

Antagonism of IL-4 Signaling by a Phosphodiesterase-4 Inhibitor, Rolipram, in Human T Cells

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Key Words

AP-1 components • cAMP • IL-4 • IL-5 • PDE4 inhibitors • PKA • Rolipram • Signal transduction • T cells

Abstract

Background: Phosphodiesterase (PDE4) inhibitors prevent breakdown of cAMP and affect the increase in cellular levels of cAMP, which is known to regulate immune cell functions. Because IL-4 plays a causal role in the pathogenesis of allergic disorders, we were interested to study the modulatory mechanisms of a PDE4 inhibitor, rolipram, in IL-4-mediated signaling in T cells. **Methods:** Human peripheral T cells were stimulated with IL-4 in combination with rolipram, and RT-PCR was performed using primers specific for IL-5. To monitor activation of transcription factors, immunostaining was employed. **Results:** Rolipram or a cAMP-analogue, 8-Br-cAMP, significantly downregulated IL-4-induced expression of IL-5 mRNA. The rolipram-induced inhibition of IL-5 mRNA was mediated by activation of protein kinase A (PKA), because rolipram-downregulated mRNA expression of IL-5 was restored by PKA inhibitors. Immunostaining revealed that rolipram interfered with IL-4-induced nuclear translocation of activator protein (AP)-1 components. **Conclusions:** This is the first demonstration of suppression of IL-4 signaling by PDE4 inhibitors via prevention of nuclear translocation of AP-1.

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Introduction

The pathogenic mechanisms underlying allergic diseases are quite complex, and the exact mode of action of immune cells is still a matter of debate. Despite the complexity, the concept that activated T cells play a central role in allergy is well accepted. T helper cells (Th), based on the cytokine profiles, are divided into two subtypes, Th1 and Th2, which are best characterized by the capacity to produce interferon- γ (IFN- γ) and IL-4, IL-5, respectively [1–3]. While Th1 may provide protection against viruses and intracellular parasites mainly via activation of delayed-type immune responses, Th2 may be a causative for immediate type allergies via the secretion of IL-4 and IL-5. Th2-derived IL-4 is mandatory for the production of antigen-specific IgE from B cells, a best-known mediator in allergic disorders such as asthma or atopic dermatitis. Owing to the pivotal role of IL-4 in immediate allergies, we previously reported that in patients with atopic dermatitis T cell reactivity to IL-4 may play a pathogenic role [4]. In light of this view, mechanisms controlling the augmented IL-4 reactivity could be a target of pharmacological intervention.

Cyclic AMP (cAMP) regulates a variety of immunological events [5–7]. It has been shown that elevated levels of cAMP inhibit the secretion of cytokines [8–10], and thus it is regarded as a negative regulator of T-cell activa-

tion [9, 11]. The intracellular levels of cAMP are controlled by adenyl cyclases and phosphodiesterases (PDEs). To date, at least eleven heterologous families of PDEs have been reported based on their substrate selectivity, enzyme kinetics and inhibitor potency [12, 13]. In particular, the cAMP-specific PDE4 is preferentially expressed in immune and inflammatory cells [14].

Rolipram [(±) 4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone] is a specific PDE4 inhibitor whose therapeutic utility has been investigated in the treatment of depression [15]. Furthermore and even more importantly, rolipram and other specific PDE4 inhibitors have the capacity to suppress inflammatory processes. A large body of evidence suggests that PDE4 inhibitors might possess anti-inflammatory effects and thus be a newer strategy to control allergic diseases [12, 16–19]. Hence, expansion of the knowledge about their mode of action may provide a newer therapeutic approach against allergies. Based on the above-mentioned concerns, we were interested to investigate whether rolipram modulates IL-4-initiated signaling in freshly isolated human T cells. In particular, we focused on the action mechanisms by which rolipram regulates IL-4 signaling.

Materials and Methods

Reagents

The following reagents were employed: Ficoll-Paque™ (Amersham Pharmacia Biotech AB, Uppsala, Sweden); human recombinant IL-4 (R & D Systems, Minneapolis, Minn., USA); rolipram, actinomycin D, 8-bromoadenosine cAMP (8-Br-cAMP), cell lysis extraction reagent and phosphatase inhibitor cocktail and casein blocking buffer (Sigma, St. Louis, Mo., USA); H-89 (Seikagaku, Tokyo, Japan); protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany); reverse transcription system and U0126 (Promega, Madison, Wisc., USA), and Taq PCR core kit 1,000 units and RNeasy Mini Kit (Qiagen, Tokyo, Japan). Vectashield® mounting medium DAPI was from Vector Laboratories (Burlingame, Calif., USA), and β -actin, IL-5 and IFN- γ primers were synthesized by TaKaRa Bio (Shiga, Japan). Rabbit polyclonal antibody raised against phosphorylated (p)-c Jun (Ser 63), rabbit polyclonal antiserum against Fra-2 (Q-20), rabbit polyclonal antiserum against Jun D (329) and RP-8-Br-cAMP were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). FITC-conjugated goat anti-rabbit IgG was from Tago (Burlingame, Calif., USA).

Preparation of Freshly Isolated Peripheral T Cells

Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood of healthy volunteers through a density gradient centrifugation method (Ficoll-Paque). PBMCs were collected from the interface and washed twice with PBS. Monocytes were eliminated through adherence on tissue

culture dishes for 1 h at 37°C twice. Human T lymphocytes were further purified by passing the non-adherent population through a nylon fiber wool column. The purity of the preparation was ascertained by staining with antibodies against CD3, CD14 or CD19 (all from BD Biosciences Pharmingen, San Diego, Calif., USA) followed by flow-cytometric analysis using a FACScan (BD Biosciences Pharmingen, San Jose, Calif., USA), demonstrating that T cells were enriched at approximately 95%. Almost all T cells were viable as determined by trypan blue exclusion assays. Compounds except for 8-Br-cAMP and RP-8-Br-cAMP, both solubilized in medium, were dissolved in dimethyl sulfoxide (Sigma) at a concentration of 10^{-2} M and prepared immediately before use. The final concentration of DMSO in each well was $\leq 0.1\%$.

T Cell Stimulation

Purified T cells were cultured in RPMI 1640 medium supplemented with 1% L-glutamine and 1% antibiotics/antimycotics (all from Gibco Invitrogen, Tokyo, Japan) but without serum at a density of 5×10^6 cells/ml for all experiments. Cells were pre-incubated for 30 min with a cAMP analogue (1 mM 8-Br-cAMP), a PKA type I inhibitor (1 mM RP-8-Br-cAMPS), a PKA inhibitor (10 μ M H-89) or an MEK/ERK inhibitor (10 μ M U0126). Various concentrations of rolipram (10^{-7} , 10^{-6} or 10^{-5} M) were added to the culture 90 min before stimulation with 10 ng/ml IL-4 or in some experiments with 10 ng/ml IL-12. Cells were harvested 6 h after cytokine stimulation.

RT-PCR

Cells were first harvested, and RNA samples prepared using the RNeasy kit. All RNA samples were subjected to reverse transcription of cDNA using a reverse transcriptase system according to the manufacturer's instructions. cDNA products were amplified with the designated primer pairs using the Taq PCR Core kit. To perform semiquantitative analysis following PCR amplification, 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. cDNA samples were then amplified using specific primers for IL-5 and IFN- γ . The following sequences were chosen for primers: β -actin (sense): 5'-CACCTTCTACAATgAgCTgC-3', β -actin (antisense): 3'-TTCATgAggTAGTCCgTCAG-3', human IL-5 (sense): 5'-gCTTCTgCATTgAgTTgCTAgCT-3', human IL-5 (antisense): 5'-Tg-gCCgTCAATgTATTTCTTTATTAAG-3', human IFN- γ (sense): 5'-TgCaggTCATTCAgATgTAg-3', and IFN- γ (antisense): 5'-Ag-CCATCACTTggATgAgTT-3'.

Cycling parameters were as follows: denaturation at 95°C for 30 s; annealing at 55°C for 45 s 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 35 cycles for IL-5 and IFN- γ), followed by a 10-minute 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 the 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.

Immunohistochemistry

Purified human T cells were cultured at a density of 5×10^6 cells/ml incubated with rolipram (10^{-5} M) or the mitogen-activated protein kinase/extracellular-regulated protein kinase (MEK/ERK) inhibitor U0126 ($10 \mu\text{M}$) and stimulated thereafter with 10 ng/ml IL-4. Cells were harvested 10 min later, washed in chilled PBS (without magnesium and calcium) twice, resuspended with RPMI (serum free), and applied and attached onto glass slides. Samples were fixed in 4% paraformaldehyde solution for 10 min, followed by washing in PBS. The nonspecific binding was blocked by incubation with PBS containing 2% goat serum for 1 h at 4°C . The primary antibody (an antibody directed against p-c Jun, Fra-2 or Jun D) was added and incubated overnight at 4°C . After extensive washing with chilled PBS, the secondary antibody (FITC-conjugated anti-rabbit IgG) was applied to the reactions and incubated overnight at 4°C . The samples were washed, covered with Vectashield mounting medium with DAPI and observed using a confocal laser scanning microscope.

Results

The Effect of a PDE4 Inhibitor, Rolipram, on the IL-4-Induced Expression of IL-5 mRNA

To first examine the IL-4-induced expression of IL-5 mRNA, T cells were stimulated with 10 ng/ml IL-4, cells were harvested at various time points thereafter, RNA extracted, and semiquantitative RT-PCR was conducted. The signal intensities of PCR transcripts for both IL-5 and β -actin and relative signal intensity were measured and calculated. As shown in figure 1a, mRNA expression of IL-5 was markedly upregulated upon IL-4 stimulation 6 h after stimulation. The IL-5 mRNA showed plateau expression between 6 and 18 h and was further upregulated to 24 h after stimulation. Thus, cells were incubated with IL-4 for 6 h throughout this study. To next determine the effect of the PDE4 inhibitor rolipram, T cells were preincubated with graded concentrations of rolipram (10^{-7} , 10^{-6} and 10^{-5} M) for 90 min, and 10 ng/ml IL-4 were added. Cells were cultured for further 6 h and harvested. RNA was extracted thereafter and reverse-transcribed, and semiquantitative RT-PCR was performed using primers specific for IL-5. In preliminary experiments, we first examined an optimal concentration and time duration of IL-4 stimulation to induce T cell activation (the induction of IL-5 mRNA was used as a readout system). Figure 1b shows that rolipram markedly downregulated IL-4-induced mRNA expression of IL-5 at all concentrations tested. To test whether IL-4 upregulated IL-5 mRNA at transcriptional levels, cells were first stimulated with IL-4, and 2 h later actinomycin D, an inhibitor of mRNA synthesis, was added. Actinomycin D almost completely abolished IL-4-upregulated expression of IL-5 mRNA (fig. 1c).

The Effect of Rolipram on IFN- γ mRNA Expression in T Cells Stimulated with IL-12

We next aimed to determine whether rolipram also modulates signals initiated by different stimuli. Therefore, cells were stimulated with IL-12, a known inducer of Th1-type immune responses, and tested for mRNA expression of IFN- γ . RT-PCR revealed that the expression of IFN- γ mRNA induced by 10 ng/ml IL-12 was not affected by rolipram (fig. 2).

The Effect of the cAMP Analogue 8-Br-cAMP on IL-4-Induced IL-5 mRNA in vitro

We next examined the possibility if increased cellular cAMP levels downregulate the expression of IL-5 mRNA. To address this question, we used 8-Br-cAMP, the cAMP analogue. The stimulation of T cells with IL-4 resulted in upregulation of IL-5 mRNA, which was markedly suppressed by 1 mM 8-Br-cAMP, similarly to rolipram (fig. 3).

The Effect of Blockade of PKA Activity on IL-4-Upregulated IL-5 mRNA

To further elucidate mechanisms by which rolipram regulates cytokine expression, we tested its effects on the cAMP/PKA signaling pathway. It is known that the effects of cAMP are mostly mediated through the binding to intracellular PKA. To explore whether cAMP inhibits IL-4-upregulated IL-5 mRNA via activation of the PKA pathway in human T cells, we employed the PKA inhibitor H-89 or PKA type I inhibitor RP-8-Br-cAMPS. T cells were preincubated for 30 min with $10 \mu\text{M}$ H-89 or 1 mM RP-8-Br-cAMP and stimulated with 10 ng/ml IL-4. RT-PCR revealed that both H-89 (fig. 4a) and RP-8-Br-cAMP (fig. 4b) successfully restored rolipram-downregulated expression of IL-5 mRNA in IL-4-stimulated cells.

Blockade of IL-4-Induced Activation of Activator Protein (AP)-1 by an ERK Inhibitor and Rolipram

We have previously shown that AP-1 is a prominent transcription factor in T cells in response to IL-4 [4, 20]. Therefore, we next checked modulations of AP-1 activation by rolipram. Therefore, we performed immunostaining with antibodies against AP-1 components (c-Jun, Jun D and Fra-2). Because in order to activate transcription of AP-1-responsive genes AP-1 ultimately translocates to nuclei, AP-1 integration in nuclei should be impaired when rolipram inhibits its activation. As shown in figure 5a, IL-4 induced nuclear translocation of AP-1 components (c-Jun, Jun D and Fra-2), indicating

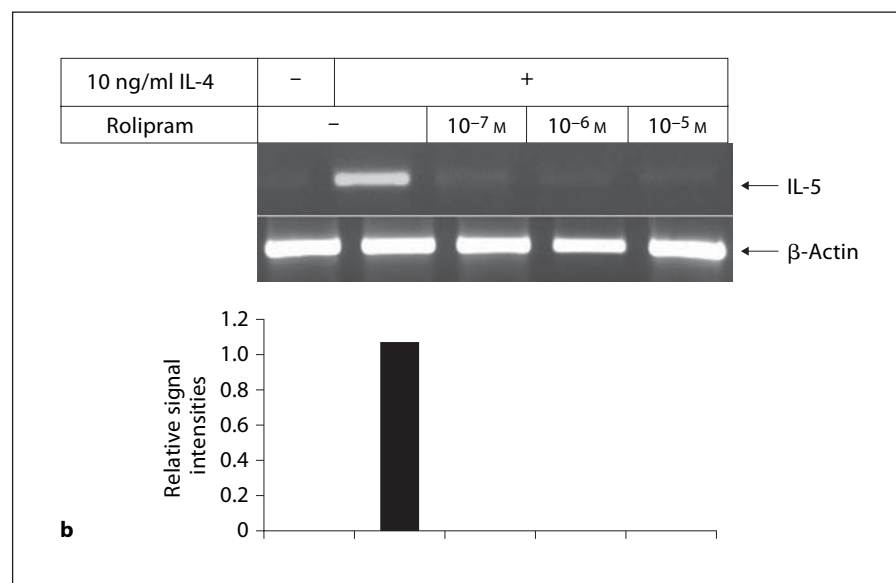
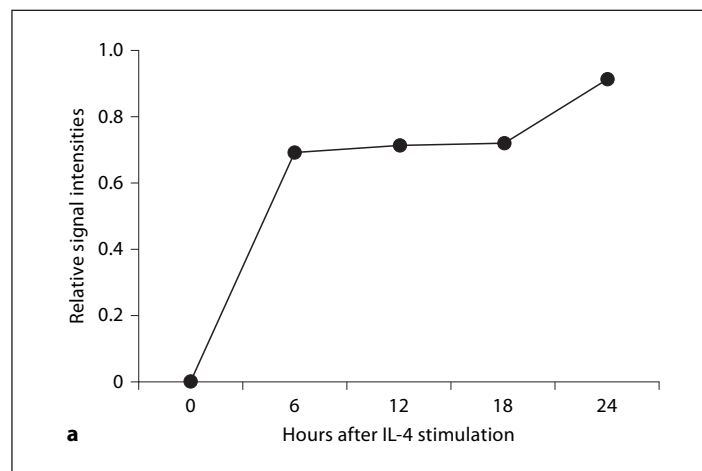


Fig. 1. Semiquantitative RT-PCR. **a** Time course of IL-5 mRNA expression in T cells stimulated with 10 ng/ml IL-4 for 0–24 h. **b** The effect of the PDE4 inhibitor rolipram on IL-4-induced IL-5 mRNA expression. Human T cells (5×10^6 cells/ml) were treated with various concentrations of rolipram (10^{-7} , 10^{-6} and 10^{-5} M) or left untreated and stimulated with 10 ng/ml IL-4; 6 h later, total cellular RNA was extracted, and RT-PCR (35 cycles) performed using primers specific for IL-5 (upper panel) or β -actin (lower panel; 30 cycles). **c** To determine if transcriptional activation occurred in IL-4-induced IL-5 mRNA expression, T cells were stimulated with 10 ng/ml IL-4 for 6 h or left unstimulated with or without preincubation of 10^{-5} M rolipram. In some experiments, an inhibitor of RNA synthesis (2 μ g/ml actinomycin D) was added 2 h following IL-4 stimulation. RNA was extracted thereafter, and RT-PCR performed with primer sets for IL-5 (upper panel) or β -actin (lower panel). PCR products were separated by 2% agarose gel electrophoresis. Representative data of three independent experiments are shown.

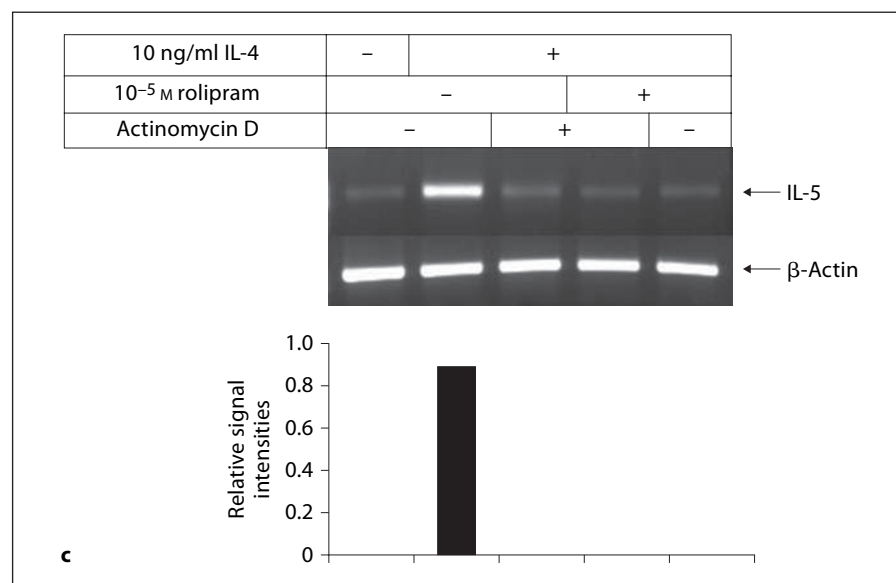


Fig. 2. Rolipram failed to modulate IL-12-induced expression of IFN- γ mRNA in T cells. T cells were first preincubated with various concentrations of rolipram (10^{-7} , 10^{-6} and 10^{-5} M) or left untreated, stimulated with IL-12 for 6 h, RNA extracted thereafter and RT-PCR performed using primers specific for IL-5 (a) or β -actin (b). Representative data of three independent experiments are shown.

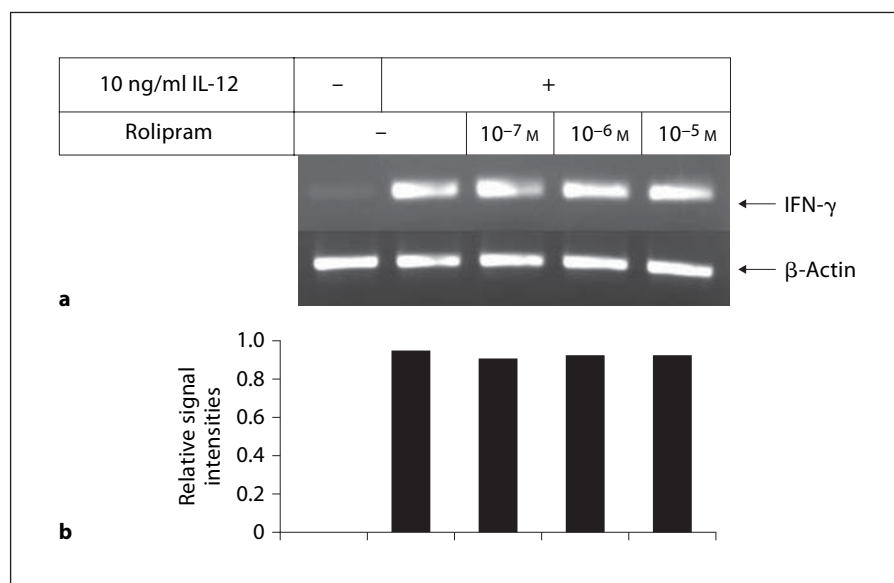
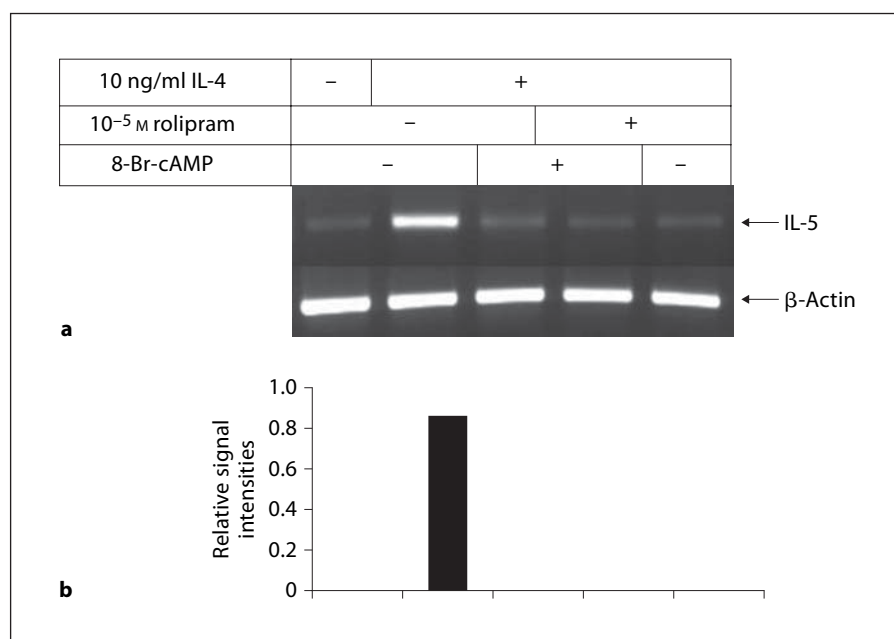


Fig. 3. The effect of a cAMP analogue, 8-Br-cAMP, on IL-4-induced mRNA expression of IL-5. T cells were preincubated with 1 mM 8-Br-cAMP or left untreated in the presence or absence of 10^{-5} M rolipram and further stimulated with 10 ng/ml IL-4 for 6 h. RNA samples were extracted thereafter, and RT-PCR performed for IL-5 (a) and β -actin (b). Representative data of three independent experiments are shown.



AP-1 activation. Rolipram, when administered prior to IL-4 stimulation, prevented its translocation (fig. 5a). Interestingly, a specific ERK/MEK inhibitor, U0126, also successfully inhibited the nuclear migration of Jun D and Fra-2, but failed to do so for c-Jun (fig. 5b), indicating that IL-4-induced activation of AP-1 is mediated, at least in part, through an MAPK/ERK pathway.

Discussion

In this study, we provided first evidence that the PDE4 inhibitor rolipram antagonizes IL-4-activated signaling. We stimulated T cells with IL-4 in our experimental setting, and the culture contained highly enriched freshly isolated peripheral T cells (approximately 95%) and only a negligible number of contaminating cells (<5%), in-

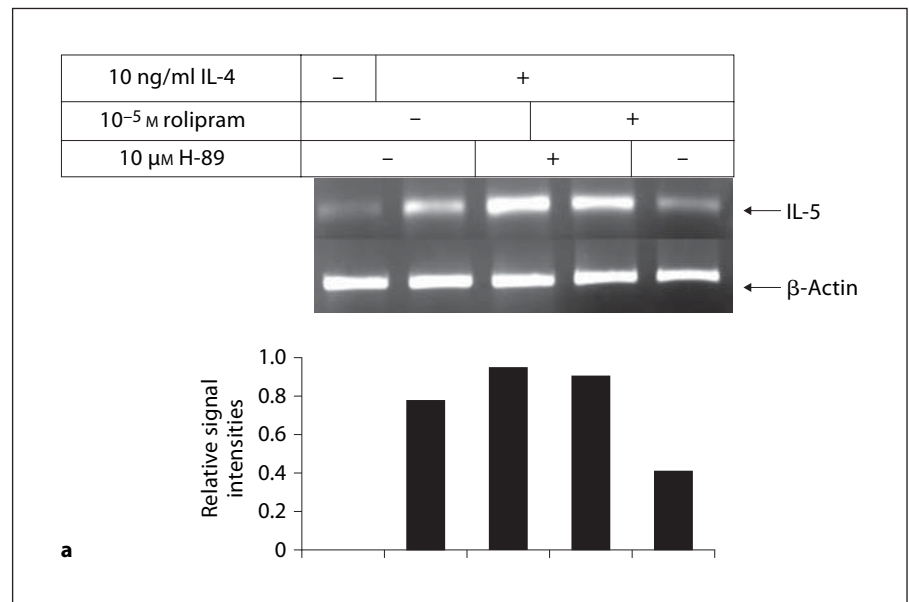
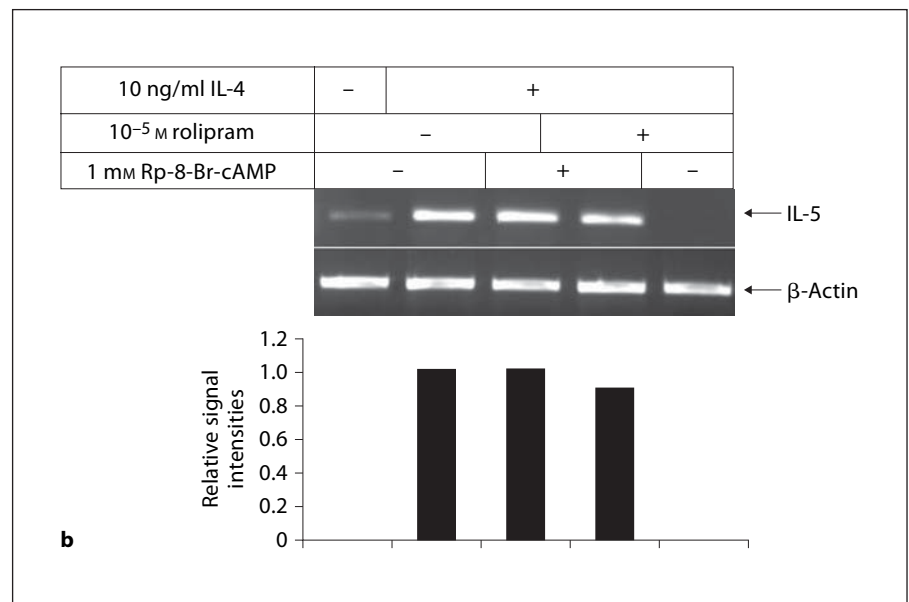


Fig. 4. Rolipram-induced downregulation of IL-4-upregulated IL-5 mRNA expression was mediated via activation of PKA. T cells were treated with 10 μM of a PKA inhibitor, H-89 (**a**), or left untreated in the presence or absence of 10⁻⁵ M rolipram and stimulated with 10 ng/ml IL-4 for 6 h. Cells were harvested, RNA extracted and RT-PCR performed using primers specific for IL-5 (upper panel) and β-actin (lower panel). Representative data of three independent experiments are shown.



cluding monocytes/macrophages, B cells or other hematopoietic cells. Since IL-5 is known to be expressed only in T cells among hematopoietic cells, our study enables us to test direct IL-4 effects on T cells. Furthermore, because the culture did not contain any antigenic peptides (not even serum), the few contaminating antigen-presenting cells apparently do not result in T cell activation through antigen presentation. This further strengthens the notion that IL-4 is only a stimulator of T cell activation in this setting. Of note, T cells were harvested 6 h

after stimulation for RNA analyses and after 10 min for immunohistochemistry. This short incubation time helps to minimize possible effects of unknown factors other than IL-5, which might be produced upon IL-4 stimulation and then affect IL-5 mRNA expression.

To date there is evidence that this compound may modulate the activation of immunocompetent cells. It was reported that rolipram inhibited the secretion of IL-5, IL-10, IL-2 and TNF-α, but poorly did so for IFN-γ from human T cells, which were stimulated with immo-

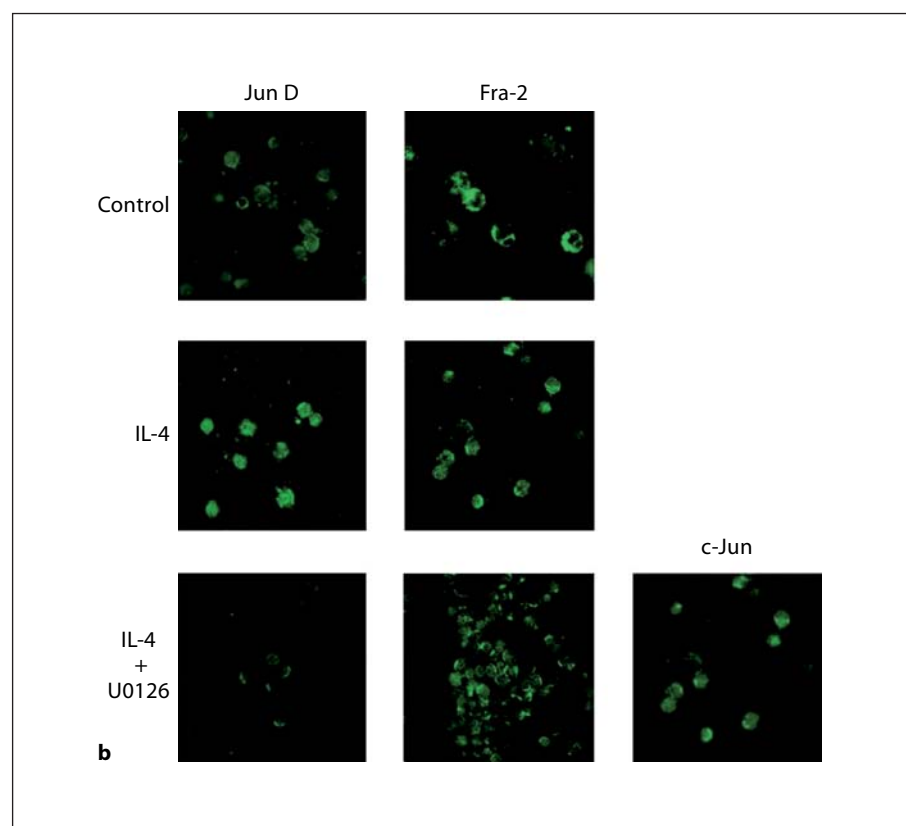
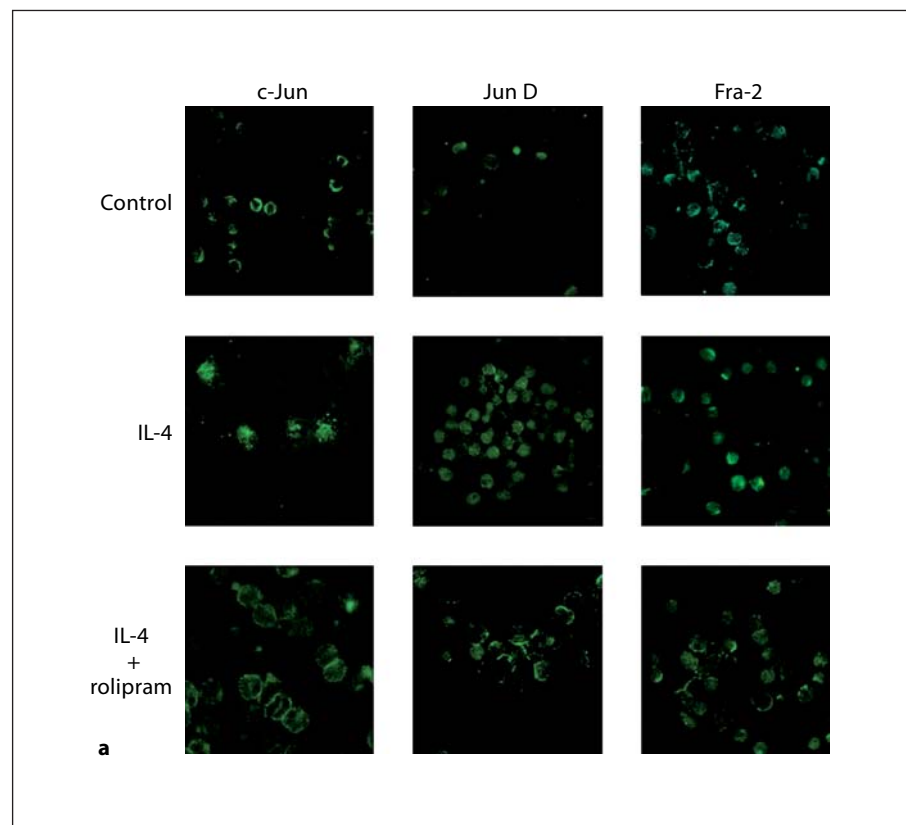


Fig. 5. Rolipram inhibited IL-4-induced activation and nuclear translocation of c-Jun, Jun D and Fra-2 transcription factors. T cells were first treated with 10^{-5} M rolipram (**a**), or $10 \mu\text{M}$ of the MEK/ERK inhibitor U0126 (**b**), or left untreated. 10 min later, the cells were harvested and attached onto glass slides. After fixation, the samples were reacted with anti-p-c-Jun, anti-Jun D or anti-Fra-2, an FITC-conjugated secondary antibody was added, and specific signals visualized under a confocal laser scanning microscope. Representative data of three independent experiments are shown.

bilized anti-CD3 [21]. Rolipram and other cAMP-elevating agents were found to inhibit the release of IL-5 from human PBMC stimulated with anti-CD3 and anti-CD28, and activation of T cells mimicked T cell receptor activation (upon immobilized anti-CD3) or stimulation plus costimulatory signals (by anti-CD28) [22, 23]. In this study, the inhibitory action of rolipram was detailed in IL-4-induced T cell activation, and therefore the message given by this study is entirely distinct and unique from others.

Employing this strict experimental condition, rolipram ranging between 10^{-7} and 10^{-5} M was shown to clearly downregulate IL-4-upregulated expression of IL-5 mRNA (fig. 1a), indicating that rolipram is involved in IL-4 signaling. Because actinomycin D completely abrogated IL-4-induced upregulation of IL-5 mRNA (fig. 1b), IL-4 almost exclusively activated this gene at a transcriptional level. Hence, it is likely that expression of IL-5 in this setting was turned on or off in a transcription-dependent manner. Also, it is worth mentioning that rolipram did not interfere with IL-12-induced mRNA expression of IFN- γ (fig. 2). This clearly indicates that rolipram-induced suppression of cytokine production is neither a universal event nor toxic, but rather specific for IL-4 signaling. Rolipram-mediated downregulation of IL-4-induced IL-5 mRNA was not affected by the cAMP analogue 8-Br-cAMP (fig. 3), but was completely restored by PKA inhibitors, H-89 (fig. 4a) and RP-8-Br-cAMP (fig. 4b), indicating that rolipram suppressed IL-4 signaling via enhancement of PKA activity. The fact that the proximal promoter regions of the human IL-5 gene lack binding sites of cAMP-responsive elements [24] readily rules out the mediation of rolipram-derived suppression of binding protein of cAMP-responsive elements. Thus, we could focus on other signaling intermediates interactive with PKA. In this context, rolipram-mediated prevention of IL-4-induced AP-1 activation may be one explanation (fig. 5a). Rolipram interfered with IL-4-activated nuclear translocation of AP-1 (c-Jun, Jun D and Fra-2) indicating that it inhibits transcriptional activation of AP-1-responsive genes (i.e. IL-5) via prevention of AP-1 migration to nuclei. Similarly and intriguingly, the specific MAPK/ERK inhibitor U0126 inhibited AP-1 activation (fig. 5b). Since U0126 only blocked Jun D and Fra-2 but not c-Jun of IL-4-activated AP-1, the observation strongly suggests that cAMP-dependent AP-1 activation (induced by rolipram) might at least partially be mediated via the MAPK/ERK pathway. This finding may be supported by observations by other authors that cAMP-inhibited activation of T cells was associated with

downregulation of MAPK pathways, ERK kinases and c-Jun N-terminal kinase (c-JNK) [25]. It remains to be elucidated whether rolipram suppression of nuclear translocation of AP-1 is also mediated via suppression of IL-4-activated c-JNK. Studies to analyze this possibility are underway.

Collectively, our observations may delineate the molecular basis how rolipram antagonizes IL-4 signaling. First, as is already known, rolipram elevates cellular cAMP levels. Binding of IL-4 to its receptor is followed by activation of early components of signaling intermediates (i.e. JAK/STAT system), while, probably independently, rolipram increases cAMP levels. The rolipram-induced increase in cAMP levels leads to activation of PKA. At the same time, activation of IL-4R results in activation of MAPK subsequently leading to phosphorylation of ERK kinases. Rolipram-activated PKA might inhibit MAPK cascades, ERK or c-JNK. Rolipram-mediated suppression of the IL-4-induced signal transducers led to interference with AP-1 activation, which consequently inhibited IL-4-induced cytokine production of AP-1-responsive genes, e.g. IL-5. The prominent role of AP-1 in IL-4 signaling has previously been shown by our group [4, 20]. Together, this is the first evidence showing that rolipram suppressed IL-4 signaling via functional suppression of AP-1 activation. However, it remains unclear if direct crosstalk exists between activated PKA, MAPK/ERK and/or JNK. This should be the subject of future investigations. As mentioned earlier, IL-4 reactivity (i.e. T-cell activation of AP-1) may constitute a proximal part in the pathogenesis of atopic disorders [4]. This study shows that PDE4 inhibitors, e.g. rolipram, may be of therapeutic benefit for allergic diseases via suppression of the reactivity. Further investigation is warranted to delineate the precise mode of action of PDE4 inhibitors at a molecular basis. This may improve the treatment and quality of life of patients suffering from long-lasting and so far therapy-resistant disorders, such as allergies.

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