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Antipruritic mechanisms of topical E6005, a phosphodiesterase 4 inhibitor: Inhibition of responses to proteinase-activated receptor 2 stimulation mediated by increase in intracellular cyclic AMP

Tsugunobu Andoh, Yasushi Kuraishi *

Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan

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ABSTRACT

Background: Phosphodiesterase 4 (PDE4), which catalyses the conversion of cyclic adenosine 3′,5′-monophosphate (cAMP) to 5′-AMP, plays a critical role in the pathogenesis of inflammatory disorders. Pruritus is the main symptom of dermatitides, such as atopic dermatitis, and is very difficult to control. Recent studies have shown that the activation of proteinase-activated receptor 2 (PAR_2) is involved in pruritus in dermatoses in humans and rodents.
Objective: To investigate the inhibitory effect of E6005, a topically effective PDE4 inhibitor, on PAR_2-associated itching in mice.
Methods: Mice were given an intradermal injection of SLIGRL-NH_2 (100 nmol/site), a PAR_2 agonist peptide, into the rostral part of the back. E6005 and 8-bromo-cAMP were applied topically and injected intradermally, respectively, to the same site. Scratching bouts were observed as an itch-related behavior, and firing activity of the cutaneous nerve was electrophysiologically recorded. Keratinocytes were isolated from the skin of neonatal mice and cultured for in vitro experiments. The concentrations of cAMP and leukotriene B_4 (LTB_4) were measured by enzyme immunoassay. The distribution of PDE4 subtypes in the skin was investigated by immunostaining.
Results: Topical E6005 and intradermal 8-bromo-cAMP significantly inhibited SLIGRL-NH_2-induced scratching and cutaneous nerve firing. Topical E6005 increased cutaneous cAMP content. Topical E6005 and intradermal 8-bromo-cAMP inhibited cutaneous LTB_4 production induced by SLIGRL-NH_2, which has been shown to elicit LTB_4-mediated scratching. E6005 and 8-bromo-cAMP inhibited SLIGRL-NH_2-induced LTB_4 production in the cultured murine keratinocytes also. PDE4 subtypes were mainly expressed in keratinocytes and mast cells in the skin.
Conclusions: The results suggest that topical E6005 treatment inhibits PAR_2-associated itching. Inhibition of LTB_4 production mediated by an increase in cAMP may be partly involved in the antipruritic action of E6005.

1. Introduction

Itch (or pruritus) is a skin sensation that provokes a desire to scratch and is the most common symptom of dermatitides (e.g., atopic dermatitis) and some systemic disorders (e.g., cholestasis). Although the use of antihistamines and short-term use of topical glucocorticosteroids are supplemented by other topical and systemic therapies in the treatment of chronic pruritic dermatitis, pharmacological therapy of chronic pruritus remains unestablished and is challenging [1]. Thus, new antipruritic agents need to be developed.

It has been reported that phosphodiesterase (PDE) 4, which catalyses the conversion of cyclic adenosine 3′,5′-monophosphate (cAMP) to 5′-AMP, plays a critical role in the pathogenesis of inflammatory disorders, and PDE4 inhibitors exert anti-inflammatory effects against these diseases [2–6]. Repeated topical application of a PDE4 inhibitor has been shown to improve the clinical dermatitis score in the lesional skin of patients with atopic

Abbreviations: cAMP, cyclic adenosine 3′,5′-monophosphate; EIA, enzyme immunoassay; IgG, immunoglobulin G; LTB_4, leukotriene B4; PDE, phosphodiesterase; PAR_2, proteinase-activated receptor 2; PBS, phosphate-buffered saline.

* Corresponding author at: Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-1094, Japan. Tel.: +81 76 434 7510; fax: +81 76 434 5045.
E-mail address: kuraisiy@pha.u-toyama.ac.jp (Y. Kuraishi).

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dermatitis [7]. Recently, a potent and selective PDE4 inhibitor, E6005, has been developed and shown to be effective after topical cutaneous dosing [8]. In patients with atopic dermatitis, twice-daily topical application of 0.2% E6005 ointment relieves atopic eczema and reduces pruritus after 12-week dosing [9]. In animal experiments, repeated topical application of 0.03% E6005 inhibits hapten-induced dermatitis in mice [8]. Interestingly, single topical application of E6005 (0.03% and lower) inhibits hapten-induced scratching in sensitized mice [8] and spontaneous scratching in mice with chronic dermatitis [10]. These findings raised the possibility that E6005 has an acute antipruritic activity, although its anti-inflammatory effect after repeated application may also contribute to the relief of pruritus. In this study, we investigated the underlying mechanisms of acute antipuritic action of E6005.

Spontaneous scratching in NC mice with chronic dermatitis is at least partly mediated by serine protease(s) and proteinase-activated receptor-2 (PAR_2) [11]. PAR_2 is also claimed to be involved in itching in patients with atopic dermatitis [12]. Therefore, we asked whether E6005-induced increase in intracellular cAMP might inhibit PAR_2-mediated itching. Leukotriene B_4 (LTB_4) is involved in PAR_2-associated scratching [13] and in spontaneous scratching in NC mice with chronic dermatitis [14]. Therefore, we also asked whether E6005-induced increase in cAMP might affect PAR_2-mediated LTB_4 production in the skin.

2. Materials and methods

2.1. Animals

Male ICR mice (Japan SLC, Hamamatsu) were used at 4–7 weeks of age except for a series of experiments, in which neonates were used to prepare primary cultures of epidermal keratinocytes. The mice were housed in a room under controlled temperature (22 ± 1 °C), humidity (55 ± 10%), and light (lights on from 07:00 to 19:00 h). Food and water were freely available. The day before the experiments, hair was removed from the rostral part of the back for intradermal injection and topical application. Procedures in the animal experiments were approved by the Committee for Animal Experiments at the University of Toyama and were conducted in accordance with the guidelines for proper conduct of animal experiments.

2.2. Materials

In in vivo experiments, SLIGRL-NH_2 (Sigma-Aldrich, St. Louis, MO, USA) and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) (Sigma-Aldrich) were dissolved in physiological saline and injected intradermally into the rostral back in a volume of 50 μL. 8-Br-cAMP was injected intradermally 10 min before SLIGRL-NH_2 injection. E6005, methyl 4-[({3-[6,7-dimethoxy-2-(methylamino)quinazolin-4-yl]phenyl}amino)carbonyl]benzoate [8] (a gift from Eisai Co., Ltd., Tokyo) was dissolved in acetone–ethanol (1:1) mixture and applied to the rostral part of the back (the application area was about 4 × 4 cm) 1 h before SLIGRL-NH_2 injection. Zileuton (Sigma-Aldrich) was dissolved in 0.5% carboxymethyl cellulose (Wako Pure Chemical Ind., Osaka) and administered orally 1 h before SLIGRL-NH_2 injection.

In in vitro experiments, SLIGRL-NH_2, E6005, and 8-Br-cAMP were dissolved in culture medium containing 0.1% dimethyl sulfoxide, which was added to increase the solubility of E6005. These three agents were administered by replacing the culture medium with the agent-containing culture medium. E6005 and 8-Br-cAMP were administered 1 h and 10 min before SLIGRUL-NH_2 administration, respectively.

2.3. Behavioral experiments

The animals were put individually in an acrylic cage composed of four cells (13 × 9 × 35 cm) for at least 1 h for acclimation. Immediately after SLIGRL-NH_2 injection, the animals were returned to the same cells, and their behaviors were videotaped for 1 h with personnel kept out of the observation room. Playback of the video served for determination of hind-paw scratching of the rostral back as an index of itching [15]. When mice scratch, they stretch their hind paw toward the treated site, lean the head toward the hind paw, rapidly move the paw several times, and then lower it back to the floor; a series of these movements was counted as one bout of scratching [16].

2.4. Electrophysiological recording

The animals were deeply anaesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal, Sigma-Aldrich) 40 min after application of E6005. The animal was laid in the prone position and the skin of the rostral back was turned inside out. The cutaneous nerve branch was then exposed, dissected free from surrounding tissues, and maintained in a mineral oil pool. Extracellular recording of nerve activity was performed using bipolar electrodes of silver wire (Unique Medical Co., Ltd., Tokyo) and an AC bioelectric amplifier (AB651; Nihon Kohden, Tokyo) with a band-pass filter (high-cut filter, 3 kHz; low-cut filter, 150 Hz). Compound action potentials were counted using a data analysis system with software to analyze the spike height histogram (PowerLab/8s; AD Instruments Pty, Castle Hill, Australia). SLIGRL-NH_2 and saline were injected intradermally into the receptive field 1 h after E6005 application.

2.5. Primary cultures of murine keratinocytes

The skin was removed from neonatal mice and treated with 0.05% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in serum-free MCDB 153 medium (Sigma-Aldrich) containing 0.67% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Dojindo, Kumamoto, Japan), 0.12% sodium bicarbonate (Wako Pure Chemical Ind.), 0.01% penicillin G (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 0.006% kanamycin (Wako Pure Chemical Ind.) at 4 °C overnight. Isolated murine keratinocytes were cultured in 200 μL of keratinocyte growth medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland).

2.6. Measurement of cAMP

The animals were anaesthetized with pentobarbital (80 mg/kg, intraperitoneal) and transcardially perfused with phosphate-buffered saline (PBS). The skin at the E6005 application site was isolated using an 8-mm diameter punch 1 h after E6005 application or 5 min after pruritogen injection. The skin was frozen with liquid nitrogen and kept at −80 °C until use. The skin samples were homogenized in a lysis buffer supplied in a cAMP enzyme immunoassay (EIA) kit (GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA) using a Polytron homogenizer (Robert Bosch Tool Corp., Mt. Prospect, IL, USA). After centrifugation at 600 × g at 4 °C for 5 min, the supernatant was analyzed for cAMP using the cAMP EIA kit. A part of the supernatant was used for protein measurement using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The concentration of cAMP was normalized to the protein amount.

2.7. Measurement of LTB_4

To measure LTB_4 content in the skin, the skin at the injection site was isolated 5 min after the injection of SLIGRL-NH_2 and stored at −80 °C, as described above. LTB_4 content in the skin was determined as previously described [14]. The skin samples were homogenized in ethanol containing 10 μM indomethacin (Sigma-Aldrich) and 10 μM zileuton (Sigma-Aldrich) to inhibit cyclooxygenase and 5-lipoxygenase, respectively. After the homogenization and centrifugation, as described above, the supernatant was diluted 1:5 with double-distilled water, and the pH adjusted to 3.5 with 1 N HCl. The sample was then applied to a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) equilibrated with methanol. After the cartridge was washed with hexane and then double-distilled water, lipids were eluted with ethanol. After the evaporation of the eluate, the residue was suspended in an EIA buffer (Cayman Chemical, Ann Arbor, MI, USA) for the assay of LTB_4. The LTB_4 content in the skin was determined using an EIA kit (Cayman Chemical) and normalized to tissue weight.

To measure LTB_4 production in keratinocytes, the culture medium (200 μL) was collected (removed) from primary cultures of murine keratinocytes 5 min after SLIGRL-NH_2 administration and assayed for LTB_4 with an EIA kit (Cayman Chemical). The remaining keratinocytes were treated with 1% Triton X-100 and used for protein determination with a protein assay kit (Bio-Rad Laboratories, Inc.). The amount of LTB_4 was normalized to the amount of protein.

2.8. Immunohistochemistry

Under anesthesia with pentobarbital (80 mg/kg, intraperitoneal), the animals were transcardially perfused with PBS and then 4% paraformaldehyde. The skin of the rostral back was isolated, postfixed with 4% paraformaldehyde, and immersed in 30% sucrose solution for 2 days. The tissue was embedded in Tissue-Tek® O.C.T. compound (Sakura Fineteck Co., Ltd., Tokyo) and kept at −80 °C until use. The frozen samples were sectioned at 20 μm with a cryostat (Leica, Wetzlar, Germany). After being washed three times with PBS, the sections were treated with 0.3% Triton X-100 in PBS and then with 0.25% fetal bovine serum to block non-specific immunoglobulin binding. The sections were treated with the first antibodies at a dilution of 1/500 at 4 °C overnight; the antibodies used were rabbit antibodies against PDE4A, 4B, 4C, and 4D (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and goat anti-mouse mast cell protease 7 (mMCP7) antibody (R&D Systems, Inc., Minneapolis, MN, USA). After washing, the preparations were incubated with Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-goat IgG antibodies (Life Technologies, Carlsbad, CA, USA) for 1 h at room temperature. Fluorescence signals were observed using a confocal laser-scanning microscope (Bio-Rad).

2.9. Statistical analysis

Data are presented as means ± standard error of the mean. Data were analyzed using a Student's *t*-test or one-way analysis of variance and a post hoc Holm–Šidák test; $p < 0.05$ was considered significant. The statistical analyses were performed using Sigmaplot graphing and statistical software (version 11; Systat Software, Ltd., Chicago, IL, USA).

3. Results

3.1. Effects of E6005 and 8-Br-cAMP on SLIGRL-NH_2-induced scratching

An intradermal injection of SLIGRL-NH_2 (100 nmol/site), a PAR_2 agonist peptide, elicited hind-paw scratching of the injection site; the effect peaked during the first 10-min period and almost subsided by 40 min (Fig. 1A). One-hour topical pretreatment with E6005 (0.003–0.03%) significantly inhibited SLIGRL-NH_2-induced scratching; the inhibition was partial and similar between 0.003%, 0.01%, and 0.03% (Fig. 1B). In order to ascertain the involvement of cAMP, we examined the effect of 8-Br-cAMP, a cAMP analog and cAMP-dependent protein kinase activator. Ten-minute intradermal pretreatment with 8-Br-cAMP (0.01–10 nmol/site) significantly attenuated SLIGRL-NH_2-induced scratching; the inhibition was dose-dependent from 0.01 to 1 nmol/site and similar between 1 and10 nmol/site (Fig. 1C). We used 0.03% E6005 and 10 nmol/site 8-Br-cAMP in the subsequent experiments.

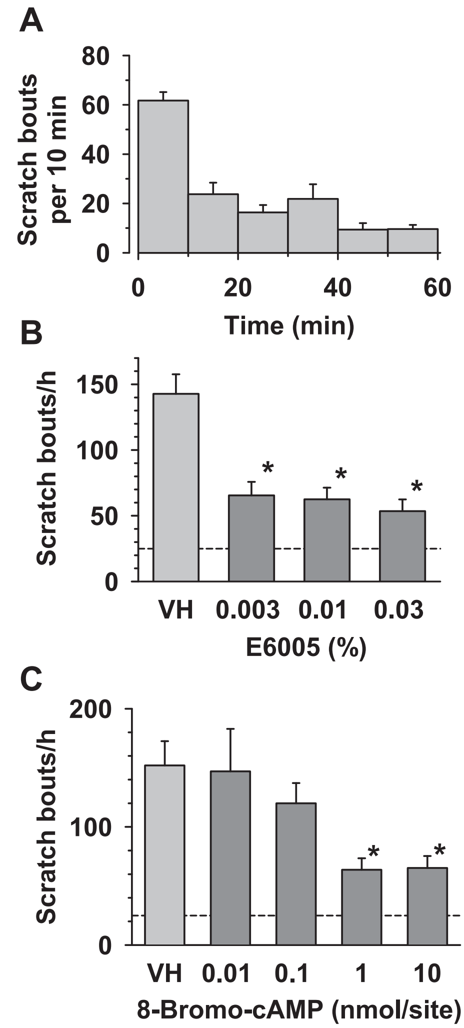

Fig. 1. Inhibitory effects of E6005 and 8-bromo-cAMP on scratching induced by SLIGRL-NH_2. SLIGRL-NH_2 (100 nmol/site) was injected intradermally into the rostral back of each mouse. (A) Time course of SLIGRL-NH_2-induced scratching. (B) Effects of E6005 on SLIGRL-NH_2-induced scratching. E6005 and the vehicle (VH) were applied topically to the injection site 1 h before SLIGRL-NH_2 injection. (C) Effects of 8-bromo-cAMP on SLIGRL-NH_2-induced scratching. 8-Bromo-cAMP and VH were injected intradermally 10 min before SLIGRL-NH_2 injection. Broken lines represent the average scratching bouts in the saline-injected group. Values represent mean ± standard error of the mean ($n = 8$). $^*p < 0.05$ vs. VH (one-way analysis of variance followed by the Holm–Šidák test).

3.2. Effects of E6005 and 8-Br-cAMP on SLIGRL-NH_2-induced cutaneous nerve firing

The activity of the cutaneous nerve innervating the rostral back was rapidly and markedly increased following intradermal injection of SLIGRL-NH_2 (100 nmol/site), decreased from 5 to 10 min, and then low and relatively constant at least until 30 min (data not shown). The nerve activity after the SLIGRL-NH_2 injection was significantly increased as compared with saline injection (Fig. 2). The SLIGRL-NH_2-induced increase of nerve activity was significantly inhibited by 1-h pretreatment with topical 0.03% E6005 and 10-min pretreatment with intradermal 8-Br-cAMP (10 nmol/site) (Fig. 2).

3.3. Effects of E6005 on cutaneous concentration of cAMP

The cutaneous concentration of cAMP was 0.25 ± 0.01 pmol/mg protein in vehicle-treated skin (n = 8). One hour after the topical application of 0.03% E6005, the cutaneous concentration of cAMP increased up to 0.31 ± 0.02 pmol/mg protein (n = 8); the increase was slight but statistically significant (Fig. 3A). An intradermal injection of SLIGRL-NH_2 (100 nmol/site) caused 33% decrease in the cutaneous concentration of cAMP 5 min after injection, and 1-h topical pretreatment of the injection site with 0.03% E6005 reversed the SLIGRL-NH_2-induced decrease of cAMP concentration; these changes were slight but statistically significant (Fig. 3B).

3.4. Effects of E6005 and 8-Br-cAMP on SLIGRL-NH_2-induced LTB_4 production

An intradermal injection of SLIGRL-NH_2 (100 nmol/site) significantly increased the cutaneous concentration of LTB_4 5 min after the injection, as compared with the saline-injected group (Fig. 4A and B). One-hour topical pretreatment of the injection site with 0.03% E6005 significantly decreased the increased concentration of LTB_4 almost down to the level in saline-injected skin (Fig. 4A). The E6005 action was mimicked by 8-Br-cAMP, which almost completely inhibited SLIGRL-NH_2 (100 nmol/site)-induced production of LTB_4 at intradermal dose of 10 nmol/site (Fig. 4B). To confirm the involvement of 5-lipoxigenase in SLIGRL-NH_2-induced itching and LTB_4 production, we examined the effect of the 5-lipoxigenase inhibitor zileuton. One-hour pretreatment with zileuton significantly (Student's t-test) suppressed SLIGRL-NH_2 (100 nmol/site)-induced scratching at an oral dose of 100 mg/kg; the number of scratching bouts following SLIGRL-NH_2 injection was 88.9 ± 9.2 and 40.5 ± 8.1 per hour (mean ± SEM, n = 8 each) in control and zileuton groups, respectively. The same dose of zileuton significantly (Student's t-test) inhibited SLIGRL-NH_2-induced LTB_4 production; the content of LTB_4 was 13.1 ± 1.2 and 6.1 ± 0.6 pg/mg tissue (mean ± SEM, n = 6 each) in control and zileuton groups, respectively.

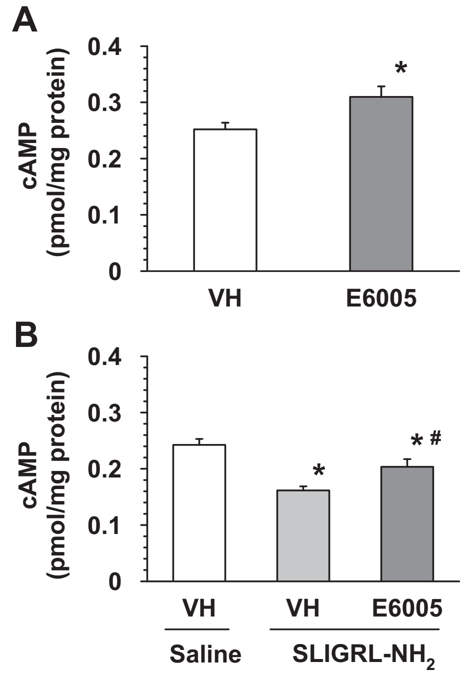

Fig. 3. Effects of E6005 on cutaneous cAMP concentration in mice. The concentration of cAMP in the skin was determined by enzyme immunoassay. (A) E6005 (0.03%) and the vehicle (VH) were applied topically to the rostral back, and the skin was isolated 1 h later. (B) E6005 (0.03%) and the vehicle (VH) were applied topically to the rostral back, and 1 h later SLIGRL-NH_2 (100 nmol/site) or saline was injected intradermally. The skin was isolated 5 min after SLIGRL-NH_2 or saline injection. Values represent mean ± standard error of the mean (n = 8). (A) $^{*}p < 0.05$ (Student's t-test). (B) $^{*}p < 0.05$ vs. VH + saline, $^{\#}p < 0.05$ vs. VH + SLIGRL-NH_2 (one-way analysis of variance followed by the Holm–Šidák test).

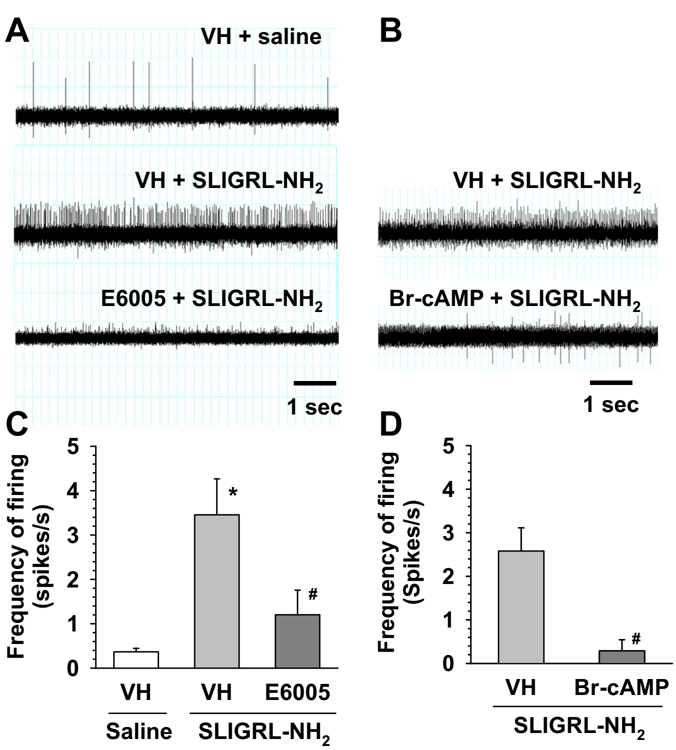

Fig. 2. Response of the cutaneous nerve branch to SLIGRL-NH_2 injection and its suppression by E6005 and 8-bromo-cAMP (Br-cAMP). SLIGRL-NH_2 (100 nmol/site) or saline was injected intradermally into the rostral back of each mouse. E6005 (0.03%) and the vehicle (VH) were applied topically to the rostral back 1 h before SLIGRL-NH_2 injection. Br-cAMP (10 nmol/site) and VH were injected intradermally to the SLIGRL-NH_2 injection site before 10 min. (A, B) Typical ongoing activity of the cutaneous nerve branch from 20 to 30 s after injection. (C, D) Average activity of the cutaneous nerve branch for 30 min from 20 s after injection. Values represent mean ± standard error of the mean. n = 6 for C and 3 for D. (C) $^{*}p < 0.05$ vs. VH + saline, $^{\#}p < 0.05$ vs. VH + SLIGRL-NH_2 (one-way analysis of variance followed by the Holm–Šidák test). (D) $^{\#}p < 0.05$ (Student's t-test).

Five-minute treatment with SLIGRL-NH_2 (100 μM) significantly increased LTB_4 production in primary cultures of murine keratinocytes (Fig. 5). One-hour pretreatment with E6005 (3 and 30 μM) inhibited SLIGRL-NH_2-induced LTB_4 production in a concentration-dependent manner, with a statistically significant inhibition observed at 30 μM (Fig. 5). Similarly, 10-min pretreatment with 8-Br-cAMP (10 and 100 μM) inhibited SLIGRL-NH_2-induced LTB_4 production in a concentration-dependent manner, with a statistically significant inhibition observed at 100 μM (Fig. 5).

3.5. Distribution of PDE4 subtypes and mMCP7 in the skin

There are four subtypes (PDE4A, 4B, 4C, and 4D) in the PDE4 isozyme family [29,33]. Fig. 6 shows a typical distribution of PDE4

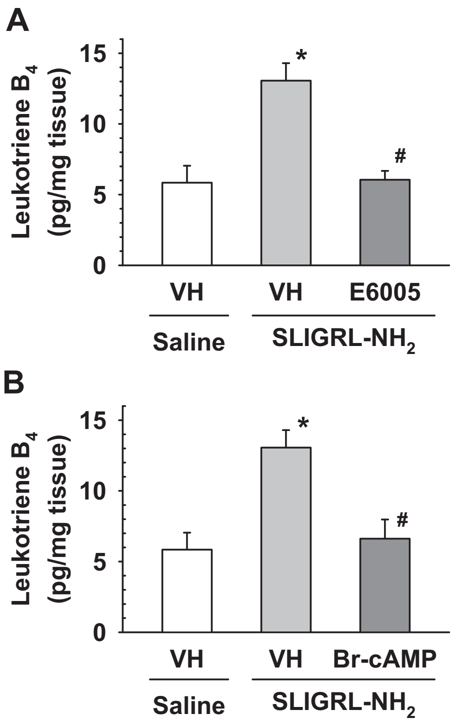

Fig. 4. Leukotriene B_4 production induced by SLIGRL-NH_2 in the mouse skin and its inhibition by topical E6005 and 8-bromo-cAMP (Br-cAMP). SLIGRL-NH_2 (100 nmol/site) or saline was injected intradermally into the rostral back, and the skin was removed after 5 min. The concentration of leukotriene B_4 was measured using an enzyme immunoassay kit (see Section 2). (A) E6005 (0.03%) and the vehicle (VH) were applied topically to the injection site 1 h before SLIGRL-NH_2 injection. (B) Br-cAMP (10 nmol/site) and VH were injected intradermally to the SLIGRL-NH_2 injection site before 10 min. Values represent mean ± standard error of the mean ($n = 6$). $^{*}p < 0.05$ vs. VH + saline, $^{\#}p < 0.05$ vs. VH + SLIGRL-NH_2 (one-way ANOVA followed by the Holm–Šidák test).

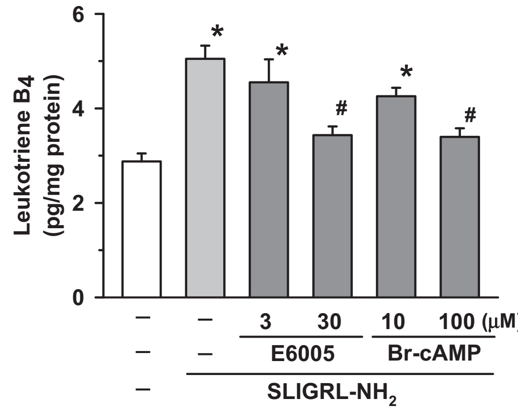

Fig. 5. Leukotriene B_4 production induced by SLIGRL-NH_2 in primary cultures of murine keratinocytes and its inhibition by E6005 and 8-bromo-cAMP (Br-cAMP). SLIGRL-NH_2 (100 μM) was administered to the cultured keratinocytes and the culture medium was collected after 5 min. Leukotriene B_4 was determined using an enzyme immunoassay kit (see Section 2). E6005 (3 and 30 μM) and Br-cAMP (10 and 100 μM) were administered 1 h and 10 min before SLIGRL-NH_2 administration, respectively. The amount of LTB_4 in the culture medium was normalized to the amount of protein in the cultured keratinocytes. Values represent mean ± standard error of the mean ($n = 6$). $^{*}p < 0.05$ vs. untreated control, $^{\#}p < 0.05$ vs. SLIGRL-NH_2-treated control (one-way ANOVA followed by the Holm–Šidák test).

subtypes in mouse skin. mMCP7 was double-immunostained as a marker of mast cells, although it was also immunostained in the epidermal keratinocytes. PDE4A, 4C, and 4D were mainly expressed in the keratinocytes and mast cells; PDE4C was expressed especially in the basal layer of epidermis (Fig. 6). PDE4B was mainly expressed in mast cells (Fig. 6).

4. Discussion

A topical application of the PDE4 inhibitor E6005 suppressed PAR_2 agonist-induced scratching and activity of the cutaneous nerve branch, suggesting that E6005 exerts antipruritic effects through a peripheral action. An intradermal pretreatment with 8-Br-cAMP also inhibited PAR_2 agonist-induced scratching and cutaneous nerve activity. Collectively, these results suggest that the increase of cAMP in the skin attenuates PAR_2-associated itching. A topical application of E6005 increased the cutaneous concentration of cAMP and inhibited the PAR_2 agonist-induced decrease of cutaneous cAMP. These results support the above-mentioned theory. Although PAR_2, a G protein-coupled receptor, was reported not to form stable complexes with $G\alpha_o$, $G\alpha_{i1}$, and $G\alpha_{i2}$ [17], SLIGRL-NH_2 has been shown to stimulate $G\alpha_{i1}$ and inhibit forskolin-stimulated cAMP formation [18]. Further studies are needed to elucidate the mechanisms of the PAR_2-mediated decrease in the cutaneous cAMP concentration.

PDE4A, 4C, and 4D subtypes were densely located in the epidermal keratinocytes. PAR_2 receptors are also densely located in the epidermal keratinocytes [11,12]. Therefore, keratinocytes are a probable site for the suppressive action of E6005 on SLIGRL-NH_2-induced scratching. In cultured mouse keratinocytes, SLIGRL-NH_2 has been shown to increase the production of LTB_4, which is inhibited by the 5-lipoxygenase inhibitor zileuton [13]. Consistent with these findings, in mouse skin, SLIGRL-NH_2 increased the production of LTB_4, which was inhibited by zileuton (the present study). LTB_4 is an endogenous potent pruritogen [19], and zileuton suppresses SLIGRL-NH_2-induced scratching [13, the present study], suggesting that LTB_4 produced in epidermal keratinocytes is involved in SLIGRL-NH_2-induced scratching. The results that E6005 inhibited SLIGRL-NH_2-induced LTB_4 production in cultured murine keratinocytes raise the possibility that the inhibition of LTB_4 production in the epidermal keratinocytes is a mechanism of antipruritic action of E6005. To test this possibility, we examined the effects of topical application of E6005 on scratching induced by intradermal injections of nociceptin and substance P in mice, because LTB_4 production in the epidermal keratinocytes is involved in scratch-inducing activity of these peptides [16,20]. Topical application of E6005 significantly inhibited scratching induced by nociceptin and substance P (Supplementary Fig. S1), supporting the idea that suppression of LTB_4 production in keratinocytes is responsible for the antipruritic activity of E6005.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2014.10.005.

SLIGRL-NH_2-induced LTB_4 production was suppressed by 8-Br-cAMP as well as E6005 in the skin and cultured keratinocytes, suggesting the involvement of an increase in intracellular cAMP in the inhibition of LTB_4 production. It has been reported that cAMP-dependent protein kinase inactivates 5-lipoxygenase through the phosphorylation of its Ser^{523} residue [21,22]. It has also been reported that the phosphorylation of Ser and Thr residues of PAR_2 results in its desensitization [23]. These activities may be involved in the inhibition of PAR_2-mediated LTB_4 production (Fig. 7). The results that topical application of E6005 reversed the SLIGRL-NH_2-induced decrease of cutaneous cAMP concentration supports the above-mentioned idea.

All four PDE4 subtypes were present in mast cells in the dermis (the present study). In humans, approximately half of cutaneous mast cells have PAR_2 receptors on the cell membrane and SLIGRL-NH_2

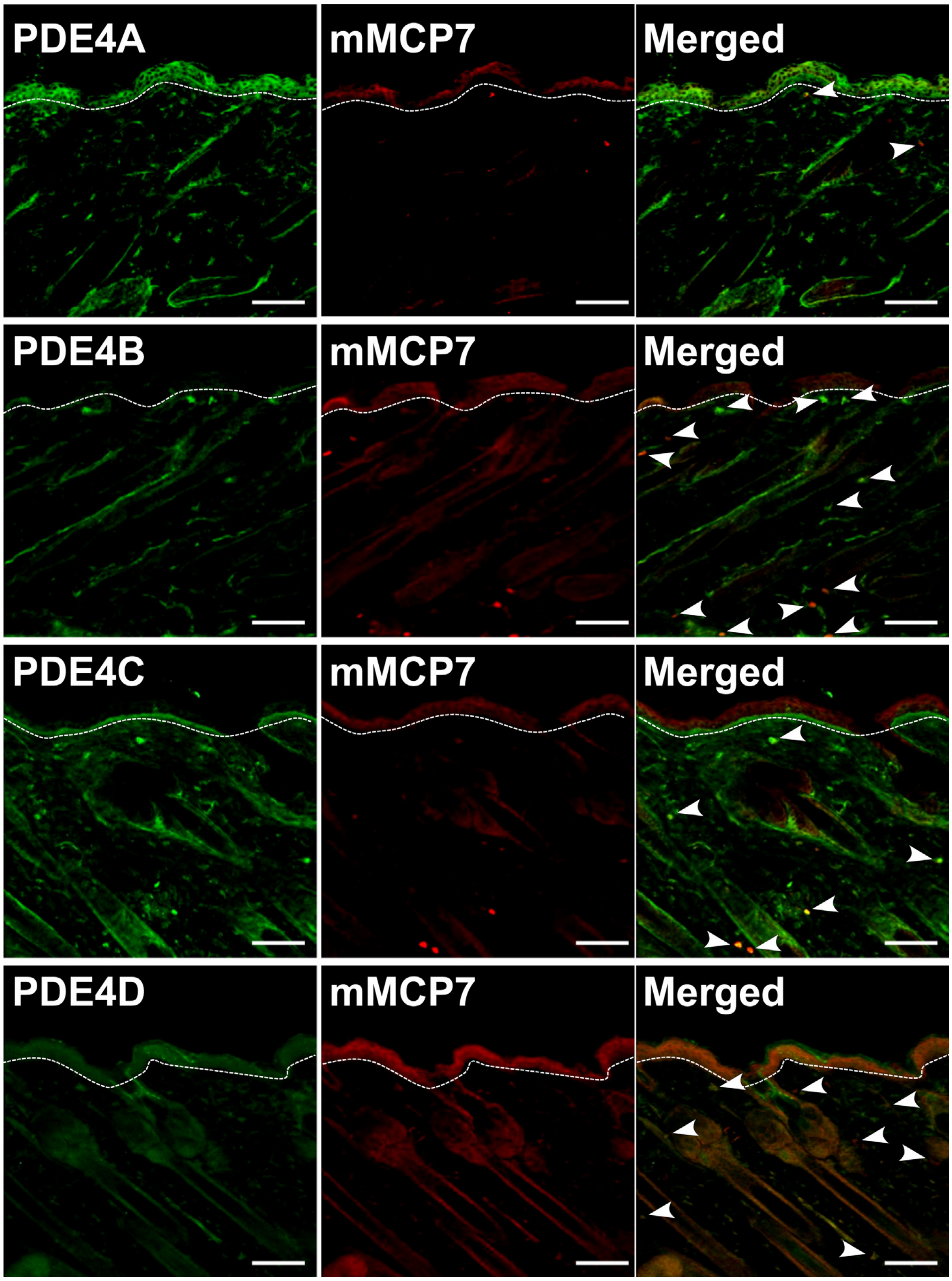

Fig. 6. Typical examples of the distribution of PDE4 subtypes and mouse mast cell protease-7 (mMCP7) in mouse skin. PDE4 subtypes (red) and mMCP7 (green) were immunostained in the rostral back skin. Arrowheads indicate PDE-positive mast cells and dotted lines boundary between the dermis and epidermis. Scale bar = 100 μm.

administration to cultured human mast cells releases histamine, the amount of which is approximately half the amount of IgE-associated histamine release [24]. Therefore, PAR_2 stimulation in the human skin releases histamine from mast cells although at low levels. PDE4 inhibitors have been shown to prevent antigen-induced histamine release from mast cells through the increase of intracellular cAMP [25]. Thus, mast cells are a conceivable site for the suppressive action of E6005 on IgE- and PAR_2-associated pruritus in humans, although higher concentrations of topical E6005 may be needed to act on mast cells than to act on epidermal keratinocytes. In contrast to humans, the PAR_2 receptor is not present in mast cells in the murine skin [11] and SLIGRL-NH_2-induced scratching is not inhibited by an H_1 histamine receptor antagonist in mice [26]. Thus, mast cells may not be a predominant site for the antipruritic action of topically applied E6005 in mice.

Although at low levels, mRNAs for all PDE4 subtypes are expressed in human dorsal root ganglia [27], raising the possibility that PDE4 subtypes are present in the primary afferents. PAR_2 has been reported to be present in neurons in the rat dorsal root ganglion [28] and in nerve fiber-like structures in human skin [12]. Thus, the primary afferents are also a conceivable site for the suppressive action of E6005 on SLIGRL-NH_2-induced scratching. However, we could not locate PDE4 subtype-positive nerve fibers in murine skin. In addition, PAR_2-positive nerve fibers were not

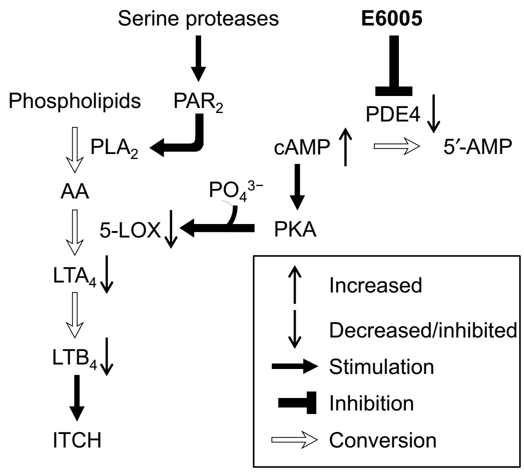

Fig. 7. Possible mechanism of the antipruritic effect of E6005. Stimulation of proteinase-activated receptor 2 (PAR_2) produces leukotriene B_4 (LTB_4), a pruritogen, in the epidermal keratinocytes. E6005 increases cyclic adenosine monophosphate (cAMP) by inhibition of phosphodiesterase 4 (PDE4). Increased cAMP activates protein kinase A (PKA), which phosphorylates 5-lipoxygenase (5-LOX). Phosphorylation of 5-LOX decreases the activity to inhibit LTB_4 production. 5′-AMP, 5′adenosine monophosphate; AA, arachidonic acid; LTA_4, leukotriene A_4; PLA_2, phospholipase A_2.

observed in murine skin [11]. Thus, the primary afferents may not be a site of the antipruritic action of E6005, at least in mice.

The details of the roles of the PDE4 subtypes are not completely understood. Many studies have focused on the role of PDE4 in inflammation [29]. Studies using gene knockout mice have suggested that an inhibitor of PDE4B, but not the other subtypes, shows anti-inflammatory effects [30–32]. In the present study, although all PDE4 subtypes were observed in the skin, PED4A, 4C, and 4D were present in the epidermal keratinocytes. These PDE4 subtypes may be important target molecule for the antipruritic action of topically applied E6005. However, we used naïve mice in this study. PDE4 is also expressed in immune cells (e.g., T cells, eosinophils, neutrophils, dendritic cells, monocytes, and macrophages) and its regulation is important for the treatment of inflammation [29,33]. These immune cells are also present in the skin of pruritic skin disease (e.g., atopic dermatitis) [34]. Thus, our results do not exclude the possibility that these immune cells are the site(s) of action of E6005 in dermatitis conditions.

In summary, topical application of the PDE4 inhibitor E6005 inhibited PAR_2-mediated scratching, which might be mediated by the inhibition of PAR_2-mediated LTB_4 production in the skin through the increase of the cutaneous concentration of cAMP (Fig. 7). Epidermal keratinocytes are suggested to be an important site for the antipruritic action of topical E6005. Topical PDE4 inhibitors, including E6005, may be useful for the treatment of PAR_2- and LTB_4-associated pruritic skin diseases.

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