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A putative anti-pruritic mechanism of the phosphodiesterase-4 inhibitor E6005 by attenuating capsaicin-induced depolarization of C-fibre nerves

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Short title: Anti-pruritic activity of E6005

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Abbreviations

AD: atopic dermatitis

PDE: phosphodiesterase

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TRPV1: transient receptor potential vanilloid 1

DRG: dorsal root ganglion

cAMP: 3',5'-cyclic adenosine monophosphate

Keywords: phosphodiesterase 4; cAMP, anti-pruritic effect; C-fibre nerve

Background

Chronic pruritus is not only a major manifestation but also a critical aggravating factor of atopic dermatitis (AD). It is, however, managed poorly with current antipruritic therapy.

The mechanism of pruritus in AD is incompletely understood, but it has become evident that a subset of C-fibre afferent neurons mediates the itch sensation (Schmelz et al, 1997). Transient receptor potential vanilloid 1 (TRPV1) is an ion channel expressed on C-fibre neurons that is relevant to the itch sensation (Imamachi et al, 2009 and S1). In the skin of patients with AD, TRPV1 is rendered more sensitive to pruritogens and plays an important role in the generation of itch (Akiyama et al. 2010)

Phosphodiesterase 4 (PDE4), an enzyme that metabolises 3',5'-cyclic adenosine monophosphate (cAMP), plays a role in AD pathogenesis (S2). E6005 is a PDE4 inhibitor developed as a topical agent for AD. We and other group have demonstrated that topical application of E6005 immediately inhibits itching behaviour as well as itch-related cutaneous nerve firing in a mouse model (Ishii et al, 2013, Andoh et al, 2014). This effect was not due to a local anaesthetic effect (Figure S1). Recently, E6005 has been shown to ameliorate itching in patients with AD (Furue et al, 2014).

Questions addressed

The mechanism underlying the immediate anti-pruritic effect of E6005 remains unknown.

The aim of this study was to examine whether this mechanism involves inhibition of activation of the dorsal root ganglion (DRG) neurons.

Experimental design

Detailed methods are described in Data S1. Briefly, small-sized DRG neurons isolated from Male Wistar rats (diameter, $<30\text{ }\mu\text{m}$), which have been reported to be relevant to itch (S3, S4), were selected for whole-cell patch clamp recording. Cells were superfused with combinations of capsaicin with vehicle or test compounds. Membrane depolarization was recorded, and the change was calculated as the difference between the membrane potential measured before and after the addition of capsaicin. cAMP levels were normalized to protein content.

Results

Under current-clamp conditions, $1\mu\text{M}$ capsaicin elicited a typical change in the membrane potential and action potential firing of DRG neurons (Figure 1a), which was attenuated by E6005 in a concentration-dependent manner (Figure 1b, 1c). There was no change in membrane potential between before and after the treatment of E6005 (Figure 1e). A structurally distinct PDE4 inhibitor roflumilast also decreased the capsaicin-evoked change in membrane potential, but anti-inflammatory agents such tacrolimus and a steroid betamethasone did not significantly affect it (Figure 1c). In contrast, the cAMP elevator forskolin reduced the magnitude of depolarization (Figure 1d). Intracellular cAMP levels in DRG neurons were increased by treatment with E6005 ($1\mu\text{M}$) (Figure 2).

Discussion and Conclusions

TRPV1 on C-fibres plays a role in generation of itch sensation (Imamachi et al, 2009, S1). Recent advances in pruritus research have revealed that a smaller sized TRPV1-positive C-fibres population has a major role in generation of itch (Schmelz et al, 1997, Han et al, 2013, S4, S5). We, therefore, used smaller DRG neurons for the electrophysiological study. DRG neurons showed rapid depolarisation in response to TRPV1 activator capsaicin. Attenuation of capsaicin-induced response by two structurally different PDE4 inhibitors suggests that inhibition of PDE4 can modulate DRG function. The cAMP elevator forskolin also diminished the depolarization, suggesting that cAMP has an inhibitory action on the activation of DRG neurons. DRG neurons expressed the PDE4B subtype (Figure S2), and intracellular cAMP levels were elevated by E6005 treatment. These results suggested that E6005 suppressed the activation of DRG neurons by increasing intracellular cAMP levels. Other anti-inflammatory compounds did not suppress the activation. The suppressive effect of TRPV1-mediated depolarization in DRG neurons seemed to be related to the immediate anti-pruritic effect of agents (Ishii et al, 2013). Tacrolimus is, however, reported to inhibit DRG activation by a longer pre-treatment than our study (Pereira et al, 2010). The lack of the effect in our study may be due to the inadequate pre-incubation time. E6005 directly affected DRG neurons. But, the mode of action seems unlike tacrolimus because E6005 exerted the suppressive effect without stimulating DRG. This was also confirmed by measuring C-fibre activity in animals with AD (Andoh et al, 2014) and absence of burning in patients with AD (Furie et al, 2014). The inhibitory effect of cAMP on depolarization was intriguing but was in contrast to that on pain, upon which it augments the sensitivity and magnitude. The reason for this difference remains to be elucidated. Han et al. reported that TRPV1-positive itch-specific DRG neurons that express the Mas-related G protein-coupled receptor (Mrgpr) generate itch but not pain upon capsaicin stimulation (Han et al, 2013). In addition, distinct

mechanisms for intracellular signalling between itch and pain generation have been suggested (Liu et al. 2013). In the present study, forskolin suppressed depolarization, instead of augmenting it, and cutaneous cAMP elevation has been shown to decrease itching in a mouse model of chronic itch (Andoh et al, 2014). Therefore, a distinct cAMP-driven inhibitory signal might be present in itch-related DRG neurons. Further experiments to study the modification of intracellular molecules by cAMP as well as Mrgpr expression in our preparation of DRG neurons are required to confirm the effect of E6005 on DRG neurons.

In conclusion, we found that E6005 has an inhibitory effect on the excitation of itch-relevant DRG neurons through activation of cAMP signalling, which may (at least in part) contribute to the immediate anti-pruritus effect. E6005 may potentially be a new agent for the management of pruritus in various skin disorders including AD.

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Author contributions

HW, MO and NI undertook the research and analysed data. IH contributed to the design. MS designed the research, interpreted the data, and wrote the manuscript.

Conflict of interests

The authors state no conflict of interest.

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Figure legends

Figure 1. Capsaicin-evoked depolarization in dorsal root ganglion (DRG) neurons and the inhibitory effect of compounds.

Tracings of membrane potential depicting capsaicin-elicited changes in membrane potential in rat DRG neurons recorded in the absence (a) or presence of E6005 (1 μ M) (b) are shown. Capsaicin was applied as indicated by the horizontal bars. E6005 was applied 2 min before capsaicin stimulation. Comparison of the effects of E6005, roflumilast, betamethasone, tacrolimus, and forskolin in DRG neurons superfused with capsaicin is presented in (c) and (d). Direct effect of E6005 on DRG neurons is shown in (e). Changes in membrane potential (Δ mV) in (c) and (d) are the mean $\pm$ S.E.M. of n = 4–7 experiments. *p < 0.05 *versus* vehicle-treated DRG neurons.

Figure 2. E6005 elevated the cAMP level in DRG neurons.

Rat DRG neurons were treated with vehicle or E6005 (1 μ M) for 10 min and lysed. cAMP levels in lysates were determined and normalized to protein content. Results represent the mean of two separate experiments.

References

- Akiyama T, Carstens MI, Carstens E (2010). Enhanced scratching evoked by PAR-2 agonist and 5-HT but not histamine in a mouse model of chronic dry skin itch. *Pain* 151: 378-83.
- Andoh T., Yoshida T., Kuraishi Y (2014). Topical E6005, a novel phosphodiesterase 4 inhibitor, attenuates spontaneous itch-related responses in mice with chronic

atopy-like dermatitis. *Experiment Dermatol* 23: 359-61.

- Furue M, Kitahara Y, Akama H, Hojo S, Hayashi N, Nakagawa H, The Japanese E6005 Study Investigators (2014). Safety and efficacy of topical E6005, a phosphodiesterase 4 inhibitor, in Japanese adult patients with atopic dermatitis: Results of a randomized, vehicle-controlled, multicenter clinical trial. *J Dermatol* 41: 1-9.
- Han L, Ma C, Liu Q, *et al* (2013). A subpopulation of nociceptors specifically linked to itch. *Nat Neurosci* 16: 174-82.
- Imamachi N, Park GH, Lee H, *et al* (2009). TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. *Proc Natl Acad Sci USA* 106: 11330-5.
- Ishii N, Shirato M, Wakita H, *et al* (2013). Antipruritic effect of the topical phosphodiesterase 4 inhibitor E6005 ameliorates skin lesions in a mouse atopic dermatitis model. *J Pharmacol Exp Ther*. 346: 105-12.
- Liu T and Ji R (2013). New insights into the mechanisms of itch: are pain and itch controlled by distinct mechanisms? *Eur. J. Physiol* 465: 1671-85.
- Pereira U., Boulais N., Lebonvallet N., Pennec J.P., Dorange G., Misery L. (2010). Mechanisms of the sensory effects of tacrolimus on the skin. *Br. J. Dermatol* 163: 70-77.
- Schmelz M, Schmidt R, Bickel A, *et al* (1997). Specific C-receptors for itch in human skin. *J Neurosci* 17: 8003-8.

Data S1

Materials and methods

Reagents

E6005 and roflumilast were synthesized in-house at Eisai Co., Ltd. (Tokyo, Japan). Forskolin, betamethasone, capsaicin, Ara-C, and type-IV collagenase were purchased from Sigma–Aldrich (Saint Louis, MO, USA). FK506 (tacrolimus) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Rat beta-nerve growth factor (β -NGF) was purchased from R&D Systems (Minneapolis, MN, USA).

Animals

Male Wistar rats (5 weeks) were purchased from Charles River Laboratories Japan. (Kanagawa, Japan). All animals were kept in a pathogen-free animal facility with temperature maintained at 20–26 °C, humidity 40–70%, and a 12-h day–night cycle, with access to food and water *ad libitum*. All procedures were undertaken at an animal facility accredited by the Center for Accreditation of Laboratory Animal Care and Use, Japan Health Sciences Foundation (Tokyo, Japan). All protocols were approved by the Institutional Animal Care and Use Committee, and carried out according to Eisai Animal Experimentation Regulations.

Isolation of DRG neurons

Primary DRG neurons were isolated as described previously with slight modification (S6). Briefly, isolated DRGs from adult Wistar rats were collected in cold Hank's balanced salt solution (HBSS), centrifuged, and then resuspended in F-12 nutrient mixture containing collagenase (2 mg/mL) and foetal calf serum (FCS, 10%). After 90 min of incubation at 37 °C with agitation, cells were centrifuged and treated with trypsin–EDTA solution for 15 min

at 37 °C. The suspension of neurons was washed and suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. Cultures were maintained for 2–4 days in a humidified atmosphere (37 °C, 5% CO₂).

Electrophysiological recordings

Small DRG neurons (diameter, <30 µm), which have been shown to exhibit high sensitivity to capsaicin (S3, S4), were selected for whole-cell patch clamp recording at room temperature (20–22 °C) by using an Axopatch 1D Amplifier (Axon Instruments, Union City, CA, USA). The micropipettes used had resistances of 2–6 MΩ. The standard pipette solution contained 4mM ATP, 130mM KCl, 2mM MgCl₂·6H₂O, 5mM EGTA, 0.5mM CaCl₂, and 10mM HEPES, with pH adjusted to 7.2 using NaOH. The standard external solution contained 130mM NaCl, 5mM KCl, 2mM MgCl₂·6H₂O, 2mM CaCl₂, and 10mM HEPES. The low-pass filter was set at 5 kHz. Cells were superfused with vehicle, E6005 alone or combinations of capsaicin with vehicle, E6005 (0.1µM and 1µM), roflumilast, betamethasone, or tacrolimus. All acquisition and analysis of data were performed using pClamp v8.02 (Axon Instruments, Sunnyvale, CA, USA). Capsaicin-evoked membrane depolarization (ΔmV) was calculated as the difference between the membrane potential measured before and after the addition of capsaicin.

Quantification of cAMP levels in DRG neurons

DRG neurons were suspended in DMEM/F12 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin, 100 ng/mL rat β-NGF, and 1M Ara-C. Cells were plated on 24-well plates. After ~7 days, cells were treated with E6005 (1µM) for 10 min at 37 °C in DMEM/F12 medium. cAMP content was measured using the cAMP-Screen™ system (Applied Biosystems, Foster, CA, This article is protected by copyright. All rights reserved.

USA) according to manufacturer instructions. cAMP levels were normalized to protein content as determined by a protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analyses

Data were analysed using unpaired *t*-tests and one-way ANOVA followed by Dunnett's multiple comparison test using SAS v6.12 (SAS Institute Japan, Tokyo, Japan). $p < 0.05$ (two-sided) was considered significant.

Supplementary figure

Figure S1. Effects of E6005, amitriptyline, bupivacaine, and procaine on Na^+ current recorded in hNav1.2-CHO cells.

hNav1.2-expressing CHO cells were used for patch clamp experiments. Nav1.2 current was evoked by application of a train of 30 pulses to 0 mV from a holding potential of -70 mV at 20 Hz and recorded automatically using IonWorks-Quattro (Molecular Devices, Sunnyvale, CA, USA). Concentration–response curves obtained after a train of 30 pulses are presented as the mean $\pm$ SEM. The Na^+ -channel blockers amitriptyline, bupivacaine, and lidocaine inhibited Nav1.2 current in a concentration-dependent manner, with half-maximal inhibitory concentration (IC_{50}) values (μM) of 1.3, 3.3, and 86.3, respectively. E6005 did not show any inhibitory activity for Nav1.2 current at ≤ 20 μM . In this experiment, the maximal attainable concentration of E6005 was 20 μM owing to its limited solubility.

Figure S2. PDE4B expression in DRG neurons

Expression of PDE4 protein in rat DRG neurons was studied by western blotting analyses. DRG neurons were collected as described in Data S1. Protein extracts of DRGs were prepared in extraction buffer comprising 50mM Tris-HCl, 133mM NaCl, 2% sodium dodecyl

sulfate (SDS), 1mM dithiothreitol (DTT), and 1mM phenylmethylsulfonyl fluoride (PMSF). Extracted proteins were electrophoresed on 10% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blotted with antibodies against PDE4B (polyclonal rabbit antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with horseradish peroxidase-conjugated secondary antibody (Dako Cytomation, Glostrup, Denmark). Antibodies against PDE4A and PDE4D (polyclonal rabbit antibody, Santa Cruz Biotechnology) were used in this experiment. Signals were detected using an ECL kit (Amersham, San Francisco, CA, USA) and visualized by exposure to X-ray film (Kodak, Rochester, NY, USA).

Lane 1; standard marker, lane 2; PDE4B signal.

Supplementary references

- S1: Tóth BI, Bíró T (2013). TRP channels and pruritus. *Open Pain Journal* 6(Suppl 1: M8): 62-80.
- S2: Hanifin JM, Chan SC, Cheng JB, *et al* (1996). Type 4 phosphodiesterase inhibitors have clinical and in vitro antiinflammatory effects in atopic dermatitis. *J Invest Dermatol* 107: 51-6.
- S3: Li C, Peoples RW, Lanthorn TH, *et al* (1999). Distinct ATP-activated currents in different types of neurons dissociated from rat dorsal root ganglion. *Neurosci Lett* 263: 57-60.
- S4: Hiura A, Nakagawa H (2011). An overview of the actions of capsaicin and its receptor, trpv1, and their relations to small primary sensory neurons. *Antiinflamm Antiallergy Agents Med Chem* 10: 2-9.
- S5: Kim SJ, Park GH, Kim D, *et al* (2011). Analysis of cellular and behavioral

responses to imiquimod reveals a unique itch pathway in transient receptor potentialvanilloid 1 (TRPV1)-expressing neurons. *Proc Natl Acad Sci USA* 108: 3371-6.

- S6: Dedov V.N., Mandadi S., Armati P.J., Verkhatsky A (2001). Capsaicin-induced depolarisation of mitochondria in dorsal root ganglion neurons is enhanced by vanilloid receptors. *Neuroscience* 103:219-226.



