliferyl-*N*-acetyl-β-D-glucosaminide. The fluorescence of the product (4-methylumbelliferone) was measured. Values represent means ± SE of four determinations. $^{***}P < 0.001$ compared with the DNP-HSA-untreated group (Dunnett). (B) Ca^{2+} responses in RBL-2H3 cells were assessed using fluo-3-AM with a fluorometric imaging plate reader (FLIPR). The IgE-sensitized RBL-2H3 cells were loaded with fluo-3-AM for 1 hr. The fluo-3-loaded RBL-2H3 cells were placed on FLIPR and stimulated with the indicated concentrations of DNP-HSA at 1 min in the figure. The change in fluorescence of fluo-3 was monitored. Each value represents the mean of the fluorescence measured in quadruplicate microplate wells.

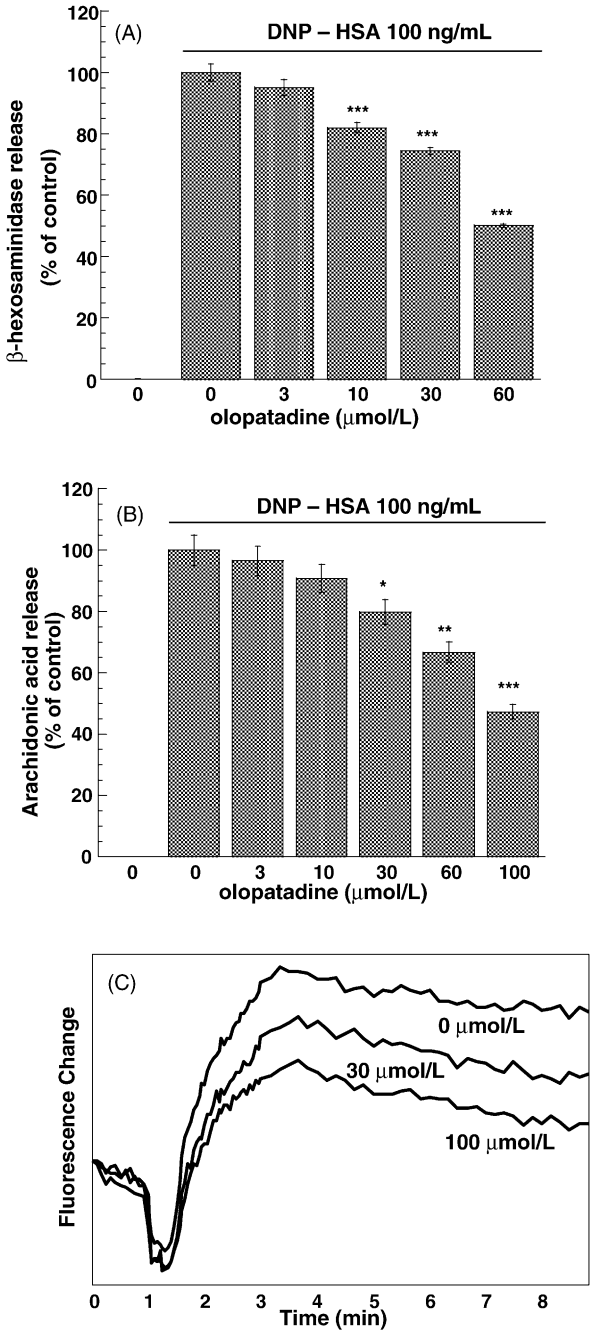

Fig. 2. Effects of olopatadine on the degranulation, arachidonic acid release and Ca^{2+} entry induced by FcεR cross-linking. The IgE-sensitized RBL-2H3 cells were treated with the indicated concentrations of olopatadine and then stimulated with 100 ng/mL DNP-HSA. (A) The enzymatic activities of released β-hexosaminidase were measured. Release of β-hexosaminidase is shown as % of control. Values represent means ± SE of four determinations. $^{***}P < 0.001$ compared with the control group (Dunnett). (B) Radioactivities of [^{3}H] arachidonic acid in the supernatant and solubilized cells were measured with a liquid scintillation counter. The results for arachidonic acid release are shown as % of control. Values represent means ± SE of three determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group (Dunnett). (C) Ca^{2+} responses were assessed using fluo-3-AM with FLIPR. The change in fluorescence of fluo-3 was monitored. Each value represents the mean of the fluorescence measured in quadruplicate microplate wells.

3.2. Effects of olopatadine on antigen-induced IL-4 mRNA expression and production

The basal level of IL-4 expression in IgE-sensitized RBL-2H3 cells was low, but the expression of IL-4 was markedly, albeit transiently, increased after antigen stimulation (Fig. 3A). The peak of IL-4 expression was observed at 1 hr. Pretreatment with olopatadine concentration-dependently suppressed the increase in IL-4 mRNA at peak time (i.e. at 1 hr; Fig. 3B and C). To examine whether olopatadine also inhibits the induction of IL-4 protein, an animal experiment was performed to assess IL-4 production by mast cells *in vivo*. Mice were passively sensitized by injection of anti-TNP IgE 24 hr before being challenged with an intravenous injection of TNP-BSA. IL-4 level peaked at 2 hr after antigen challenge. A similar increase in plasma IL-4 was detected in nude mice, although the increase in plasma IL-4 was not detectable in mast cell-deficient W/W^v mice (data not shown), suggesting that IL-4 was derived from mast cells. Oral administration of olopatadine (10 mg/kg) 30 min before antigen challenge significantly prevented the increase in plasma IL-4 caused by antigen–antibody reaction (Fig. 3D).

3.3. Effects of olopatadine on the induction of IL-2, IL-3, IL-6, IL-13, TNF-α and GM-CSF mRNA in RBL-2H3 cells

Expression of the cytokines IL-2, IL-3, IL-6, IL-13, TNF-α and GM-CSF was induced following antigen stimulation in IgE-sensitized RBL-2H3 cells. The time of maximal mRNA induction differed among the cytokines (see Fig. 4A). Up-regulation of the mRNA expression of IL-3, TNF-α and GM-CSF was much greater than that for IL-2, IL-6 and IL-13 (Fig. 4A). The effect of olopatadine on the induction of these cytokines at 1h after antigen stimulation was investigated: olopatadine significantly suppressed induction of IL-2 and GM-CSF expression and marginally inhibited IL-3 up-regulation in a concentration-dependent manner, but had no apparent effect on the induction of IL-6, IL-13 and TNF-α (Fig. 4B).

3.4. Effects of some anti-allergic drugs on degranulation, arachidonic acid release and IL-4 mRNA expression in RBL-2H3 cells

The effects of olopatadine and other anti-allergic drugs on β-hexosaminidase and arachidonic acid release, and IL-4 mRNA induction are recorded in Table 1. As reported by others, ketotifen [18], astemizole [14], loratadine [19], DSCG [17], tranilast [21] and amlexanox [22] inhibitedβ-hexosaminidase release concentration-dependently. However, cetirizine [20] did not inhibit release of β-hexosaminidase. The potency of each drug in inhibiting β-hexosaminidase release was similar to that of inhibiting arachdonic acid release, but the effects of the tested drugs on IL-4 mRNA expression differed (Table 1). For example,

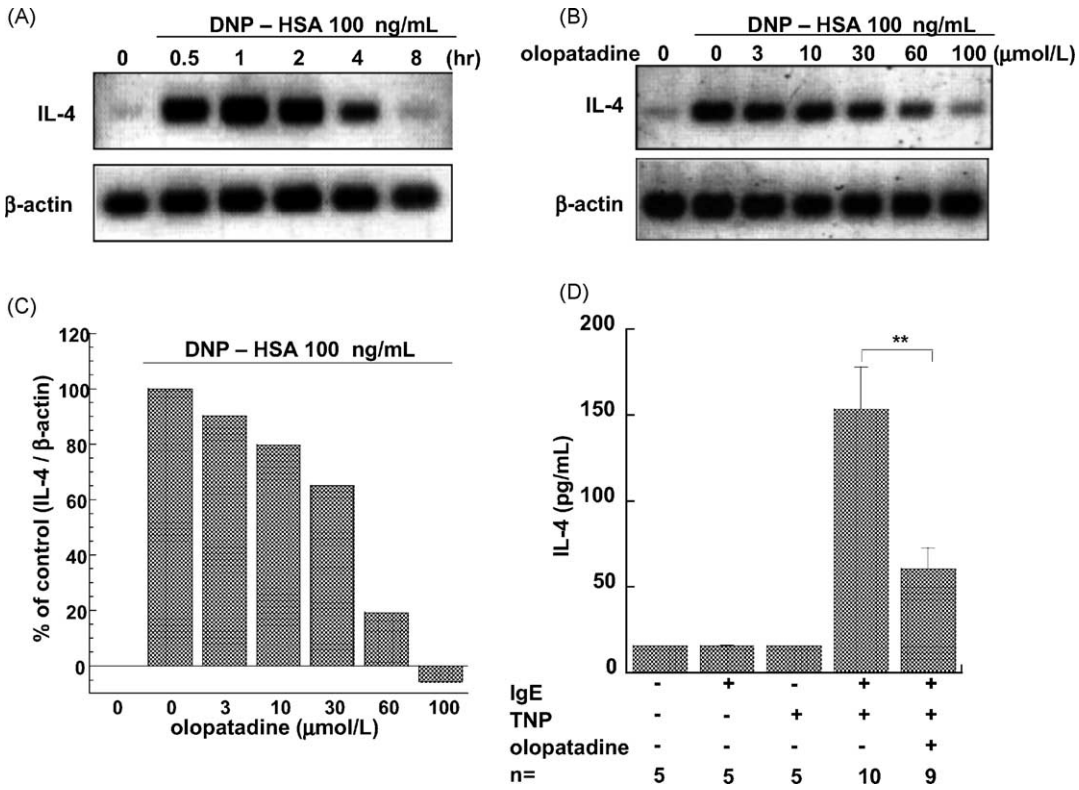

Fig. 3. Effects of olopatadine on the increases in IL-4 mRNA and IL-4 production induced by FcεR cross-linking. (A) The IgE-sensitized RBL-2H3 cells were stimulated with 100 ng/mL DNP-HSA. Time course of change in IL-4 mRNA levels was analyzed with the semi-quantitative RT–PCR method. Synthesis of first-strand cDNA and PCR reaction are described in Section 2. (B) The IgE-sensitized RBL-2H3 cells were treated with the indicated concentrations of olopatadine and then stimulated with 100 ng/mL DNP-HSA for 1 hr. (C) Results of densitometric analysis are shown as % of control (IL-4 mRNA/β-actin mRNA ratio). One representative result of two independent experiments is shown. (D) IL-4 production in passive-sensitized mice induced by antigen challenge. Olopatadine (10 mg/kg) was orally administered 30 min before stimulation. Values represent means ± SE. Statistical analysis was performed by the Aspin–Welch test. $^{**}P < 0.01$.

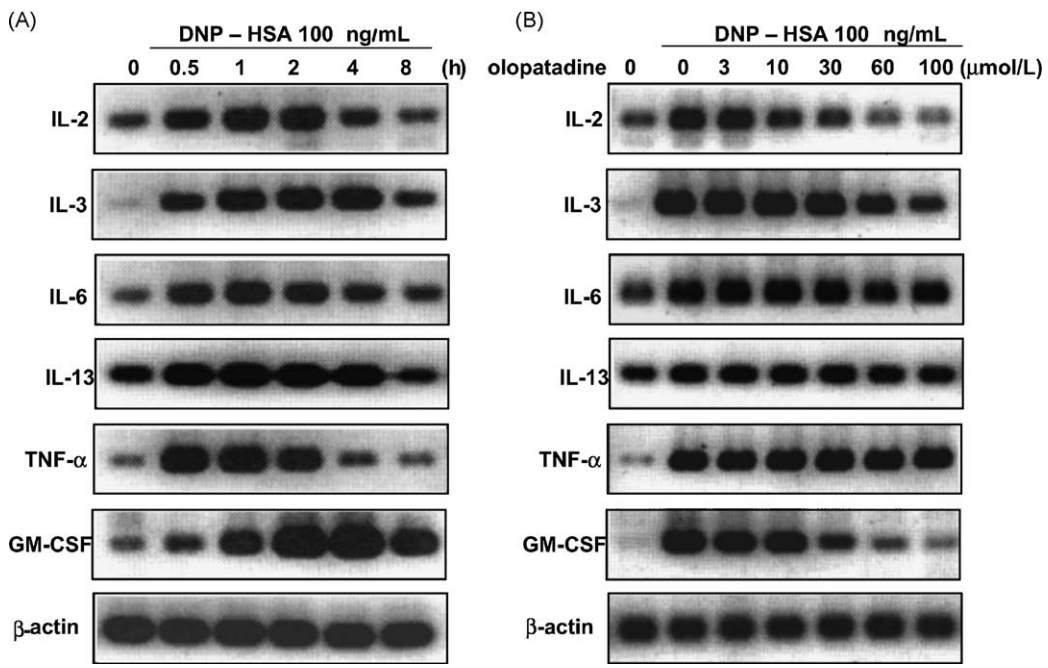

Fig. 4. Effects of olopatadine on levels of mRNA various cytokines induced by FcεR cross-linking. (A) Time course of change in mRNA levels of various cytokines following antigen-stimulation in RBL-2H3 cells. (B) The IgE-sensitized RBL-2H3 cells were treated with the indicated concentrations of olopatadine and then stimulated with 100 ng/mL DNP-HSA for 1 hr. Total RNA was isolated and used for semi-quantitative RT–PCR analysis. One representative result of two independent experiments is shown.

Table 1
Effects of anti-allergic drugs on degranulation, arachidonic acid release and IL-4 induction by FcεR cross-linking

Drug (μmol/L)	% of control			
	3	10	30	60
(A) β-Hesosaminidase release				
Olopatadine	Not determined	82.2 ± 2.6	71.6 ± 1.6	54.1 ± 1.3
DSCG	98.4 ± 2.4	87.6 ± 2.7	86.7 ± 1.0	67.1 ± 2.6
Tranilast	90.1 ± 7.1	89.8 ± 2.7	65.4 ± 1.3	37.5 ± 2.6
Amlexanox	86.8 ± 2.5	81.9 ± 2.3	57.9 ± 1.9	17.1 ± 10.2
Ketotifen	97.6 ± 7.6	78.7 ± 4.9	77.6 ± 4.2	54.4 ± 5.1
Loratadine	80.0 ± 2.5	70.2 ± 1.3	62.1 ± 8.3	29.4 ± 2.4
Astemizole	95.9 ± 1.4	85.0 ± 1.8	73.5 ± 2.6	32.1 ± 2.5
Cetirizine	109.8 ± 3.3	89.4 ± 2.2	120.1 ± 5.9	96.3 ± 6.8
(B) Arachidonic acid release				
Olopatadine	93.9 ± 2.2	86.4 ± 1.9	76.4 ± 2.5	56.7 ± 1.1
DSCG	83.6 ± 0.4	80.9 ± 3.2	75.7 ± 1.8	57.9 ± 2.7
Ketotifen	92.6 ± 3.6	91.8 ± 2.1	66.7 ± 1.0	51.5 ± 1.2
Loratadine	79.8 ± 3.8	61.0 ± 2.4	58.3 ± 2.2	42.6 ± 1.9
Cetirizine	102.6 ± 2.3	103.2 ± 1.2	90.4 ± 4.8	78.4 ± 2.1
(C) IL-4 mRNA/β-actin mRNA				
Olopatadine	98.2	57.6	13.6	3.3
DSCG	80.3	61.2	32.7	16.9
Ketotifen	83.3	89.6	23.9	3.6
Loratadine	97.9	114.0	106.4	134.3
Cetirizine	90.2	81.0	58.5	53.2
Tranilast	101.6	45.7	56.7	35.8

The IgE-sensitized RBL-2H3 cells were treated with indicated concentrations of anti-allergic drugs. Treated cells were then stimulated with 100 ng/mL DNP-HSA. (A) The enzymatic activities of released β-hexosaminidase were measured. Values represent means ± SE of four determinations in the same experiment. (B) Radioactivities of [^{3}H] arachidonic acid were measured. Values represent means ± SE of three determinations in the same experiment. (C) The change in IL-4 mRNA levels were analyzed with semi-quantitative RT–PCR analysis. One representative result of two or three RT–PCR is shown in the same experiment.

loratadine did not inhibit IL-4 induction although it did inhibit release of β-hexosaminidase and arachidonic acid, whereas cetirizine did reduce the induction of IL-4 but did not inhibit release of β-hexosaminidase.

3.5. Effects of some anti-allergic drugs on Ca^{2+} entry in RBL-2H3 cells

The anti-allergic drugs ketotifen, astemizole, loratadine, DSCG, tranilast and amlexanox suppressed Ca^{2+} influx in response to antigen (Fig. 5) but cetirizine did not inhibit Ca^{2+} influx at a concentration of 300 μmol/L. The degrees of inhibition of Ca^{2+} influx produced by the tested drugs corresponded to their effects on β-hexosaminidase release but not their suppression of IL-4 expression. Thus, whereas inhibition of β-hexosaminidase release appears to be related to the effect of drugs on the influx of Ca^{2+}, the present findings imply that other mechanism(s) in addition to inhibition of Ca^{2+} influx are involved in the suppression of IL-4 expression.

3.6. Effects of the ROC blocker SKF-96365 on degranulation and IL-4 mRNA expression

To test the hypothesis mentioned above, SKF-96365, a well-known ROC blocker [10], was applied to the β-hexosaminidase release and IL-4 mRNA induction assay. SKF-96365 suppressed Ca^{2+} influx (Fig. 6A) and inhibited β-hexosaminidase release (Fig. 6B) by antigen-stimulated RBL-2H3 cells. Although the inhibition of Ca^{2+} influx by SKF-96365 was greater than that caused by olopatadine, the inhibitory effect of SKF-96365 on IL-4 expression was only partial even at 100 μmol/L (Fig. 6C and D). This finding further supports the hypothesis that IL-4 expression is regulated not only by Ca^{2+} influx but other, unknown mechanism(s) as well.

3.7. Effects of olopatadine on Ca^{2+} release from intracellular stores

A transient increase in Ca^{2+} was observed after antigen stimulation of sensitized RBL-2H3 cells in the absence of extracellular Ca^{2+}. Pretreatment with olopatadine did not modify the release of Ca^{2+} from intracellular stores (Fig. 7).

3.8. Effects of olopatadine on the phosphorlyation of p38 MAK and JNK1

Examination of the effect of olopatadine on the protein phosphorylation recognized by PY-20 revealed a band at about 70 kDa molecular weight, which may result from the phospholyation of Syk, as suggested in a previous report [23]. Pretreatment with olopatadine altered protein

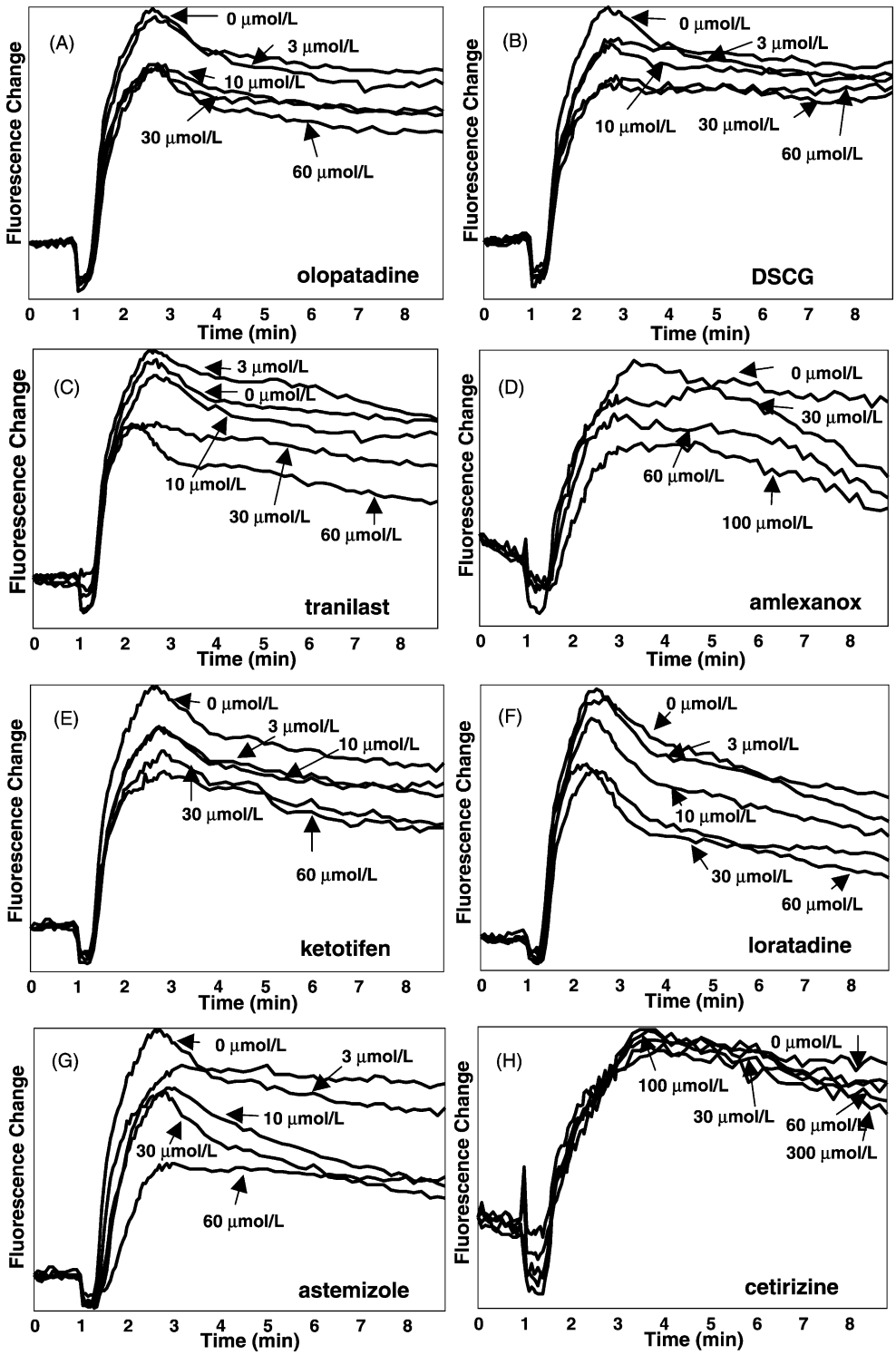

Fig. 5. Effects of anti-allergic drugs on Ca^{2+} entry by FcεR cross-linking. The IgE-sensitized RBL-2H3 cells were treated with the indicated concentration of anti-allergic drugs. Treated cells were then stimulated with 100 ng/mL of DNP-HSA. Ca^{2+} response was assessed using fluo-3-AM with FLIPR. The change in fluorescence of fluo-3 was monitored. Each value represents the mean of the fluorescence measured in quadruplicate microplate wells.

phosphorylation dramatically in neither unstimulated nor stimulated cells (Fig. 8A). A similar result was obtained using another phosphotyrosine antibody (P-Tyr-102, data not shown).

The report that p38 MAPK activation is required for Ca^{2+} influx in human basophils [24] prompted us to test the effect of the ROC blocker SKF-96365 on this activation. As shown in Fig 8B, SKF-96365 completely inhibited the phosphorylation of both proteins, consistent with the previous studies [24]. On the other hand, anti-allergic drugs, including olopatadine, only partially inhibited it up to 100 μmol/L (Fig. 8B). Since olopatadine also partially

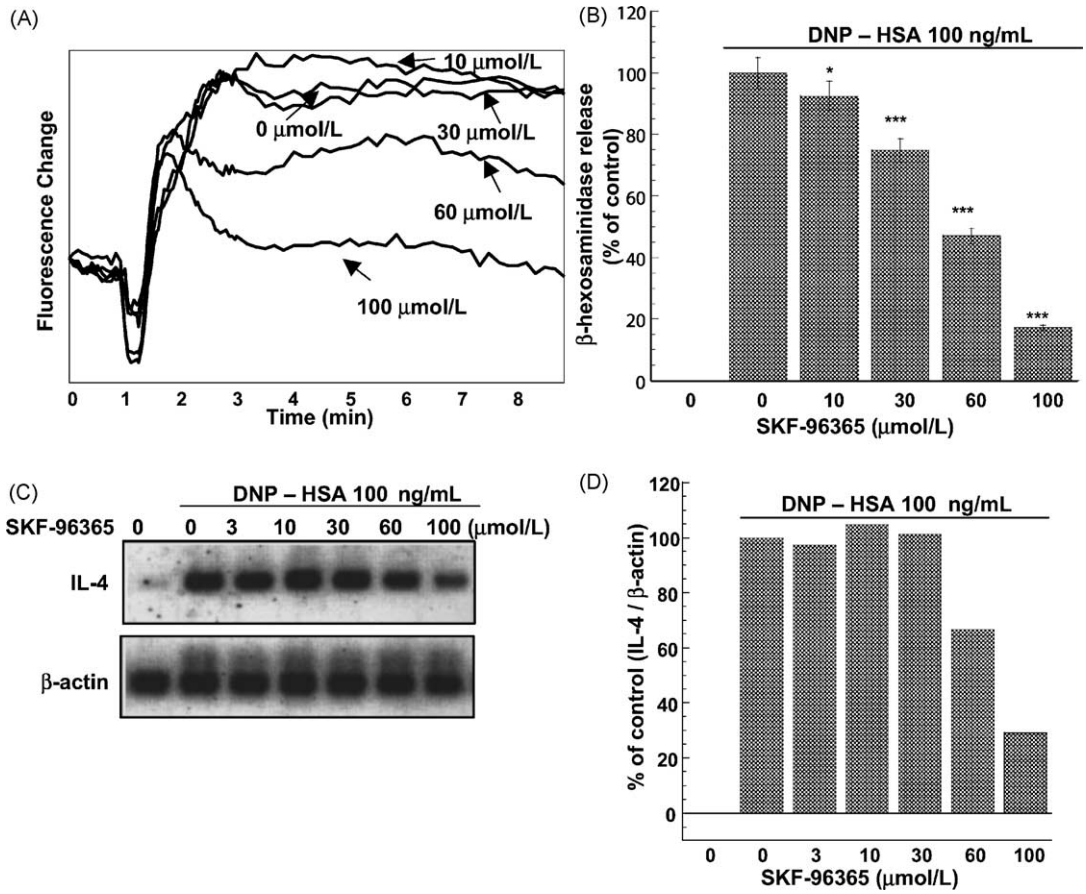

Fig. 6. Effects of SKF-96365 on Ca^{2+} entry, degranulation and induction of IL-4 mRNA by FcεR cross-linking. The IgE-sensitized RBL-2H3 cells were treated with the indicated concentration of SKF-96365 and then stimulated with 100 ng/mL DNP-HSA. (A) Ca^{2+} response was assessed using fluo-3-AM with FLIPR. The change in fluorescence of fluo-3 was monitored. Each value represents the mean of the fluorescence measured in quadruplicate microplate wells. (B) The enzymatic activities of released β-hexosaminidase were measured. Release of β-hexosaminidase is shown as % of control. Values represent means ± SE of four determinations. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with the control group (Dunnett). (C) IL-4 mRNA level was determined by semi-quantitative RT–PCR analysis. One representative result of two independent experiments is shown. (D) Results of densitometric analysis are shown as % of control (IL-4 mRNA/β-actin mRNA ratio).

inhibited Ca^{2+} influx compared with SKF-96365 at concentrating up to 100 μmol/L, the inhibitory effects of olopatadine on the activation of p38 MAPK and JNK1 may be due to inhibition of Ca^{2+} influx.

3.9. Effects of olopatadine on intracelluar cAMP

cAMP-elevating drugs and agents suppress Ca^{2+} influx and inhibit histamine release from mast cells [25,26]. Consistent with these observations, we confirmed that the phosophodiesterase (PDE) inhibitor IBMX reduced both β-hexosaminidase release and Ca^{2+} influx (data not shown). A recent study [27] demonstrated that the β_2 agonsit salmeterol and the PDE inhibitor theophylline inhibited IL-4 production as well as histamine and LTs release from human basophils. It is conceivable that the observed inhibitory effects on olopatadine were due to an increase in cAMP in RBL-2H3 cells. However, as shown in Fig. 9, olopatadine did not increase cAMP levels even in the presence of IBMX (1 mmol/L) or the adenylate cyclase activator forskolin (100 μmol/L).

4. Discussion

In the present study, we investigated whether olopatadine suppresses the up-regulation of expression of some cytokines caused by aggregation of FcεRI in a rat mucosal mast cell line, RBL-2H3. Our results show that olopatadine inhibited not only degranulation and arachidonic acid release but also the induction of cytokine (e.g. IL-4 and GM-CSF) genes in antigen-stimulated RBL-2H3 cells. Since IL-4 production was not detected in RBL-2H3 cells *in vitro*, we investigated whether oral administration of olopatadine would inhibit IL-4 production by mast cells in an animal model *in vivo*, and found that olopatadine did indeed reduce serum levels of IL-4. We have calculated the plasma peak of olopatadine (10 mg/kg p.o.) to be nearly reached at 10 μmol/L (unpublished observation). Accordingly, this dosage was tested to examine the effect of olopatadine on IL-4 production *in vivo*. In the present study, DSCG and ketotifen each inhibited IL-4 expression in RBL-2H3 cells as potently as olopatadine. Both drugs are reported to inhibit IL-4 production by human peripheral

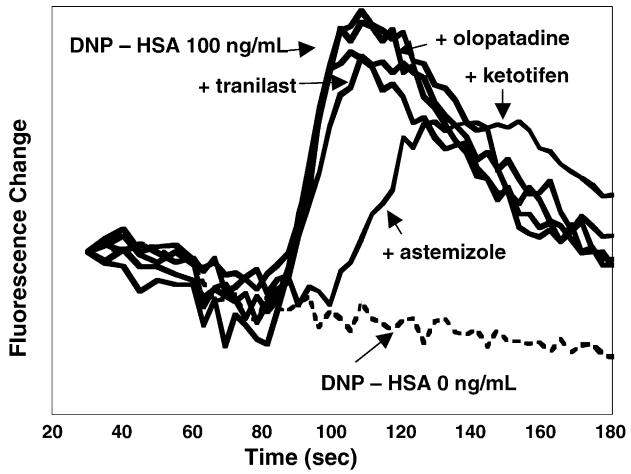

Fig. 7. Effects of olopatadine on Ca^{2+} release from intracellular stores by FcεR cross-linking. Ca^{2+} responses in RBL-2H3 cells were assessed using fluo-3-AM with FLIPR. The IgE-sensitized RBL-2H3 cells were loaded with fluo-3-AM for 1 hr and treated with 60 μmol/L of the indicated anti-allergic drugs under extracellular Ca^{2+}-free conditions (in the presence of the same concentration of EGTA instead of 2.54 mol/L Ca^{2+}). The cells were then placed on FLIPR and stimulated with 100 ng/mL DNP-HSA under extracelluar Ca^{2+}-free conditions. The change in fluorescence change of fluo-3 was monitored. Each value represents the mean of the fluorescence measured in quadruplicate microplate wells.

blood leukocytes stimulated by concanavalin A [28]. Moreover, ketotien inhibits both early and late signs of allergy in animal models [2,29], while DSCG is widely used to prevent the early and late bronchoconstriction induced by a variety of stimuli in treatment of bronchial asthma [30]. Various cytokines (e.g. IL-4 and GM-CSF) derived from mast cells are believed to mediate late allergic symptoms [6]. Thus, these findings suggest that the alleviation of late allergic symptoms by olopatadine might be attributable to this drug's ability to suppress cytokine expression.

It has been argued that the clinical efficacy of olopatadine is mainly due to H_1 antagonism rather than the above mast cell stabilization, since it is necessary to use comparatively high concentrations to inhibit IL-4 expression *in vitro*. However, olopatadine inhibits nasal blockage in experimental allergic rhinitis [3], while the classical H_1 antagonist chlorphenilamine has little effect on nasal blockage [31]. In fact, oral administration of olopatadine exhibited clinical efficacy in treatment of the nasal obstruction associated with allergic rhinitis [1]. This clinical effect of olopatadine cannot be simply explained by H_1 antagonism, as mentioned above, suggesting that the histamine-unrelated anti-allergic effects of olopatadine are of clinical importance. In the present study, the oral dose of olopatadine that prevented IL-4 production by mast cells *in vivo* was nearly equivalent to the dosage that inhibited allergic symptoms in experimental animal models [1–3], suggesting that determination of inhibition of IL-4 expression may offer new insights into the mechanisms of action of existing anti-allergic drugs.

The mechanisms involved in the inhibition of degranulation, lipid mediator release and induction of IL-4

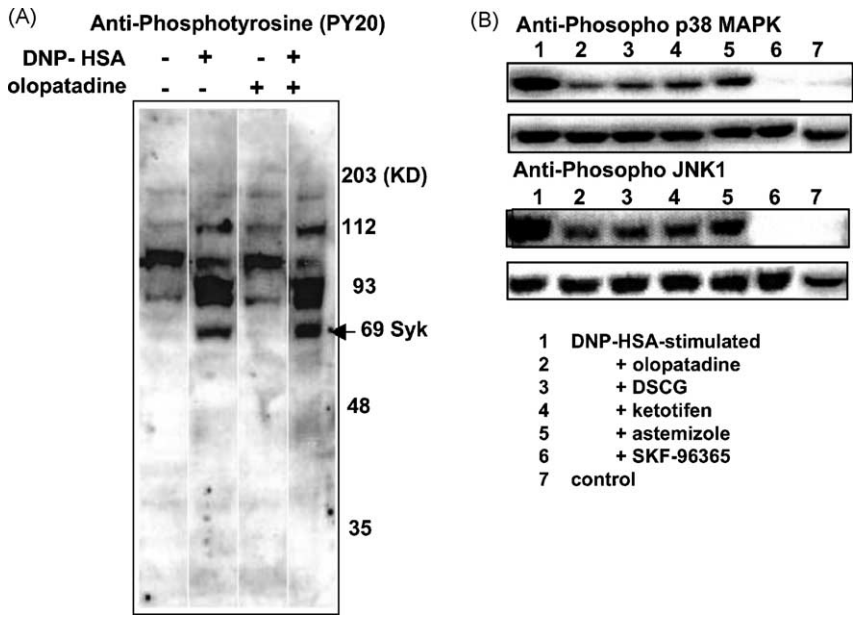

Fig. 8. Effects of olopatadine on the phosphorylation of cellular proteins in RBL-2H3 cells. The IgE-sensitized RBL-2H3 cells were treated with 60 μmol/L of olopatadine. The cells were then stimulated or not with 100 ng/mL DNP-HSA for 30 min. (A) Phosphorylation of cellular proteins was analyzed by Western blotting using an anti-phosphotyrosine antibody (PY-20). The arrow corresponds to the apparent molecular weight of Syk, which is phosphorylated after FcεR crosslinking (a similar result was obtained using another anti-phosphotyrosine antibody P-Tyr-102; data not shown). (B) The IgE-sensitized RBL-2H3 cells were treated with 100 μmol/L of the indicated anti-allergic drugs and SKF-96365 and stimulated with 100 ng/mL DNP-HSA for 30 min. Phosphorylation of p38 MAPK and JNK1 was detected with phospho-protein specific antibodies in the upper panel. After detection, the phospho-protein specific antibodies used were stripped. Total p38 MAPK and JNK1 were detected with their specific antibodies in the lower panel. One representative result of two or three independent experiments is shown.

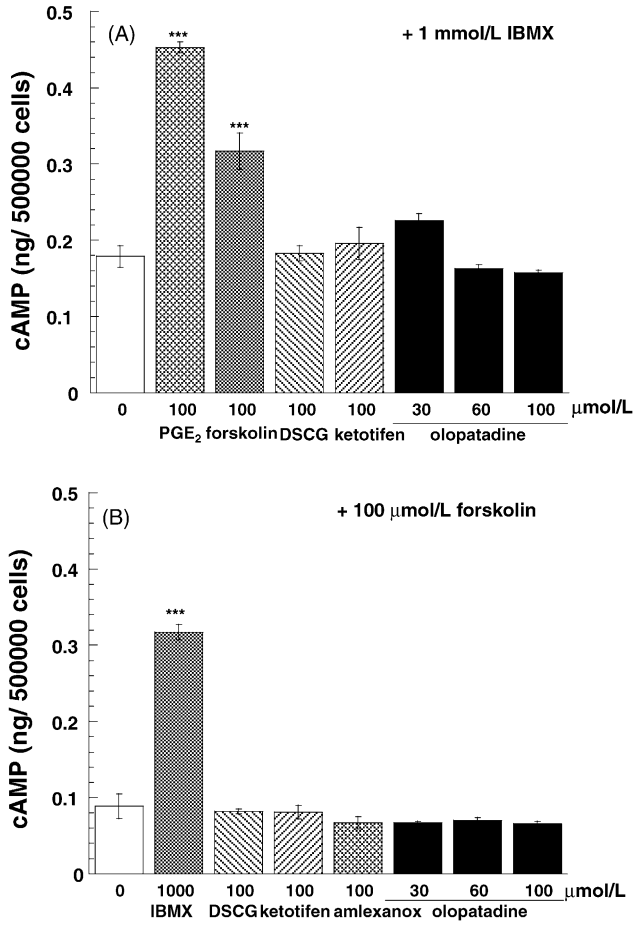

Fig. 9. Effects of anti-allergic drugs on cAMP accumulation in RBL-2H3 cells. (A) Effects of anti-allergic drugs on cAMP synthesis were determined. The IgE-sensitized RBL-2H3 cells were preincubated with 1 mmol/L IBMX for 10 min, and then incubated with the indicated concentrations of anti-allergic drugs, 100 μmol/L forskolin or 100 μmol/L PGE_2 in the presence of 1 mmol/L IBMX for 15 min. (B) Effects of anti-allergic drugs on cAMP degradation were determined. The IgE-sensitized RBL-2H3 cells were preincubated for 15 min, and then incubated with the indicated concentrations of anti-allergic drugs or 1 mmol/L IBMX in the presence of 100 μmol/L forskolin for 15 min. cAMP measurements were carried out as described in the manufacturer's manual. Values represent means ± SE of three determinations. $^{***}P < 0.001$ compared with control (Dunnett).

expression by olopatadine were investigated. Ca^{2+} influx through ROCs participates in a triggering of degranulation, and there is ample evidence that inhibition of Ca^{2+} influx suppresses of degranulation by anti-allergic drugs [14,17–19]. In the present study, olopatadine inhibited Ca^{2+} influx. Findings of a comparative study of olopatadine with other anti-allergic drugs and the ROC blocker SKF-96365 suggest that inhibition of Ca^{2+} influx is responsible for the suppression of degranulation. Since olopatadine did not affect Ca^{2+} release from intracellular stores, this drug may prevent Ca^{2+} influx via ROCs. Since it has been suggested that ROCs are transient receptor potential (TRP) channels [10], it is noteworthy that expression of TRP3, TRP4 and TRP6 has been detected in RBL-2H3 cells [32].

Olopatadine suppressed IL-4 expression more potently than it affected degranulation and lipid mediator release. Ketotifen and DSCG have similar profiles of activity. In this respect, desloratadine also inhibited IL-4 production via suppression of gene expression more effectively than it blocked release of histamine and LTC_4 by human basophils [33]. We therefore hypothesize that in mast cells distinct mechanism(s) are involved when anti-allergic drugs inhibit (a) IL-4 expression and (b) degranulation and arachidonic acid release. Olopatadine produced different degrees of inhibition of various cytokines, indicating that the mechanisms involved in the induction of cytokine expression are complex.

Since p38 MAPK is reported to partially regulate IL-4 expression in RBL-2H3 cells [13], we examined the effects of olopatadine and SKF-96365 on the activation of p38 MAPK. Olopatadine did not affect phosphorylation of intracellular proteins recognized by PY-20, but partially inhibited the phosphorylation of p38 MAPK and JNK induced by antigen stimulation. In the present study, the ROC blocker SKF-96365 completely inhibited the phosphorylation of p38 MAPK and JNK1, suggesting that activation of both proteins is dependent on Ca^{2+} influx via ROC in RBL-2H3 cells. These results are consistent with a previous report that the removal of extracellluar Ca^{2+} reduces phosphorylation of p38 MAPK in human basophils [24]. Accordingly, inhibition by olopatadine of the activation of p38 MAPK and JNK1 may be due to inhibition of Ca^{2+} influx. SKF-96365 suppressed IL-4 expression less potently than it affected Ca^{2+} influx and phosphorylation of p38 MAPK. These observations indicate the existence of an unknown mechanism(s) of action of anti-allergic drugs, in addition to inhibition of Ca^{2+} influx and p38 MAPK activation, in IL-4 expression in RBL-2H3 cells.

The β_2 agonist salmeterol and the PDE inhibitor theophylline each elevate cAMP levels and also inhibit IL-4 production as well as degranulation and lipid mediator release by human basophils [27]. Moreover, these cAMP-elevating drugs prevent Ca^{2+} influx and inhibit the phospholylation of p38 MAPK activated by antigen stimulation [24], suggesting that elevation of cAMP may suppress IL-4 expression. However, this was not the mechanism whereby olopatadine affected IL-4 expression, since olopatadine did not increase cAMP levels. The latter finding indicates that olopatadine acts neither as an agonist for a G protein-coupled receptor such as a β_2 adrenergic receptor to activate adenylate cyclase, nor as a PDE inhibitor.

In conclusion, the inhibition of Ca^{2+} influx by anti-allergic drugs plays a crucial role in the suppression of degranulation in mast cells. However, the suppression of IL-4 induction is not simply explained by the inhibition of Ca^{2+} influx. Further research is needed to fully elucidate alternative mechanisms by which anti-allergic drugs, including olopatadine, suppress IL-4 expression in mast cells.

Acknowledgments

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