

Rolipram Inhibits Phosphorylation and Activation of ERK/MAP Kinase Signalling Pathways in Allergen-activated Human Peripheral Mononuclear Cells

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Abstract: Phosphodiesterase (PDE) enzymes catalyze breakdown of cAMP and cGMP, which is known to have potent immunomodulatory capacity. Here, we aimed at clarifying whether PDE4 is involved in allergen-induced activation of immunocompetent cells. To address this issue, human peripheral blood-derived mononuclear cells (PBMC) were cultured with mite antigen, *dermatophagoides farinae* (Der-f), in the presence or the absence of a specific PDE4 inhibitor, rolipram or a non-selective PDE inhibitor theophylline. Semiquantitative RT-PCR analysis demonstrated that stimulation of cells with Der-f led to upregulated expression of IL-5 mRNA, which was clearly downregulated by both inhibitors. Downregulation of Der-f-induced IL5 mRNA was similarly observed when cells were treated with U0126, a specific MEK/ERK inhibitor. This suggests that ERK signaling pathways are involved in this event. This was further supported by the observation that rolipram as well as theophyllin interfered with phosphorylation of ERK1/2 induced by Der-f as determined by Western blot analysis using an antibody directed against phosphorylated ERK1/2. Finally, immunostaining showed that stimulation to PBMC with Der-f led to nuclear translocation of AP-1 components (JunD, Fra-2), whose migration was inhibited by rolipram, indicating the interference with activation of AP-1 transcription factors. Together, the present study indicates that allergen-induced activation of immunocompetent cells was prevented by rolipram through interference with ERK1/2 signaling.

Key words: Immune modulation, antigen presentation, phosphodiesterase inhibitors, allergic disorders

INTRODUCTION

It has been thought that incidence of allergies by inhalant allergens has been increased in last decades possibly due to worldwide popularization of modern lifestyle, consisting of airtight and humid living circumstances in which aerosol antigen such as mites may favorably grow (Sublett, 2005). In this context, it is well-known that atopic disorders including allergic rhinitis, asthma and eczema are, at least in part, pathogenically attributed to hypersensitivity to mites (Gaffin and Phipatanakul, 2009). After invasion to target organs, mite antigens may activate antigen-specific T cell responses via activation of antigen presenting cells (macrophages, tissue specific dendritic cells). Hence, immune cascades leading to allergies may be attributed to crosstalk between different types of immunocompetent cells.

Adenosine 3'5'-cyclic monophosphate (cAMP) is widely accepted to exert a broad suppressive effect on the

activation of most inflammatory cells (Lugnier, 2006). Agents which elevate cAMP levels thus have the potential to inhibit cellular activation. The phosphodiesterase (PDE) enzymes catalyze the breakdown of cAMP and guanosine 3'5'-cyclic monophosphate (cGMP). Among at least 15 genes encoding over 30 different isoforms of heterologous members of PDE family reported to date (Torphy, 1998; Houslay and Kolch, 2000), PDE type 3 and 4 are the predominant isoforms found in immune cells (Ekholm *et al.*, 1997; Bielkova *et al.*, 2000). Rolipram ((±) 4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone), is a specific PDE4 inhibitor, whose therapeutic utility has been investigated in treatment of depression (Zhu *et al.*, 2001). Furthermore, rolipram and other specific PDE4 inhibitors have the capacity to suppress inflammatory processes (Herve *et al.*, 2008; Hirose *et al.*, 2008). A large body of evidence suggests that PDE4 inhibitors might possess anti-inflammatory effects and thus be a newer

strategy to control allergic diseases (Lugnier, 2006; Torphy, 1998; Zhu *et al.*, 2001; Herve *et al.*, 2008; Hirose *et al.*, 2008; Spina *et al.*, 1998; Torphy and Page, 2000). Recently, it was reported that rolipram in combination with lovastatin may suppress severity of experimental autoimmune encephalomyelitis of mice, which is well-known to be a human analogue of multiple sclerosis (Goldhoff *et al.*, 2008). Together, to control allergic as well as immune disorders PDE4 inhibitors, such as rolipram, might be of therapeutic interest.

We have previously shown that the PDE4 inhibitor rolipram may inhibit IL-4-induced activation of T cells via interference with IL-4-specific signal transduction, i.e., prevention of AP-1 activation (Markova *et al.*, 2007). However, the experimental system in the report did not include antigen, so that we do not know yet whether rolipram is able to modulate antigen-induced activation of immune systems. Here, we used peripheral blood mononuclear cells (PBMC), since this may include antigen presenting cells (peripheral macrophages, monocytes) and antigen specific responder cells (T-cells) and enabled us to examine immune responses to mite antigen.

Keeping in mind these notions, we were interested to investigate whether PDE4 inhibitor rolipram may modulate Der-f-induced activation of immune cascades in freshly isolated human PBMC.

MATERIALS AND METHODS

Study: This research project was conducted from April 1, 2003 to March 31, 2008 at Department of Pharmacology in Kinki University School of Medicine.

Reagents: The following reagents were obtained from manufactures described as follows: Ficoll-Paque™ from Amersham Pharmacia Biotech AB, Uppsala, Sweden. Rolipram and theophylline (Wako Pure Chemical Industries, Osaka, Japan), RP-8-Br-cAMP and MEK/ERK inhibitor U0126 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Cell lysis extraction reagent and phosphatase inhibitor cocktail, casein blocking buffer were all from Sigma, St. Louis, Missouri, USA. Protease inhibitor cocktail was from Boehringer Mannheim, Mannheim, Germany. Dermatophagoides farinae (Der-f) antigen from Seikagaku Biobusiness Corporation, Tokyo, Japan. Phospho-p44/42 MAP kinase (Thr 202/Tyr 204) and p44/42 MAP kinase antibodies were obtained from Cell Signaling Technology, Beverly, MA, USA. We also purchased RNeasy kit and Taq PCR Core kit from Qiagen, Hilden, Germany, reverse transcriptase system from Promega, Madison, WI, USA,

primers for human IL-5 (Tanabe *et al.*, 1987) and human β -actin (Gunning *et al.*, 1983) from Takara Bio Inc., Shiga, Japan. The following sequences were chosen for PCR primers:

- **Human β -actin (sense):** 5'-CACCTTCTACAATGAG CTGC-3'
- **Human β -actin (antisense):** 5'-TCCATGAGGTAGTC CGTCAG-3'
- **Human IL-5 (sense):** 5'-GCTTCTGCATTTGAGTTTG CTAGCT-3'
- **Human IL-5 (antisense):** 5'-TGGCCGTCATGTATT TCTTTATTAAG-3'

Preparation of freshly isolated peripheral blood mononuclear cells: Human Peripheral Blood Mononuclear Cells (PBMC) were obtained from heparinized venous blood of healthy volunteers through a density gradient centrifugation method using Ficoll-Paque®. PBMC were collected from the interface and washed twice with phosphate-buffered saline (PBS).

PBMC stimulation: PBMC were cultured in RPMI1640 medium supplemented with 1% L-glutamine and 1% antibiotics/antimycotics (all from Invitrogen Japan, Tokyo, Japan), at a density of 5×10^6 cells mL^{-1} throughout this study. Rolipram was dissolved in DMSO at a concentration of 10^{-2} M and prepared immediately before use. The final concentration of DMSO in each well was no greater than 0.1%. All other compounds were solubilized in medium. Cells were pre-incubated for 30 min with rolipram (10^{-7} to 10^{-5} M), theophylline (10^{-7} to 10^{-5} M) or MEK/ERK inhibitor U0126 ($10 \mu\text{M}$) before stimulation with $1 \mu\text{g mL}^{-1}$ Der-f antigen.

Semiquantitative RT-PCR: Eighteen hours after addition with Der-f, cells were harvested and RNA samples prepared by use of RNeasy kit. All RNA samples were subjected to reverse-transcription of cDNA using a reverse transcriptase system according to the manufacturer's instructions. The cDNA products were amplified with the designated primer pairs using Taq PCR Core kit. To perform following PCR amplification in a semi-quantitative way (Markova *et al.*, 2007), serially diluted samples were amplified with β -actin primers with different cycle numbers and PCR conditions yielding exponential increase in PCR signals were used for further study. The cDNA samples were then amplified using specific primers for IL-5. Cycling parameters were as follows: denaturation at 95°C for 30 sec; annealing at 55°C for 45 sec and extension at 72°C for 1 min for the appropriate number of cycles giving a visible signal on

agarose gels without reaching saturation (30 cycles for β -actin and 40 cycles for IL-5), followed by a 10 min extension at 72°C. PCR products were subsequently size-fractionated on 2% agarose gels, stained with ethidium bromide and visualized under UV light. Signal intensity was assessed using semiautomated software NIH Image, version 1.62 (National Institutes of Health, Bethesda, MD, USA), on an Apple Macintosh Power PC (Mac OS 7.5.5). Relative signal intensity was calculated as the measured intensity of the IL-5 mRNA signal/the measured intensity of the β -actin mRNA signal yielded from the same sample.

Western blot analysis: PBMC were seeded in serum free medium, treated with drugs and stimulated with 1 $\mu\text{g mL}^{-1}$ Der-f antigen. Cells were harvested in 15 min, lysed with cell lysis extraction reagent containing phosphatase inhibitor cocktail and protease inhibitor cocktail and sonicated. Cell debris was removed by centrifugation at 15,000 rpm for 15 min at 4°C. Protein concentrations of the supernatants were determined by the Bio-Rad protein assay reagent and 40 μg of protein diluted with equal volumes of 2 x sodium dodecyl sulfate (SDS) sample buffer was denatured by heating at 95°C for 5 min, which were resolved in 10% SDS-polyacrylamide gels. After completion of electrophoresis, fractionated protein samples were further transferred to nitrocellulose membranes (Bio-Rad) in a buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% SDS and 10% methanol. Blots were blocked in casein blocking buffer for 12 h at 4°C and incubated with primary antibody diluted 1:1,000 in casein blocking buffer overnight at 4°C. After extensive washing in PBS containing 0.1% Tween 20, blots were incubated with secondary anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) diluted 1:10,000 in casein blocking buffer at room temperature for 1 h. After extensive washing, specific signals were visualized by using the ECL-plus® Western Blotting Detection System (Amersham).

Immunostaining: Immunostaining was performed as described elsewhere (Aragane *et al.*, 1998). Briefly, human PBMC were cultured with 10^{-5} M rolipram or 10^{-5} M theophyllin and stimulated with 1 $\mu\text{g mL}^{-1}$ Der-f. Cells were harvested 15 min later, washed twice in ice-cold PBS (without Mg^{++} and Ca^{++}), resuspended in serum-free RPMI1640 and attached on glass slides by centrifugation. The samples were fixed in 4% paraformaldehyde for 10 min, followed by extensive washing twice in ice-cold PBS. Non-specific binding was blocked by incubation with PBS containing 2% goat serum for 1 h at 4°C. The

primary antibody (an antibody directed against Fra-2 or JunD) was added and incubated overnight at 4°C. After extensive washing with ice-cold PBS, the secondary antibody (FITC-conjugated anti-rabbit IgG from the goat) was applied to the reactions and incubated overnight for 4°C. The samples were washed, covered with Vectashield® mounting medium with DAPI (Vector laboratories, Burlingame, CA) and observed using a confocal laser scanning microscope.

Statistical analysis: Statistical analysis was performed using student t-test and $p < 0.05$ is referred to be significant.

RESULTS

The PDE4 inhibitor, rolipram, inhibited allergen-induced IL-5 mRNA expression in human PBMC: Previously, we showed the ability of rolipram to interfere with IL-4-induced activation of AP-1 transcription factors leading to downregulation of IL-4-upregulated IL-5 mRNA expression (Markova *et al.*, 2007). In this study, we addressed the question whether PDE4-specific inhibitor, rolipram, may interfere with antigen-induced activation of immunocompetent cells. As is well-known, IL-5 plays a pivotal role in many allergic disorders (Kouro and Takatsu, 2009), including atopic dermatitis, bronchial asthma and allergic rhinitis, all of which are thought, at least in part, to be caused by hypersensitivity reaction induced by inhalant allergens, such as house dust mites (Gaffin and Phipatanakul, 2009). In this context, it is worth to examine the above mentioned question regarding the immunomodulatory activity of rolipram. Throughout this study, PBMC were freshly prepared and cultured with Der-f, since these cells include some fractions of immune cells necessary to initiate immune cascades. Accordingly, the cells were incubated with or without PDE4-specific inhibitor, rolipram or non-selective PDE inhibitor, theophyllin. The 18 h later, cells were harvested, RNA was prepared and reverse-transcribed and semiquantitative RT-PCR was conducted using primers specific for human IL-5 or human β -actin. As shown in Fig. 1, Der-f markedly induced IL-5 mRNA expression. Graded concentrations of rolipram ranging from 10^{-7} to 10^{-5} M significantly downregulated Der-f-upregulated IL-5 mRNA (Fig. 1a). Similarly, theophylline at a concentration of 10^{-5} M also significantly downregulated IL-5 mRNA induced by Der-f (Fig. 1b).

Effect of blockade of ERK1/2 signaling pathway on Der-f-induced expression of IL-5 mRNA: We have previously shown that rolipram may interfere with activation of ERK1/2 kinases in purified human T-cells that were

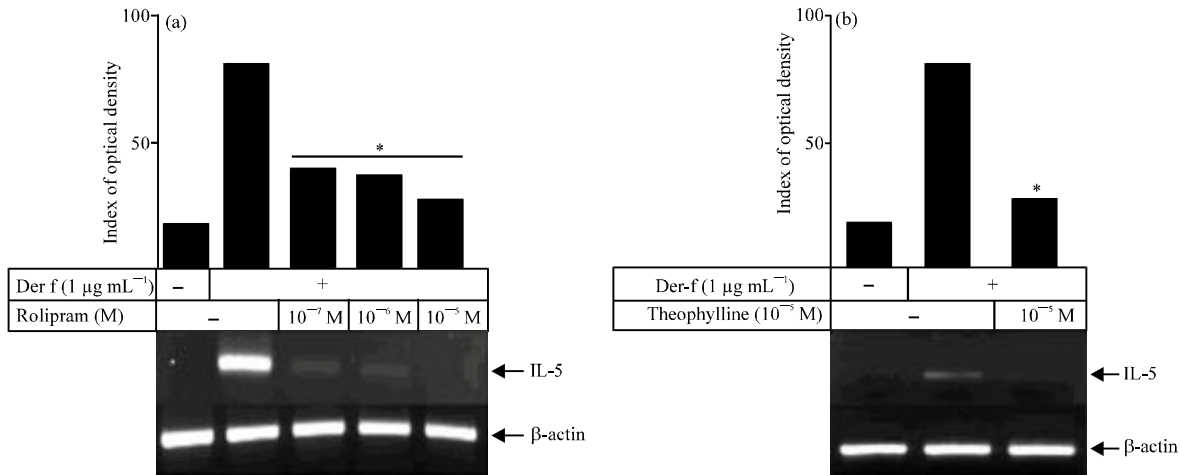


Fig. 1: Downregulation of mite-antigen-induced expression of IL-5 mRNA in PBMC by phosphodiesterase inhibitors. Human PBMC (5×10^6 cells mL⁻¹) were cultured in the presence or the absence of 1 μg mL⁻¹ Der-f antigen with or without the indicated concentrations of rolipram (a) or of theophylline (b) 18 h later, cells were harvested, RNA extracted, cDNA synthesized and semiquantitative RT-PCR performed using primers specific for IL-5 or β-actin. In both (a) and (b), the lower column shows raw PCR results, which were densitometrically scored and compared in the upper column. Densitometric data are depicted for IL-5 for all subjects. Data were normalized to Der-f-treated cells: nonstimulated drug-free controls and with background values subtracted. *p<0.05 when compared with Der-f-treated but drug-untreated cells. Note that densitometric score of Der-f-treated cells was significantly higher than that of non-treated control cells (p<0.001), which were however not depicted in the figure. The data presented are one of representative of four independent experiments

activated by IL-4 without the presence of antigens (Markova *et al.*, 2007). Here, we checked whether Der-f-induced upregulation of IL-5 mRNA in PBMC involves ERK kinases. Therefore, cells were treated or left untreated with a specific MEK/ERK inhibitor, U0126 (Favata *et al.*, 1998) at a concentration of 10 μM 30 min before stimulation with Der-f. The 18 h later, cells were harvested and processed as done in Fig. 1. Consequently, Fig. 2 shows that U0126 alone markedly downregulated Der-f-upregulated expression of IL-5 mRNA, strongly suggesting that ERK signaling pathways are involved in Der-f-induced activation of T-cells.

Prevention of phosphorylation of ERK1/2 by rolipram:

Based on the results in Fig. 2, we further address the question if rolipram interferes with activation of ERK1/2 kinases. Therefore, PBMC were serum-starved and treated with Der-f in the presence or the absence of rolipram, cAMP analogue 8-Br-cAMP or theophylline. Fifteen minute later, cells were harvested and protein extracts prepared. Equal amounts of cell lysates were subjected to SDS-PAGE and Western blot analysis performed using an antibody directed against phospho-p44/42 MAP kinase (Thr 202/Tyr 204), specific for the activated (phosphorylated) form of ERK1/2. This antibody

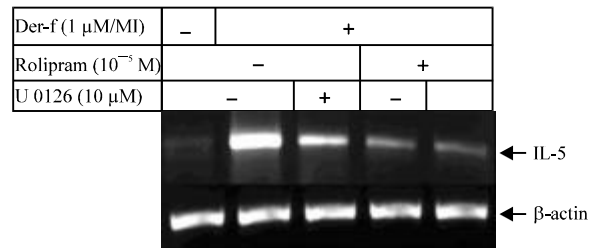


Fig. 2: Specific downregulation of mite-antigen-induced expression of IL-5 mRNA in PBMC by a specific MEK/ERK inhibitor, U0126. Human PBMC (5×10^6 cells mL⁻¹) were cultured in the presence or the absence of 1 μg mL⁻¹ Der-f antigen with or without 10⁻⁵M rolipram and/or 10 μM a highly specific MEK/ERK kinase inhibitor U0126. The 18 h later, cells were harvested and semiquantitative RT-PCR done as performed in Fig. 1. The data presented are one of representative of four independent experiments

recognizes ERK only when it is phosphorylated on both threonine and tyrosine residues. By using this specific antibody, it appears that two faint bands were detected at the expected sizes (42 and 44 kD) when cells were left

untreated (the leftmost lane of the upper column in Fig. 3). Cells treated with Der-f exhibited detectable phosphorylation of ERK1/2 15 min poststimulation. As compared to the positive control (cells treated with Der-f), rolipram as well as theophylline or 8-Br-cAMP interfered with Der-f-induced phosphorylation of ERK kinases. Together with Fig. 2, these data indicate that Der-f-induced activation of immunocompetent cells was mediated via ERK signaling pathways, which can be inhibited by rolipram.

Interference of Der-f-induced activation of AP-1 components by rolipram: We previously could show that AP-1 may be a prominent transcription factor in T-cells in response to IL-4 (Yamazaki *et al.*, 2002; Maeda *et al.*, 2003). Rolipram inhibited activation of AP-1 by means of inhibition of its nuclear migration, in human T-cells stimulated with IL-4 (Markova *et al.*, 2007). These findings attempted us to examine whether rolipram interferes with activation of AP-1 when activated in Der-f-stimulated immune cells. To do so, immunostaining was conducted using antibodies raised against JunD- or Fra-2-components of AP-1. Because AP-1 ultimately translocates in nuclei to activate transcription of AP-1-responsive genes, AP-1 integration in nuclei should be interfered when rolipram has suppressive activity on

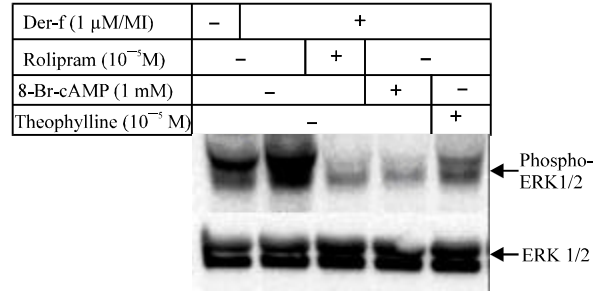


Fig. 3: Interference with Der-f-induced phosphorylation of ERK1/2 kinase by rolipram, cAMP analogue 8-Br-cAMP, theophylline. Human PBMCs (5×10^6 cells mL^{-1}) were pretreated or left untreated (the leftmost lane) for 30 min with 10^{-5} M rolipram (the third lane from the leftmost), 1 mM 8-Br-cAMP (the fourth lane from the leftmost), or 10^{-5} M theophylline. The cells were then cultured without or with 1 $\mu\text{g mL}^{-1}$ Der-f antigen for 15 min and lysed. The lysates were electrophoresed in 10% tris-glycine polyacrylamide gels, transferred to nitrocellular membranes and immunoblotted with an anti-phosphorylated ERK1/2 antibody (the upper panel). The same blot were reprobed with anti-ERK1/2 antibody to ensure the equal loading among the lanes (the lower panel)

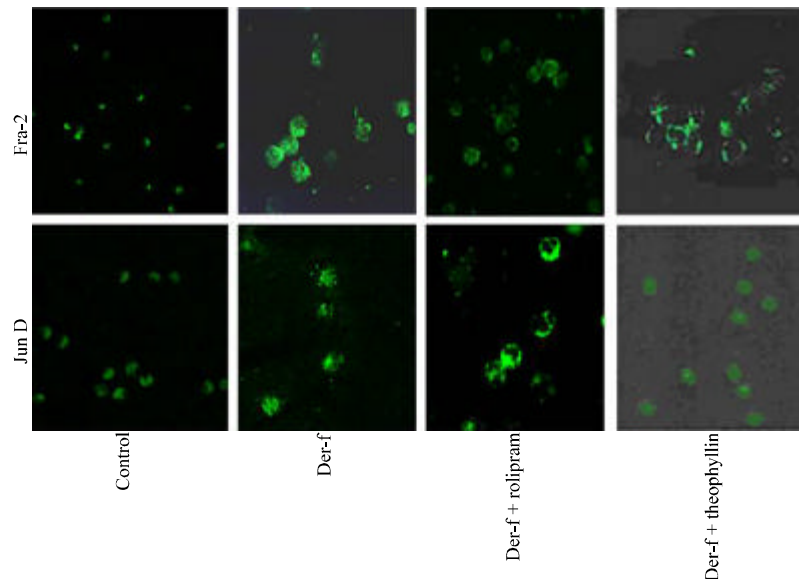


Fig. 4: Effect of rolipram and theophylline on nuclear migration of Der-f-activated components (JunD, Fra-2) of AP-1 transcription factor. Human PBMC (5×10^6 cells mL^{-1}) were cultured in the presence of 1 $\mu\text{g mL}^{-1}$ Der-f with or without 10^{-5} M rolipram or 10^{-5} M theophylline. Cells were harvested 15 min later and attached onto glass slides. After fixation, non-specific reaction was blocked with goat serum and after extensive washing a primary antibody directed against JunD or Fra-2 was applied and incubated overnight at 4°C . The reaction was then visualized using FITC-conjugated anti-rabbit Ig and observed under a confocal laser scanning microscope

activated AP-1. The cells were treated or left untreated with rolipram or theophyllin in the presence of Der-f. 15 min later, cells were harvested, extensively washed twice with ice-cold PBS, fixed on slide glasses and immunostaining was performed using antibodies raised against AP-1 components, JunD (Kobierski *et al.*, 1991) or Fra-2 (Suzuki *et al.*, 1991). Figure 4 shows that in untreated cells JunD located in cytoplasm, which in turn migrated into nuclei when stimulated with Der-f. The same applied for Fra-2 (Fig. 4). The selective PDE4 inhibitor rolipram prevented Der-f-induced migration of JunD and Fra-2 to nuclei, while non-selective PDE inhibitor theophyllin did so for Fra-2 but not for JunD (Fig. 4).

DISCUSSION

In this study, we were trying to elucidate the effects of rolipram on the Der-f-induced lymphocyte activation (immune activation). Throughout this study, we stimulated human PBMC with Der-f in our experimental setting. To date there is evidence that rolipram may modulate activation of immunocompetent cells. In this context, it is worth to mention that cAMP-elevating agents induce rather than block IL-5 production in T cells stimulated by mitogens or via the TCR-CD3 complex (Betz and Fox, 1991; Snijdwint *et al.*, 1993; Lee *et al.*, 1993; Siegel *et al.*, 1995; Schmidt *et al.*, 1995). It was observed that PDE-related pharmacological agents capable of the increase in intracellular cAMP inhibited Th1 cytokine production (IL-2 and IFN- γ), but had no effect on Th2 cytokine (IL-4, IL-5 and IL-10) production (Munoz *et al.*, 1990; Katamura *et al.*, 1995). Altogether, these previous observations were seemingly contradictory to our present data shown here, however, but generated by use of somewhat more artificial systems than the use of freshly isolated human PBMC.

By contrast, it was shown previously that stimulation of T cells with immobilized anti-CD3 resulted in secretion of IL-2, IL-5, IL-10 and TNF β all of which were inhibited by rolipram (Jimenez *et al.*, 2001). Similarly to our present work, other reports showed that rolipram as well as cAMP-elevating agents inhibited IL-5 release from human PBMC when stimulated with anti-CD3 plus anti-CD28 (Secchiero *et al.*, 2000). Stimulation of T cells with immobilized anti-CD3 leads to activation of T-Cell Receptor (TCR), which may result in T cell proliferation and cytokine synthesis. Furthermore, stimulation with anti-CD3 and anti-CD28 activates TCR and co-stimulatory molecule CD86 (and/or CD80), respectively. Together, both of above mentioned ways of stimulation mimic antigen-induced activation of T-cells, but not are identical to antigen-induced activation of T-cells, because

antibody-mediated activation of T-cells does not restrict to any antigens and is not antigen-specific. Hence, throughout this study we stimulated PBMC only with Der-f, since, theoretically, antigenic information of Der-f expressed on antigen presenting cells (i.e., peripheral macrophages) may activate T cells specific for Der-f. We thought that this experimental system is less artificial and more closed to that seen in periphery in allergic individuals when compared to those with the antibodies. Although, the stimulation index of Der-f is much lower than that of the antibodies (data not shown), this point was successfully overcome by use of sensitive methods, PCR and immunostaining. Furthermore, since dermatophagoides is known to be one of causal inhalant allergens in atopic disorders (Gaffin and Phipatanakul, 2009) (bronchial asthma, non-seasonal allergic rhinitis, atopic dermatitis, etc.), use of this antigen may be of clinical relevance.

By using this experimental system, we showed that Der-f upregulated the expression of IL-5 mRNA in PBMC (Fig. 1). We convince that IL-5 producing cells in this system is T cells by following reasons, 1) IL-5-producing cells should be able to recognize Der-f allergen, that is mediated by specific receptor (TCR), 2) T cells are the major internal source of IL-5 (it was originally called as T-cell replacing factor (TRF) (Takatsu, 1992). Together, we think that peripheral antigen presenting macrophages or other equivalents (i.e., dendritic cells) may express antigenic peptides of Der-f driven on their Major Histocompatibility Antigen Complex (MHC), present antigenic information to Der-f-specific T cells, which synthesized IL-5. This pathway may not be mutually exclusive, but may cover the majority. Upregulation of IL-5 mRNA was almost completely inhibited by specific PDE4 inhibitor, rolipram or non-selective PDE inhibitor theophylline (Fig. 1a and b, respectively). Because among at least 11 different PDE enzymes PDE3 and 4 are the predominant ones (Ekholm *et al.*, 1997; Bielkova *et al.*, 2000), suppression of Der-f-induced IL-5 mRNA expression by rolipram (PDE4-specific inhibitor) makes much sense.

Downregulatory effects of rolipram on Der-f-induced expression of IL-5 mRNA is mediated via the induction of ERK1/2 kinase activation, because the specific MEK/ERK inhibitor, U0126 downregulated Der-f-induced expression of IL-5 mRNA similarly as rolipram did (Fig. 2). This notion was further confirmed by the observation that phosphorylation of ERK1/2 kinases induced by Der-f was inhibited by rolipram as well as cAMP elevating agent, 8-Br-cAMP as shown by Western blot analysis (Fig. 3). Based on these results, the signaling pathway involved is thought to be as follows, 1) ligation of MHC to TCR by

mediation of antigenic Der-f-peptides leads to activation of Raf kinase possibly mediated via Ras kinase, resulting in activation of MEK kinases, which further activates ERK kinases. Deactivation of Raf-1 may result in deactivation of downstream signaling intermediates, such as MEK kinases and/or ERK kinases, as similarly demonstrated in Fig. 3. It remains unclear if rolipram interferes with antigen-induced activation of Raf-1 or does so for downstream signaling intermediates. This should be a target of future study. Functional consequence of rolipram-deactivated ERK kinases was shown in Fig. 4 that nuclear migration of JunD and Fra-2 components of AP-1 was interfered by rolipram, while that of Fra-2 but not JunD was done so with theophylline. It was previously reported that JunD forms a heterodimer with Fra-2 that possesses the potent transcriptional activation leading to cell differentiation and others (Hernandez *et al.*, 2008). This might be possible that rolipram at least in part suppresses the activation of JunD/Fra-2 heterodimers of AP-1 complex. In contrast, theophylline only did so for Fra-2 but not JunD, suggesting that AP-1 complexes suppressed by theophylline is composed of Fra-2 bound to Jun family of transcription factors other than JunD. It remains to be clarified yet which one is the case.

Previously, it was reported that cAMP-inhibited activation of T cells was associated with suppression of MAPK pathways, ERK kinases and c-Jun N-terminal kinase (c-JNK) (Tamir *et al.*, 1996). Our present study clearly demonstrates that rolipram may interfere with mite-antigen-induced T-cell activation, such as downregulation of antigen-induced IL-5 mRNA expression, phosphorylation of ERK1/2 and nuclear migration of the AP-1 complex, although exact target of rolipram in this event remains to be determined.

Together, this study for the first time shows that phosphodiesterase inhibitors, rolipram via suppression of ERK signaling pathway may suppress the mite-antigen-induced activation of human PBMC, presumably of T-cells. Further investigation is warranted to elucidate more precise mode of actions of phosphodiesterase inhibitors at a molecular basis. This may contribute to welfare and health of patients suffering from long-lasting and so far therapy-resistant disorders, such as allergies.

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