Doctoral Thesis

.

# Involvement of lysophosphatidic acid-evoked TRPA1 and TRPV1 activation in peripheral itch sensation in mice

Hiroki Kittaka

Department of Physiological Sciences

School of Life Science

The Graduate University for Advanced Studies (SOKENDAI)

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## *Introduction*

### *Peripheral itch system*

The sensation of itching, formally called pruritus is defined as ‘an unpleasant cutaneous sensation that provokes a desire to scratch’. Current understanding of the peripheral itch pathway is as follows. When a subset of C-fibers, originating superficially in the skin, is activated by a pruritogen that invokes an itching sensation, the impulses are transmitted to the dorsal horn neurons of the spinal cord through the spinothalamic tract to the thalamus, and to the somatosensory cortex.

Histamine is the most-studied pruritogen. It evokes peripheral itching sensations in humans and induces itch-related behaviors in mice. Conversely, itching that is thought intractable and independent of histamine is also reported. Lysophosphatidic acid (LPA) was identified in cholestatic patients with pruritus and proven to cause scratching behaviors in mice, and the mechanism is not well elucidated.

### *Transient receptor potential channels involved in itching sensations*

Transient receptor potential (TRP) is a protein superfamily of calcium-permeable ion channels, currently composed of 6 subfamilies (ankyrin, canonical, melastatin, mucolipin, polycystin and vanilloid) in mammals. Of all the TRP channels, TRPA1 and TRPV1 have been mainly reported to be involved in pruritus. Both TRPA1 and TRPV1 are expressed in dorsal root ganglion (DRG) neurons. They are greatly important to the physiology of pruritus in addition to pain sensation. In addition to their roles as sensors for painful stimuli, recent studies have shown that both channels are also essential for the sensation of itching.

The peripheral mechanism of pruritus involving TRPA1 and/or TRPV1 appears to

be very complex. For example, several intracellular signals following the activation of multiple pruritogenic receptors have been reported. In this study, I hypothesized that LPA activates primary sensory neurons through TRPA1 and/or TRPV1. Moreover, this study examined the physiological role and the mechanism of the LPA-induced itch sensation.

***Methods and Materials***

*Animals*—Male C57BL/6NCr mice (6–10 weeks old; SLC, Japan) were housed in a controlled environment (12 h light/dark cycle; 22–24℃; 50–60% humidity) with food and water provided *ad libitum*. TRPA1KO and TRPV1KO mice were kindly provided by Dr. David Julius (University of California, San Francisco). TRPA1/TRPV1 double KO (A1V1DKO) mice were obtained by crossbreeding TRPA1KO and TRPV1KO mice. All procedures involving and care and use of animals were approved by the institutional Animal Care and Use Committee of National Institute of Natural Sciences and carried out in accordance with the National Institutes of Health *Guide for the care and use of laboratory animals* (NIH publication No. 85–23. Revised, 1985).

*Behavioral test*—A cheek injection model was used in this study. After a 30-min habituation, 10 μl of saline as a vehicle or LPA (Avanti Polar Lipids, USA) dissolved in saline (100 nmol/10 μl) was intradermally injected into the right cheek and behaviors were recorded for 30 min. Thereafter, scratching and wiping responses toward the injection site were counted.

*Cultures of dissociated DRG neurons*—Dorsal root ganglia from all spinal levels of adult C57BL/6NCr mice were rapidly dissected and dissociated by incubation for 20 min at 37℃ in Earle's balanced salt solution (EBSS, Sigma) containing 10% fetal bovine serum (FBS; Biowest, France), 2 mM $_{L}$-glutamine (GulutaMAX, Gibco), 1% vitamin mixture, 50 U $ml^{-1}$ penicillin/ 50 μM $ml^{-1}$ streptomycin (Invitrogen) and 2.5 mg/ml collagenase. After changing the salt solutions by centrifugation (RT, 1000 rpm, 4 min), DRG were gently triturated with a fire-polished Pasteur pipette and filtered through 70 μm nylon mesh (BD). The solution was changed to Dulbecco's modified Eagle's medium (DMEM, Wako, Japan) containing 10% FBS, 50 U $ml^{-1}$ penicillin/ 50 μM $ml^{-1}$ streptomycin and 2 mM $_{L}$-glutamine. DRG neurons were re-suspended in DMEM, inoculated in a drop on poly-$_{D}$-lysine (100 μg/ml, Sigma) pre-coated coverslips and incubated for 30 min at 37℃ in 5% $CO_2$ followed by medium addition. DRG neurons were used in 14–24 h after dissection.

*Calcium imaging*—Calcium-imaging experiments were conducted with the fluorescent indicator Fura 2-acetoxymethyl ester (Fura 2-AM; Molecular Probes). Standard bath solution contains 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM $_{D}$-glucose at pH 7.4 adjusted with NaOH. $Ca^{2+}$-free bath solution was prepared by omitting 2 mM $CaCl_2$ from the solution and adding 5 mM EGTA. Ionomycin (5 μM) was applied to confirm cell viability. Fura 2 loaded in the cells was excited with 340- and 380- nm wavelength and emission was monitored at 510 nm with a digital CCD camera. Data were analyzed by using *ImageJ* software (National Institute of Health); values of the (340/380) ratio were normalized to the values

obtained in the ionomycin application.

*Construction of mutant mouse TRPA1* － mTRPA1 cDNA in the pcDNA5/FRT expression vector was used. Vectors containing the full-length mouse TRPA1 mutants were constructed by PCR with synthesized oligonucleotide primer sets that have specific substitutions that cause amino acid substitutions.

*Maintenance and transient transfection of HEK293T cells*－Human embryonic kidney 293T (HEK293T) cells were maintained at 37℃ in 5% $CO_2$ in the supplemented DMEM described above. Transient transfection of HEK293-cells was achieved in PLUS™ Reagent (Invitrogen), Lipofectamine™ Regent (Invitrogen) and Opti-MEM® I Reduced Serum medium (Invitrogen) following the manufacturer's protocol. 0.5 μg mTRPA1 or mutated mTRPA1 in the pcDNA5/FRT and 0.1μg pGreen Lantern 1 were transfected into HEK293T cells.

Electrophysiology－HEK293T cells expressing mTRPA1 or mutant mTRPA1 were used for single channel recording with standard patch pipettes (3-5 MΩ resistance) made of borosilicate glass capillaries. The solution was changed before the excision and the pipette solution contains 140 mM KCl, 5 mM EGTA and 10 mM HEPES at pH 7.4, adjusted with KOH.

*Statistics* － All results are expressed as means ± sem. Statistical analysis was performed with Mann-Whitney U test or Kruskal-Wallis tests followed by Steel's tests. $p < 0.05$ was considered significant.

## *Results*

### *Effects of LPA on mouse scratching behaviors*

LPA can induce scratching responses and pain-related responses in mice, indicating the ambiguity of LPA actions in mice. In order to clarify the physiological role of LPA in mice, a cheek-injection model was chosen. LPA markedly induced scratching responses but not wiping behaviors compared with vehicle-treatment. Thus, it was confirmed that LPA caused itching sensations upon injection into the mouse cheek.

### *Effects of LPA on intracellular $Ca^{2+}$ concentrations in mouse DRG neurons*

To examine whether LPA directly affected mouse DRG neurons, the cells were examined using a $Ca^{2+}$-imaging method. Application of 5 μM of LPA robustly increased intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) in DRG neurons. About half of total neurons were LPA-responding ($LPA^+$). This effect was LPA dose-dependent. The LPA-induced $[Ca^{2+}]_i$ increases were not observed when the extracellular bath solution was free of $Ca^{2+}$, indicating that the LPA-induced $[Ca^{2+}]_i$ increases were caused by $Ca^{2+}$ influx from the extracellular space. To identify the DRG neuron subpopulation sensitive to LPA, selective agonists for TRPA1 and TRPV1 were applied after LPA. Among the $LPA^+$ neurons, most (87.2 ± 2.7 %, 184/213) functionally expressed TRPA1 and/or TRPV1, suggesting that TRPA1 and/or TRPV1 contributed to the effect of LPA on DRG neurons. To examine whether activation of TRPA1 and/or TRPV1 was involved in the $[Ca^{2+}]_i$ increases induced by LPA application, a TRPA1 inhibitor or a TRPV1 inhibitor was applied before LPA application. These results

suggested that activation of both TRPA1 and TRPV1 was involved in the LPA-induced $[Ca^{2+}]_i$ increases. $LPA^+$ neurons were significantly decreased in TRPA1KO and/or TRPV1KO DRG neurons. Taken together, it was concluded that most of the LPA-induced $[Ca^{2+}]_i$ increases in DRG neurons occurred through activation of TRPA1 and/or TRPV1.

***Involvement of TRPA1 and TRPV1 in LPA-induced scratching responses in mice***

Scratching behaviors decreased in the TRPA1KO, TRPV1KO and A1V1DKO mice. No changes in the wiping behaviors were observed among mouse strains, suggesting both TRPA1 and TRPV1 were involved in the LPA-induced scratching behaviors.

***Involvement of LPA receptors in LPA-induced increases of intracellular $Ca^{2+}$ concentrations in DRG neurons***

At least 6 G protein-coupled LPA receptors have been reported. Among them, $LPA_1$, $LPA_3$ and $LPA_5$ are expressed in DRG neurons. To investigate the involvement of these LPA receptors in the LPA-induced $[Ca^{2+}]_i$ increases in DRG neurons, H2L 5765384 (an antagonist of $LPA_1$, $LPA_3$ and $LPA_5$) was used. Ten μM of H2L 5765834 inhibited the LPA-induced $[Ca^{2+}]_i$ increases of the $LPA^+$-DRG neurons, 71.5 ± 4.3 % of which expressed TRPA1 and/or TRPV1, suggesting possible involvement of LPA receptors in the LPA-induced $[Ca^{2+}]_i$ increases in DRG neuron.

***Direct activation of TRPA1 by LPA***

Direct activation of TRPV1 by LPA binding was shown using biochemical and electrophysiological methods. Here, it was hypothesized that LPA could directly

activate TRPA1 similar to TRPV1. LPA application induced opening of the single-channel TRPA1 currents at +30 mV in a dose-dependent manner, indicating that LPA activated TRPA1 in a membrane-delimited manner. The LPA-activated currents were inhibited almost completely by the application of 1 μM A967079, a specific TRPA1 inhibitor, further indicating the activation of TRPA1 by LPA.

***Identification of the amino acids involved in the LPA-mediated activation of TRPA1***

To explore the amino acid residues involved in LPA-mediated activation of TRPA1, I constructed single or double mutants in which lysine or arginine residues were replaced with glutamine. The functions of the mutant TRPA1 channels in the $Ca^{2+}$-imaging experiments were confirmed as well. Among the mutants tested, two mutants showed markedly reduced $NP_o$ values upon LPA application compared with WT TRPA1. The two mutants showed similar large responses to other known TRPA1 agonists, carvacrol and AITC, suggesting that these basic residues were involved in the activation of TRPA1 by LPA.

***Discussion***

Pruritus in rodents has been evaluated by quantifying scratching behaviors induced by the injection of pruritogens into the back, a response that is not induced by algogens. Although this method is very useful when studying known pruritogens, injection of capsaicin also can induce scratching responses in mice, suggesting that scratching of the back (and neck) in rodents could be unsuitable for evaluating the ability of new compounds to cause pruritus. An alternative cheek-injection model was recently proposed. LPA causes scratching behaviors, as well as licking behaviors, resulting in a

controversy whether the physiological role of LPA is to induce itching and/or pain. In the preceding studies, LPA was injected into the back and hind paws of mice to evaluate itch-related scratching behaviors and pain-related licking behaviors. In the present study, a cheek-injection model was utilized to clarify these issues and it was shown that LPA significantly induced itch-related scratching behaviors, but not pain-related wiping behaviors.

Peripheral targets of LPA are assumed to be mainly sensory nerve endings, keratinocytes or mast cells. In this study, the focus was on the effects of LPA on DRG neurons. LPA induced increases of $[Ca^{2+}]_i$ in DRG neurons and proportion of $LPA^+$-DRG neurons, which was dose-dependent. The mechanism by which LPA increases $[Ca^{2+}]_i$ is unknown, although LPA-mediated $[Ca^{2+}]_i$ increases were observed in rat DRG neurons. This study is the first to show clarified that extracellular $Ca^{2+}$ is necessary for the LPA-mediated $[Ca^{2+}]_i$ increases in mouse DRG neurons. These data suggest that molecules associated with the plasma membrane are involved in $Ca^{2+}$ influx. In the present analysis, experiments with agonists and antagonists for TRPA1 and/or TRPV1 revealed that the majority (~90%) of mouse DRG neurons functionally expressed TRPA1 and/or TRPV1 and that activation of these two channels mediated the LPA-induced $[Ca^{2+}]_i$ increases. However, it is not clear which channel is more important for the LPA-induced $[Ca^{2+}]_i$ increases. They might contribute to the LPA-mediated $[Ca^{2+}]_i$ increases separately or in concert because there are several reports showing functional interaction between the two channels.

Consistent with the results that TRPA1 and TRPV1 were involved in the LPA-induced $[Ca^{2+}]_i$ increases in mouse DRG neurons, LPA-induced scratching behaviors were reduced in TRPA1KO, TRPV1KO and A1V1DKO mice, suggesting that

both TRPA1 and TRPV1 were involved in the LPA-induced scratching responses. LPA-induced scratching behaviors in TRPA1KO, TRPV1KO and A1V1DKO mice were reduced to levels similar to that of WT mice treated with control vehicle. Those results suggest that the functional interaction between TRPA1 and TRPV1 in causing scratching behaviors is similar to the results observed in the $Ca^{2+}$-imaging experiments.

One of the candidate targets of LPA action is an LPA receptor, which is supported by the fact that an LPA receptor antagonist inhibited the LPA-induced $[Ca^{2+}]_i$ increases in DRG neurons while the LPA receptor antagonist did not inhibit the AITC- or capsaicin-induced $[Ca^{2+}]_i$ increases. These results suggest the possible intracellular signaling to cause $Ca^{2+}$ influx via TRPA1 and TRPV1 downstream of LPA receptor activation. LPA receptors are G protein-coupled receptors currently known as $LPA_1$-$LPA_6$. Among the 6 receptors, $LPA_1$, $LPA_3$ and $LPA_5$ were reported to be expressed in DRG neurons. All these receptors couple to $G_{q/11}$/PLC (phospholipase C) signaling that is generally thought to be involved in intracellular $Ca^{2+}$ release from the endoplasmic reticulum (ER) store. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG), and $IP_3$ activates ionotropic $IP_3$ receptor mainly expressed in ER, which releases $Ca^{2+}$ into cytoplasm. However, $Ca^{2+}$-imaging experiments using DRG neurons in extracellular $Ca^{2+}$-free condition didn't represent $[Ca^{2+}]_i$ increases, suggesting that cytosolic $Ca^{2+}$ release is not involved in the LPA-induced $[Ca^{2+}]_i$ increases.

Pruritus induced by histamine, α-methyl-5-hydroxytryptamine and BAM8-22 were reported to be PLC-dependent. In addition, involvement of another phospholipase, phospholipase $A_2$ ($PLA_2$) in histamine-induced pruritus was reported, in which H1 receptor activation was followed by activation of $PLA_2$ and lypoxygenase (LOX).

$PLA_2$ hydrolyzes membrane glycerophospholipids to arachidonic acids (AA) and lysophospholipids. AA is metabolized to bioactive, fatty acid substances. LOX-dependent fatty acid metabolites were reported to directly activate TRPV1. Cyclooxygenase (COX)-dependent fatty acid metabolites were reported to directly activate TRPA1. Therefore, if $PLA_2$ is activated, following activation of LPA receptors, metabolites derived from AAs produced by $PLA_2$ could activate TRPA1 and TRPV1. However, activation of $PLA_2$ follow1ing GPCRs activity hasn't been well established. It would be definitely necessary to examine the involvement of $PLA_2$ in the LPA-induced activation of TRPA1 and TRPV1 by using $PLA_2$ inhibitors such as quinacrine.

This study also asked another possibility that LPA directly activates TRPA1 or TRPV1 without involvement of LPA receptors. TRPV1 has already been reported to be directly activated by LPA. Therefore, the present analysis focused on the effects of LPA on TRPA1. I performed the single-channel recordings of the patch membrane excised from HEK293T cells expressing mouse TRPA1, which revealed that mouse TRPA1 was directly activated by LPA in a membrane-delimited manner. The possible interaction between TRPA1 and LPA receptors could be hardly imagined in the inside-out configuration because LPA was applied to the intracellular side. Even when LPA applied to the intracellular side activates LPA receptors upon crossing the lipid bilayers, the downstream substances upon LPA receptor activation should not be abundant. Therefore, it is unlikely that LPA receptor activation causes TRPA1 channel opening in the inside-out configuration. In addition, the amino acid residues of TRPA1 interacting with LPA were identified. This conclusion was based on the results that the open probability was decreased upon LPA application while channel activation was

preserved upon application of other TRPA1 agonists (carvacrol and AITC) in mutants in which those residues were altered. Thus, I conclude that TRPA1 can be directly activated by LPA without involvement of LPA receptor activation. If that is the case, LPA should reach the intracellular action sites upon penetrating the lipid bilayers in the physiological conditions. Alternatively, LPA can be produced in the cytosol by the enzymes described above, in which upstream events would be necessary. Because the identified residues were basic amino acids, it is possible that negatively charged LPA interacted with the positively charged lysine and arginine residues. These results suggest that these two basic regions are important for the action of lipid-like molecules with negative charges. The residues are conserved between mouse and human TRPA1, further supporting the importance of the residues for the TRPA1 function.

The results presented in this study suggest that the action of LPA on TRPA1 could be involved in the pathophysiological responses in humans. LPA has been identified as one of the most likely candidate pruritogens for cholestatic pruritus observed in diseases such as primary biliary cirrhosis (PBC). LPA concentrations between 1 and 10 μM are attainable in cholestasis. Therefore, the LPA-induced itch-related behaviors observed in mice could explain the itch sensation observed in such conditions in humans. PBC-model mice made with TRPA1KO or TRPV1KO strains could exhibit less itch-related behaviors. Although further experiments are necessary, it is possible that antagonizing TRPA1 or TRPV1 function could lead to novel anti-itch therapies.