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The effects of S1319, a novel marine sponge-derived β_2-adrenoceptor agonist, on IgE-mediated activation of human cultured mast cells

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Abstract. *Objective:* This study aimed to evaluate the ability of S1319 (4-hydroxy-7-[1-(1-hydroxy-2-methylamino) ethyl]-1,3-benzothiazol-2(3*H*)-one acetate), a novel β_2-adrenoceptor selective agonist derived from marine sponge, to inhibit IgE-mediated activation of human cultured mast cells (HCMC) *in vitro*.
Materials and Methods: We examined the effect of S1319 (racemate) on tryptase release and tumor necrosis factor- α (TNF-α) production in HCMC generated from human cord blood cells, after cross-linking of high affinity immunoglobulin E receptors (FcεRI), compared with those of the non-selective β-adrenoceptor agonist, isoproterenol (R-isomer), the selective β_2-adrenoceptor agonist, salbutamol (racemate), and the selective and long-acting β_2-adrenoceptor agonist, formoterol (racemate). We also evaluated the effect of S1319 on the intracellular cAMP level, inositol phosphate production and protein tyrosine phosphorylation in HCMC.
Results: S1319 and β-adrenoceptor agonists inhibited the IgE-mediated release of tryptase. Approximate IC_{50} values of S1319, formoterol, isoproterenol and albuterol for the inhibition of tryptase release were 0.51 ± 0.12, 0.15 ± 0.1, 0.80 ± 0.09, and 28 ± 32.4 nM, respectively. S1319 and β-adrenoceptor agonists also inhibited TNF-α production by HCMC in a concentration-dependent manner. Approximate IC_{50} values of S1319, formoterol and isoproterenol for the inhibition of TNF-α production were 0.19 ± 0.03, 0.28 ± 0.02 and 0.32 ± 0.03 nM, respectively. S1319 caused a concentration-dependent increase in total cell cyclic AMP levels in HCMC. On the other hand, S1319 inhibited the accumulation of inositol 1,4,5-triphosphate and IgE-mediated protein tyrosine phosphorylation of 42-kDa protein, p42 mitogen activated protein (MAP) kinase (ERK-2).
Conclusion: These results indicate that S1319 and β-adrenoceptor agonists are potent inhibitors of the IgE-mediated release of mediators from HCMC.

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Key words: β-adrenoceptor agonist – Mast cells – Tryptase – TNF-α – Protein tyrosine phosphorylation

Introduction

β-adrenoceptor agonists are the most widely prescribed bronchodilator drugs for the symptomatic treatment of reversible airway disease [1]. While the primary effect of bronchodilators is to relax airway smooth muscles, additional effects may include the stabilization of inflammatory cell activity. Because the release of pro-inflammatory mediators from cells in the airway could contribute to both the progression and the severity of asthma, suppression of the release of these mediators by β_2-adrenoceptor agonists could represent an important anti-asthma activity of these drugs. However, controversy exists about the therapeutic mode of action of β_2-adrenoceptor agonists, especially whether or not they have significant anti-inflammatory activities. Among the identified anti-inflammatory effects, β_2-adrenoceptor agonists have been shown to inhibit plasma exudation in the airways in response to inflammatory mediators [2] and to reduce late cutaneous reactions [3]. Furthermore, β_2-adrenoceptors have been identified on inflammatory cells such as eosinophils, neutrophils, lymphocytes, mast cells, and macrophages [4]. β_2-adrenoceptor agonists inhibit the release of mediators from eosinophils [5] and neutrophils [6] and inhibit IL-2 synthesis and IL-2 receptor expression by lymphocytes [7, 8]. Also, β_2-adrenoceptor agonists prevent T helper 1 type development by the selective inhibition of IL-12 production in both macrophages and dendritic cells [9]. As concerns mast cells, β_2-adrenoceptor agonists inhibit the release of histamine [10–15], prostaglandin D_2 [11, 12] and leukotriene C_4 [11] from human lung mast cells and dispersed skin mast cells [13].

Mast cells could also be an important target for the anti-asthma actions of β_2-adrenoceptor agonists, because mast cells have been identified as a source of several cytokines

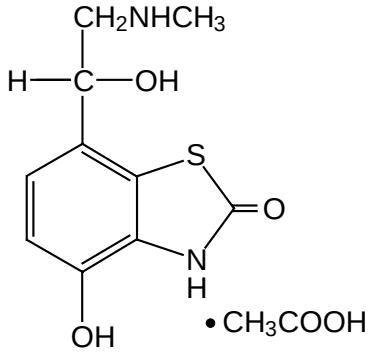

Fig. 1. Chemical structure of racemic S1319.

[16, 17] believed to be important to inflammation and allergic responses. Recently, a method for developing human cultured mast cells (HCMC) by culturing cord blood mononuclear cells for more than 12 weeks in the presence of recombinant human stem cell factor (rh-SCF) and IL-6 has been established [18, 19]. The phenotypic characterization of HCMC revealed considerable similarities with human lung mast cells [20]. Furthermore, HCMC have a functional signal transduction mechanism mediated via high affinity immunoglobulin E receptors (FcεRI) [21]. HCMC may therefore be useful for the biological and pharmacological study of human lung mast cells. Though there is evidence that β_2-adrenoceptor agonists inhibit the release of mediators from human mast cells, little is known about the modulation of mediator release and cytokine production by β_2-adrenoceptor agonists. Given the importance of mast cells in inflammation and allergic diseases and the evidence that β_2-adrenoceptor agonists inhibit mast cell activation, we investigated the effect of β_2-adrenoceptor agonists on the degranulation and production of tumor necrosis factor- α (TNF-α) from HCMC. Furthermore, HCMC enabled us to study the mechanism of inhibitory effects of β_2-adrenoceptor agonists in human mast cells such as protein tyrosine phosphorylation, because it is difficult to obtain more than 10^7 highly purified mast cells from human tissues.

S1319 (4-hydroxy-7-[1-(1-hydroxy-2-methylamino)ethyl]-1,3-benzothiazol-2(3*H*)-one acetate) (Fig. 1) is a potent β_2-adrenoceptor agonist with a short duration of action on airways smooth muscle in vitro [22, 23]. In the present study, we describe the effect of S1319 (racemate) as an inhibitor of IgE-induced mediator and cytokine release from HCMC, comparing with the non-selective β-adrenoceptor agonist, isoproterenol (R-isomer), the selective β_2-adrenoceptor agonist, albuterol (racemate), and the selective and long-acting β_2-adrenoceptor agonist, formoterol (racemate).

Materials and methods

Cell culture

HCMC were raised from human umbilical cord blood cells by the method described by Yanagida et al. [19]. Briefly, mononuclear cells were obtained from heparinized umbilical cord blood and suspended in α-MEM supplemented with 12.5% FCS, 10 µg/ml deoxyadenosine, deoxyguanosine, deoxycytidine, adenosine, guanosine, cytidine, thymidine, and uridine, 100 ng/ml rh-SCF, and 10 ng/ml rh-IL-6. Non-adherent cells were harvested weekly after gently pipetting the culture media, half of which was replaced. The purity of mast cells was determined by staining with May-Grünwald and Giemsa reagents. Beyond 8 weeks, the purity of HCMC reached almost 100%.

Analysis of mast cells degranulation

Tryptase release assay was used to evaluate mediator release from HCMC upon cross-linking of FcεRI [24]. HCMC (1×10^6 cells/ml) were passively sensitized overnight with 1 µg/ml human IgE at 37 °C. After being washed, the cells (2×10^4) were appropriate with the indicated reagents at 37 °C for 30 min in Tyrode's/HEPES solution and then with 3 µg/ml anti-IgE antibody (Chemicon, Temecula, CA) for 30 min. After centrifugation, tryptase release was quantified by measurement of the tryptase activity in cell supernatants by the hydrolysis of benzoyl-DL-arginine-*p*-nitroanilide as detailed elsewhere [25]. Tryptase release and histamine release were significantly well correlated with each other (data not shown). The percentage of release relative to negative controls was calculated using the following formula: (stimulated release – spontaneous release)/(total amount of tryptase – spontaneous release) × 100.

IgE-dependent TNF-α release

HCMC (1×10^6 cells/ml) were passively sensitized overnight with 1 µg/ml human IgE at 37 °C following culture with IL-4 (10 ng/ml) for 7 days. After being washed, the cells (2×10^6) were incubated with the appropriate reagents at 37 °C for 30 min in the complete medium and then with 3 µg/ml anti-IgE antibody (Chemicon, Temecula, CA, USA) for 16 h. After centrifugation, the TNF-α content in cell-free supernatants was measured with an ELISA kit (Biosource International Inc., Camarillo, CA, USA) with a sensitivity of 115 fg/ml.

Quantification of cyclic AMP levels

HCMC (2×10^5 cells/ml) suspended in 55 µl of the Tyrode's/HEPES solution were incubated at 37 °C for 10 min in the presence of various concentrations of β-adrenoceptor agonists. The reaction was stopped by addition of 1 ml of acidic ethanol (1 ml of 1 M HCl/100 ml of ethanol) followed by freezing in dry ice/acetone. The reaction mixture was dried under nitrogen. Cyclic AMP was determined by double antibody radio immunoassay using a cyclic AMP (^{125}I) assay kit (Amersham Pharmacia Biotech).

Determination of IP_3

Measurement of inositol 1,4,5-triphosphate (IP_3) was carried out using a commercially available kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's protocol. Briefly, HCMC were extracted with chloroform/methanol (1:2) on ice for 15 min. Methanol fractions containing phosphorylated inositol were lyophilized and mixed with bovine adrenal IP_3-binding proteins in the presence of limiting amounts of the tracer D-myo-[^{3}H]inositol 1,4,5-triphosphate. The mixtures were centrifuged at 2000 × g for 15 min and radioactivity bound to IP_3 -binding protein was measured in a β-scintillation counter.

RT-PCR analysis of β-adrenoceptor mRNA expression

Poly(A) + mRNA was isolated using a QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. First strand cDNA was synthesized using a SuperScript Preamplification System (GIBCO BRL, Rockville, MD, USA). cDNA equivalent to 20 ng of total Poly(A) + mRNA was amplified in a 50 µl reaction volume using a TaKaRa LA PCR kit (Takara Shuzo, Shiga, Japan). Amplification of cDNA was performed as described

[25]. Sequences of the sense and antisense oligonucleotides, respectively, were: 5′-TCGTGTGCACCGTGTGGGCC-3′ and 5′-AGGAAACG-GCGCTCGCAGCTGTCG-3′ (amino acids 178-265) for β_1, 5′-GCCT-GCTGACCAAGAATAAGGCC-3′ and 5′-CCCATCCTGCTCCACCT-3′ (amino acids 143-252) for β_2, and 5′-GCTCCGTGGCCTCACGA-GAA-3′ and 5′-CCCAACGGCCAGTGGCCAGTCAGCG-3′ (amino acids 2-106) for β_3. As a control, primers for G3PDH (Clontech, Palo Alto, CA, USA) were used. Using these primers, the lengths of the fragments, calculated from the structure of the corresponding genes, were 265, 329, 314 and 450 bp for β_1-, β_2-, and β_3-adrenoceptors and G3PDH, respectively. PCR was performed as follows: 1) denaturation at 94 °C for 5 min; 2) 30 cycles of 92 °C for 1 min (melting), 57 °C for 1.5 min (annealing), and 72 °C for 1.5 min (extension); 3) incubation at 72 °C for 10 min (final extension). Reaction products were resolved on 2% agarose gels and visualized with ethidium bromide under UV light.

Preparation of cell lysate and immunoblotting

After stimulation for a predetermined period, cell lysates were then either used directly for SDS-PAGE or for immunoprecipitation. In the case of anti-ERK, the lysate was denatured, and then immunoprecipitated as described previously [21]. Immune complexes were pelleted with Pansorbin (Calbiochem, San Diego, CA). Extracted proteins (equivalent to 3×10^5 cells/lane) were separated by SDS-PAGE, and transferred onto PVDF membranes. The membranes were blocked with 2% gelatin in PBS-T buffer (0.1% Tween-20 in phosphate-buffered saline), and probed with the appropriate primary antibody. After washing, the membranes were treated with horseradish peroxidase (HRP)-conjugated anti-immunoglobulin antibody, and immunoreactivity was detected by using enhanced chemiluminescence detection reagents (ECL, Amersham Pharmacia Biotech) and by exposure to X-ray film.

Materials

S1319 (4-hydroxy-7-[1-(1-hydroxy-2-methylamino)ethyl]-1,3-benzothiazol-2(3*H*)-one acetate) was synthesized in our laboratory. S1319 was a racemic, composed of 50:50 mixtures of R and S isomaers. Other drugs used were as follows: (R)-isoproterenol hydrochloride, albuterol sulphate (Research Biochemicals, Inc., Natick, MA), histamine hydrochloride, propranolol and forskolin (Sigma Chemical Co., St. Louis, MO, USA), and formoterol fumarate (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan). All other chemicals used were of reagent grade. The following was used as primary antibody for immunoblotting; anti-phosphotyrosine mAb (4G10) (Upstate Biotechnology Inc., Lake Placid, NY, USA) and for immunoprecipitation; anti-ERK-1 (cross-reactive with ERK-1 and ERK-2, Santa Cruz Biotechnology Inc., Santa Cruz, CA). As secondary antibodies, HRP-conjugated donkey anti-rabbit immunoglobulin antibody and HRP-conjugated sheep anti-mouse immunoglobulin antibody were purchased from Amersham Pharmacia Biotech.

Data analysis

IC_{50} values and the standard error of the means (SEM) were calculated using Microsoft Excel (Microsoft). The statistical significance of the difference between two groups was determined by the Student *t* test.

Results

Effect of β-adrenoceptor agonists on the tryptase release from HCMC

The effect of β-adrenoceptor agonists on IgE-mediated trypase release from HCMC was assessed. S1319 (racemate), isoproterenol (R-isomaer), albuterol (racemate) and formoterol (racemate) inhibited the release of tryptase from HCMC in a concentration-dependent manner (Fig. 2). The rank order of potency for the inhibition was formoterol > S1319 > isoproterenol > albuterol. Approximate IC_{50} values of formoterol, S1319, isoproterenol and albuterol for the inhibition of tryptase release were 0.15 ± 0.1 nM (n = 6), 0.51 ± 0.12 nM (n = 6), 0.80 ± 0.09 nM (n = 4), and 28 ± 32.4 nM (n = 6), respectively (Table 1). S1319 also inhibited the IgE-mediated histamine release from HCMC and this inhibition was significantly well correlated with tryptase release (data not shown). To further examine the role of β-adrenoceptors in the inhibition of tryptase release, the effect of propranolol, a β-adrenoceptor antagonist, on the observed inhibition of HCMC degranulation by S1319 was determined. The concentration-dependent inhibitory effect of S1319 on IgE-mediated release of tryptase from HCMC was diminished by pre-incubation with 1 µM propranolol for 10 min (Table 2).

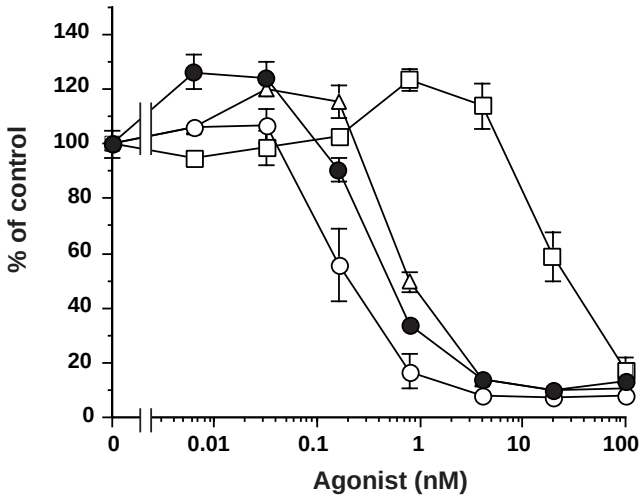

Fig. 2. Effect of β-adrenoceptor agonists on IgE-mediated tryptase release from HCMC. Sensitized HCMC were incubated with either S1319 (closed circle), isoproterenol (triangle), albuterol (square) and formoterol (open circle) for 30 min before challenge with anti-IgE (3 µg/ml) for a further 30 min for the release of tryptase. The results were normalized to the values in the absence of agonists as 100%. The control tryptase release was 25 ± 2%. Values are presented as the mean ± SEM from 3 independent experiments.

Table 1. The effect of β-adrenoceptor agonists (IC_{50} value) on IgE-mediated mediator release from HCMC.

	IC_{50} value (nM)	
β-adrenoceptor agonists	tryptase	TNF-α
S1319	0.51 ± 0.12	0.19 ± 0.03
isoproterenol	0.80 ± 0.09	0.32 ± 0.03
albuterol	28.0 ± 32.4	ND*
formoterol	0.15 ± 0.10	0.28 ± 0.02

Values are represented as the mean SEM.
* An IC_{50} value for albuterol could not be estimated reliably because maximal responses beyond 50% inhibition were not observed over the concentration range employed.

Table 2. Antagonism by 1 μM propranolol of the inhibitory effect of S1319.

Treatment	Tryptase release (%)	
	S1319	S1319 with propranolol
Control	42.8 ± 15.0	
S1319 (nM) 10	12.9 ± 6.8	42.8 ± 9.0
3.3	14.5 ± 6.3	44.4 ± 10.4
1.1	21.9 ± 10.4	43.5 ± 10.2
0.4	40.3 ± 14.4	43.5 ± 10.0

Sensitized HCMC were incubated without or with for 10 min, and then incubated with indicated concentration of S1319 for 30 min before challenge with anti-IgE (3 μg/ml) for a further 30 min for the release of tryptase. Values are presented as the mean ± SEM from 3 independent experiments.

mRNA expression of β_2-adrenoceptors in HCMC

The presence of β-adrenoceptors on HCMC has not been demonstrated previously. Therefore, non-quantitative RT-PCR of β_1-, β_2-, and β_3-adrenoceptor mRNA was performed in HCMC. As shown in Figure 3, only β_2-adrenoceptor message (329 bp) was amplified in HCMC.

Effect of β_2-adrenoceptor agonists on TNF-α production by HCMC

The effects of β_2-adrenoceptor agonists on IgE-mediated TNF-α production by HCMC were assessed. When we stimulated HCMC through FcεRI, we did not detect the production of TNF-α by the cells in our ELISA system. However, cells co-cultured with IL-4 for one week over the course of generating HCMC, showed a remarkable enhancement of IgE-mediated TNF-α production. Thus, we used the IL-4 treated HCMC for this experiment. The maximum production of TNF-α was detected at 16 h after activation and we therefore used this incubation period. S1319, isoproterenol and formoterol (0.1–10 nM) each inhibited the production of TNF-α ($p<0.01$) by HCMC in a concentration-dependent manner (Fig. 4). Approximate IC_{50} values of S1319, formoterol and isoproterenol for the inhibition of TNF-α production were 0.19 ± 0.03 nM, 0.28 ± 0.02 nM and 0.32 ± 0.03 nM (n = 3), respectively (Table 1). An IC_{50} value for albuterol could not be estimated reliably because maximal responses beyond 50% inhibition were not observed over the concentration range employed. Albuterol inhibited TNF-α production only at 10 nM ($p<0.01$). The rank order of potency for the inhibition was S1319 > formoterol > isoproterenol > albuterol, but there was no significant difference among the potency of S1319, isoproterenol and formoterol.

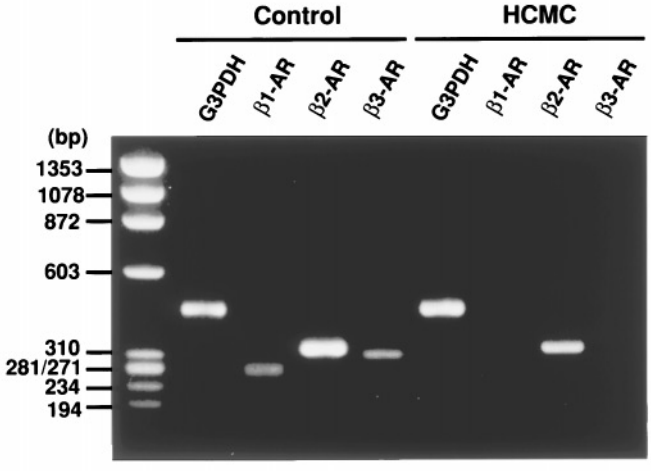

Fig. 3. RT-PCR studies of β-adrenoceptor-mRNA expression in HCMC. RT-PCR analysis was performed as described in Materials and Methods. The lengths of the fragments, calculated from the structure of the corresponding genes, were 265, 329, 314 and 450 bp for β_1-, β_2-, β_3-adrenoceptors and G3PDH, respectively. Human heart cDNA was used as positive control. The results presented are representative of two different experiments.

The effects of S1319 on intracellular cyclic AMP in HCMC

Previous studies have shown that isoproterenol induces an increase in total cyclic AMP levels in purified human lung mast cells [27]. Thus, if S1319 is acting through β-adrenoceptors to inhibit tryptase release and TNF-α production, then it is anticipated that S1319 would also increase intracellular cyclic AMP levels in HCMC. HCMC were incubated for 10 min with the indicated concentrations of S1319 and then samples were analyzed for cyclic AMP (Fig. 5). The unstimulated level of cyclic AMP in HCMC was 1.9 ± 0.3 pmol per 10^6 cells (n = 4) and S1319 caused concentration-dependent increases in intracellular cyclic AMP levels which

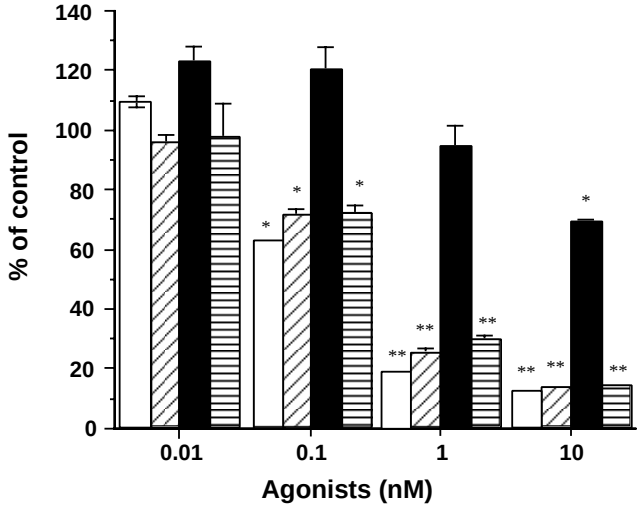

Fig. 4. The effect of β-adrenoceptor agonists on IgE-mediated TNF-α production by HCMC. Sensitized HCMC pre-treated with rh-IL-4 (10 ng/ml) for 7 days were incubated with either S1319 (open column), formoterol (diagonally hatched column), albuterol (closed column), or isoproterenol (horizontally hatched column), and for 30 min before challenge with anti-IgE (3 μg/ml) for a further 16 h for TNF-α production. Cell-free supernatants were assessed for TNF-α content by ELISA. Values show the concentration-dependent inhibition of the control TNF-α production which was 41.5 ± 2.0 (pg/10^6 cells). Data are presented as the mean ± SEM from 3 independent experiments. Asterisk denote statistically significant difference compared to the control (*; $p<0.01$, **; $p<0.001$).

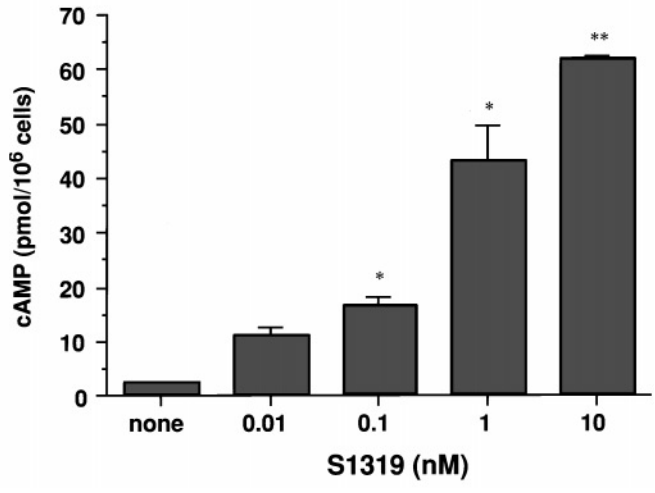

Fig. 5. Effect of S1319 on cyclic AMP levels in HCMC. Sensitized HCMC were incubated for 10 min with S1319 after which time the reaction was terminated and intracellular cyclic AMP levels were measured as described in Materials and Methods. Data are presented as the mean ± SEM from 4 independent experiments. Asterisks denote a significant difference compared to the no-treatment group (*; $p < 0.01$, **; $p < 0.001$).

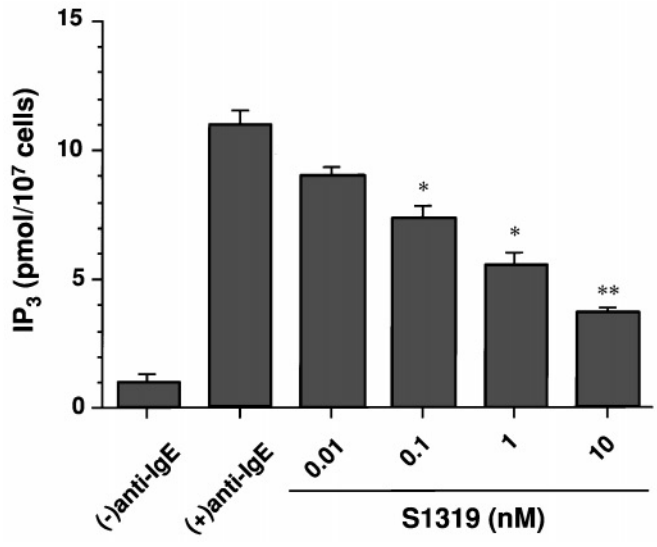

Fig. 6. The effect of S1319 on IgE-mediated IP_3 accumulation in HCMC. Sensitized HCMC were incubated with S1319 for 30 min before challenge with anti-IgE (3 μg/ml) for a further 15 s after which time the reactions were terminated and IP_3 levels were measured as described in Materials and Methods. Data are presented as the mean ± SEM from 4 independent experiments. Asterisks denote statistically a difference compared to the (+)anti-IgE group (*; $p < 0.01$, **; $p < 0.001$).

attained statistical significance at 0.1 nM. S1319 (10 nM) induced a 29 fold increase in intracellular cyclic AMP levels (61.4 ± 0.5 pmol per 10^6 cells, n = 4) and there was an evident correlation between the increases in intracellular cyclic AMP levels and the inhibition of tryptase release by S1319.

The effects of S1319 on intracellular IP_3 generation in HCMC

Along with the elevated intracellular Ca^{2+} concentration, additional primary signals are needed: for the degranulation and secretion of newly formed TNF-α, activation of PKC; and for the production of TNF-α, the co-activation of PKC and p42 MAP kinase [28]. And the major events in IgE-mediated signaling involve tyrosine phosphorylation of cellular proteins. However, little is known about the effects of β_2-adrenoceptor agonists on IgE-mediated IP_3 generation and protein tyrosine phosphorylation in human mast cells. First, we investigated the effect of S1319 on the generation of IP_3 in HCMC. The production of IP_3 reached a maximum at 15 s after activation and decreased to the basal level within 30 s in HCMC (data not shown). In this experimental condition, the enhancement of IgE-mediated IP_3 generation (10.9 ± 0.5 pmol per 10^7 cells, n = 4) was observed after activation for 15 s when the basal IP_3 generation was 0.9 ± 0.3 pmol per 10^7 cells (n = 4). As shown in Figure 6, S1319 produced a concentration-dependent inhibition of IgE-mediated IP_3 generation which attained statistical significance at 0.1 nM. There was an evident correlation between the inhibition of IP_3 generation and the inhibition of tryptase release by S1319.

The effect of S1319 on IgE-mediated protein tyrosine phosphorylation in HCMC

As shown in Figure 7a, immunoblotting analysis revealed rapid tyrosine phosphorylation of a number of proteins upon cross-linking of FcεRI, with the most prominent signals appearing at molecular masses of 180, 104, 84, 80, 42–40 and 36 kDa (Fig. 7a, lane 2). When HCMC were pretreated with 0.01 to 1 nM of S1319 for 30 min before activation, the tyrosine phosphorylation of 42 kDa protein (indicated by an arrow) was reduced (Fig. 7a, lane 3 to 5) in a concentration-dependent manner. S1319 (1nM) completely inhibited the

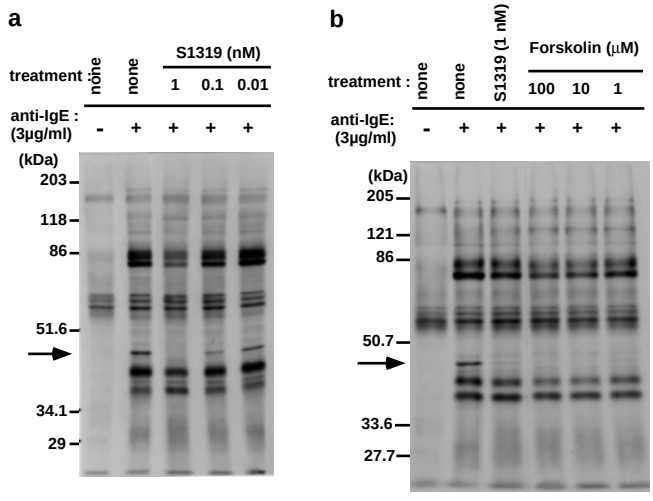

Fig. 7. Effects of S1319 and forskolin on protein tyrosine phosphorylation upon cross-linking of FcεRI in HCMC. Sensitized HCMC were pretreated with S1319 (**a**) and forskolin (**b**) for 30 min before challenge with anti-IgE (3 μg/ml) for a further 3 min. Protein tyrosine phosphorylation of total cellular proteins was analyzed as described in Materials and Methods. The arrow indicates the de-phosphorylated p42 MAP-kinase. The results presented are representative of two different experiments.

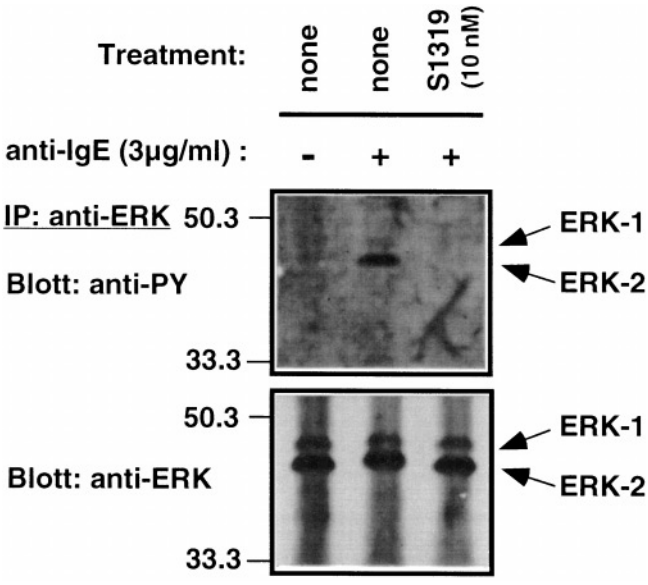

Fig. 8. Effects of S1319 on protein tyrosine phosphorylation of ERK-2 upon cross-linking of FcεRI in HCMC. Sensitized HCMC were pretreated with S1319 (10 nM) for 30 min before challenge with anti-IgE (3 μg/ml) for a further 3 min. The lysates were prepared as described in Materials and Methods, and immunoprecipitated with anti-ERK Ab and the precipitate were analyzed by immunoblottig with anti-phosphotyrosine Ab (anti-PY, upper panel) or anti-ERK Ab (anti-ERK, lower panel). The results presented are representative of two different experiments.

tyrosine phosphorylation of 42 kDa protein. The same result was obtained on pretreatment with isoproterenol and formoterol (data not shown). Furthermore, forskolin, which elevates cyclic AMP by direct activation of adenylate cyclase, inhibited the tyrosine phosphorylation of 42 kDa proteins in a concentration-dependent manner (Fig. 7b). Our previous study indicated that a possible candidate for 42 kDa proteins was p42 MAP-kinase, ERK-2 [21, 24]. Therefore, we next assessed the effects of S1319 on tyrosine phosphorylation of ERK-2 upon cross-linking of FcεRI. HCMC treated for the indicated conditions were lysed, and immunoprecipitated with anti-ERK Ab and were analyzed by immunoblotting with anti-phosphotyrosine Ab. As shown in Figure 8, whereas S1319 had no effect on the expression of ERK-2, at 10 nM, S1319 inhibited the tyrosine phosphorylation of ERK-2 almost completely.

Discussion

In the present study, we used the HCMC, which are suitable for analysis of human mast cells because phenotypic characterization of HCMC revealed considerable similarities with normal human mast cells [18–20] and HCMC possess functional signaling pathways via FcεRI [21]. Therefore, HCMC enabled us to evaluate the effects of β-adrenoceptor agonists on IgE-mediated activation without the possible actions of β-adrenoceptor agonists on contaminating cells. It was shown that HCMC resemble lung mast cells from the pharmacological point of view [29]. It is now generally agreed that HCMC will be useful for the analysis of the function of lung mast cells and the pharmacological study of lung mast cells. Still, the debate continues concerning the difference between HCMC and native human lung mast cells.

S1319 and a number of β-adrenoceptor agonists were found to inhibit the IgE-mediated release of tryptase from HCMC. Furthermore, a concentration of β-adrenoceptor agonists except albuterol as low as 1 nM was able to significantly inhibit the TNF-α production by HCMC. Here, we report on the inhibitory effects of β_2-adrenoceptor agonists on protein tyrosine phosphorylation in human mast cells. From the analysis of the inhibitory effects of S1319 on early and late events in IgE-mediated signal transduction in HCMC, it was suggested that S1319 inhibited the activation of both PLC and MAP kinase. Therefore, this inhibition of signal transduction pathways might account for some of the anti-inflammatory actions of S1319 and β_2-adrenoceptor agonists.

It is well known that β-adrenoceptor agonists are potent inhibitors of mediator release from human mast cells. It has been reported that β-agonists inhibit the release of IgE-mediated mediators such as histamine [10–15, 30], prostaglandin D_2 [11, 12] and leukotriene C_4 [11] from human lung mast cells and dispersed skin mast cells [13]. Recently, Shichijo et al. [29] showed that β-adrenoceptor agonists inhibited the release of mediators from cultured human mast cells generated by culturing cord blood mononuclear cells in the presence of SCF, IL-6 and prostaglandin E_2. Because prostaglandin E_2 influences the elevation of intracellular cyclic AMP levels, HCMC generated without prostaglandin E_2 seem to be more suitable for analyzing the role of β-adrenoceptor agonists which cause the elevation of intracellular cyclic AMP levels (*vide infra*). In this study, similar to previous results obtained using dispersed human lung mast cells, β-adrenoceptor agonists inhibited tryptase release from HCMC generated by a culture system. For example, isoproterenol inhibited the IgE-mediated histamine release from dispersed human lung mast cells [30] and HCMC (Fig. 2) with an IC_{50} of 6.3 and 0.8 nM, respectively. Furthermore, S1319 (Fig. 1), a novel β_2-selective agonist [22, 23], inhibited the tryptase release at the same potency as isoproterenol and formoterol (Fig. 2) and the inhibitory effect of S1319 on the tryptase release was blocked by addition of propranolol (Table 2). Although isoproterenol is a non-selective β-adrenoceptor agonist and propranolol is a non-selective β-adrenoceptor antagonist, that formoterol, albuterol and S1319, which are highly selective β_2-adrenoceptor agonists [23], inhibited tryptase release suggests that the inhibitory activity is mediated through β_2-adrenoceptors. Furthermore, RT-PCR analysis of β-adrenoceptor mRNA indicated that only β_2-adrenoceptors are present in HCMC (Fig. 3). These results suggest that the inhibitory effect of S1319 and β-adrenoceptor agonists is mediated by β_2-adrenoceptors.

The β_2-adrenoceptor agonist employed clinically are racemic mixture of R and S enantiomers [31]. It is shown that intravenous infusion of the S enantiomers of isoproterenol and albuterol into guinea pigs induced airways hyperresponsiveness and studies conducted in patient with mild asthma have suggested that a deleterious effect for the S enantiomer of albuterol in human [32]. Furthermore, the fact that S enantiomer of albuterol sinificantly enhanced IL-5 induced superoxide production from human eosinophils suggests that S enantiomer of β-adrenoceptor agonist may have pro-

inflammatory effects [33]. In this study, low concentration of β-adrenoceptor agonists enhanced the IgE-mediated tryptase release from HCMC (Fig. 2). In apparent agreement with these findings, the present results would appear to support the contention that pro-inflammatory effects of β-adrenoceptor agonists can develop in HCMC. The low concentration of β-adrenoceptor agonists may attribute to the pro-inflammatory effects, because R isomer is more rapidly metabolized than S isomer and then the anti-inflammatory effect by R isomer may be masked by the pro-inflammatory effect by S isomer [33]. It needs further investigation to reveal the difference of the effects of R and S enantiomer of β-adrenoceptor agonists.

Although there was some evidence that β_2-adrenoceptor agonists inhibited TNF-α production by human monocytic cells [34, 35] and human dispersed skin mast cells [15], the effect of β_2-adrenoceptor agonist on TNF-α production by human lung mast cells had not been well defined. As shown in Figure 4, both short-acting (S1319) and long-acting (formoterol) full β_2-adrenoceptor agonists inhibited the TNF-α production by HCMC, but a partial β_2-adrenoceptor agonist (albuterol) was significantly less potent than S1319 and formoterol. The tendency toward inhibitory activity of TNF-α production by β-adrenoceptor agonists is similar to that of tryptase release. However, the low IC_{50} value of isoproterenol (IC_{50} = 0.3 nM) in the present study contrasts with that (IC_{50} = 50 nM) for dispersed human skin mast cells [15]. The reason for this discrepancy is not clear, but it is possible that the reduced inhibitory effect of isoproterenol on dispersed human lung mast cells was due to the fact that these cells were subjected to a purification process resulting in a decrease in the number of functional receptors.

The activation status of mast cells, measured by the release of pro-inflammatory mediators, can be suppressed by rolipram and selective PDE inhibitors as well as by β_2-adrenoceptor agonists [36]. While the precise intracellular mechanism by which these agents inhibit mediator release from mast cells is not clear, an increase in intracellular cyclic AMP levels is widely associated with an inhibition of the activation status of a number of different inflammatory cell types and this is considered to be the mechanism by which β_2-adrenoceptor agonists and PDE inhibitors suppress inflammatory cell activation [37]. The present results demonstrate that increases in intracellular cyclic AMP levels, induced by β_2-adrenoceptor agonist, correlate with the inhibition of tryptase release and TNF-α production in HCMC. This is consistent with previous observations that isoproterenol induced increases in total cell cyclic AMP levels [27] and that the non-hydrolysable cyclic AMP analogue, dibutyryl-cyclic AMP, and the adenylate cyclase activator, forskolin, inhibited the IgE-mediated histamine release from human lung mast cells [36]. These results suggest that increases in intracellular cyclic AMP levels play an important role in the inhibitory effect of S1319 and β_2-adrenoceptor agonists on tryptase release and TNF-α production in HCMC.

Though there is much evidence to support the idea that increases in intracellular cyclic AMP levels are important to the inhibitory effect of β_2-adrenoceptor agonists, little is known about the effect of β_2-adrenoceptor agonists on the IgE-mediated signal transduction pathways in human lung mast cells. A major limitation in this area of research is the difficulty in obtaining a large number of highly purified human lung mast cells. Previous studies have shown three functional responses of mast cells to antigen stimulation as follows. Along with an elevated intracellular calcium ion concentration, the primary signals are as follows: for degranulation, activation of protein kinase C (PKC) [38]; for cytosolic phospholipase A_2-mediated release of arachidonic acid, activation of p42 MAP kinase (ERK-2) [39]; and for production of TNF-α, the co-activation of PKC and p42 MAP kinase [28]. In the present study, S1319 inhibited the IgE-mediated generation of IP_3 and there was a correlation between the inhibition of IP_3 generation and the inhibition of tryptase release (Fig. 2 and 6). In rodent mast cells, PLC-γ catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in the generation of IP_3 and diacylglycerol which activate PKC [34]. It is further evident that PLC-γ1 is activated upon IgE-mediated activation in HCMC [21]. Therefore, the fact that IP_3 generation is inhibited by S1319 suggests that the activation of PLC-γ1 is inhibited by S1319, and in turn, that the activation of PKC and the elevation of intracellular Ca^{2+} may also be inhibited by S1319. Thus, it is likely that S1319 and β_2-adrenoceptor agonists inhibit the IgE-mediated activation of the PLC/PKC cascade of the signal transduction pathway in human mast cells.

The present results demonstrated that the IgE-mediated tyrosine phosphorylation of 42 kDa protein was almost completely inhibited by S1319 (Fig. 7a). Our previous studies indicated that one candidate for the 42 kDa protein was ERK-2 [21, 25]. This is consistent with our result that tyrosine phosphorylation of ERK-2 was inhibited by the addition of S1319 (Fig. 8). These results suggest that S1319 inhibits the IgE-mediated activation of the MAP kinase cascade of the signal transduction pathway in human mast cells. In T lymphocytes, forskolin inhibited anti-CD3-induced MAP kinase activation and this is due to Raf-1 phosphorylation by protein kinase A which is activated by cyclic AMP [41]. This is consistent with our result that forskolin inhibited the tyrosine phosphorylation of 42 kDa protein, which is probably p42 MAP kinase (Fig. 7b, 8). In point of fact, it would seem more likely that the elevation of intracellular cyclic AMP results in the regulation of Raf-1 activity [42–44]. Although, in RBL-2H3 (the rat mast cell line), the Raf-1/MAP kinase kinase (MEK)/p42 MAP kinase pathway regulated the production of both TNF-α and arachidonic acid [28], there was no evidence that the production of TNF-α was regulated by the Raf1/MEK/p42 MAP kinase pathway in human mast cells. The present results that S1319 and forskolin inhibited the tyrosine phosphorylation of p42 MAP kinase may suggest that the IgE-mediated Raf-1/MEK/p42 MAP kinase pathway is inhibited resulting in the inhibition of TNF-α production in HCMC although, clearly, more work would be required to substantiate this.

Controversy exists about the therapeutic mode of action of β_2-adrenoceptor agonists, especially whether or not they have significant anti-inflammatory activities in the treatment of asthma. The inhibition of the release of histamine [10–15], prostaglandin D_2 [11, 12] and leukotriene C_4 [11] by β_2-adrenoceptor agonists from human lung mast cells, dispersed skin mast cells and HCMC (in the present study) has been suggested by *in vitro* studies. However, β_2-adrenoceptor agonists have generally been demonstrated not to inhibit the late

allergic reaction after antigen-challenge or the accompanying rise in non-specific bronchial responsiveness [45]. There is evidence that mediator release from both human alveolar macrophages [46] and human eosinophils [47] is not inhibited by β_2-adrenoceptor agonists. They are thought to be important effector cells in the pathogenesis of asthma, and the lack of influence of β_2-adrenoceptor agonists on these cell type may explain in part the poor efficacy of these agents as monotherapy in asthma. It is also demonstrated that the β_2-adrenoceptor is susceptible to functional desensitization in the human lung mast cells [48]. Two recent studies have suggested that downregulation of these β_2-adrenoceptor on mast cells could results in increased susceptibility to inhaled allergen in allergic asthmatics [49, 50]. Finally, it has been proposed that the reason for the lack of clinical significance could be that the period during which the conventional agents remain in the body is too short to counteract the potent pro-inflammatory effect of continuous allergen exposure [12]. With the extended duration of action, it is possible that the new generation of long-acting β_2-adrenoceptor agonists would provide better control of the underlying airway inflammation in asthma.

In summary, the present work has established that S1319, a novel β_2-adrenoceptor agonist, attenuates the IgE-mediated response of HCMC by interfering with the activation of both the PLC/PKC cascade of signals resulting in degranulation and the MAP kinase/phospholipase A_2 pathway resulting in the production of TNF-α. These findings define the importance of β_2-adrenoceptor agonists not only as a bronchodilator but also as an anti-inflammatory agent by modulation of the degree of mast cell activation and cytokine production.

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