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Full Paper

Histamine enhances ATP-induced itching and responsiveness to ATP in keratinocytes

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ABSTRACT

Mechanical stimulation of cultured keratinocytes and a living epidermis increases intracellular calcium ion concentrations ($[Ca^{2+}]_i$) in stimulated cells. This action propagates a Ca^{2+} wave to neighboring keratinocytes via ATP/$P2Y_2$ receptors. Recent behavioral, pharmacological studies revealed that exogenous ATP induces itching via $P2X_3$ receptors in mice. We previously showed that alloknesis occurs when an external stimulus is applied to the skin with increased epidermal histamine in the absence of spontaneous pruritus. Based on these results, we investigated the effects of histamine at a concentration that does not cause itching on ATP-induced itching. The mean number of scratching events induced by the mixture of ATP and histamine increased by 28% over the sum of that induced by histamine alone or ATP alone. A317491, a $P2X_3$ receptor antagonist, suppressed the mixture-induced scratching more often than the ATP-induced scratching. Next, we examined the ATP-induced $[Ca^{2+}]_i$ change before and after histamine stimulation using normal human epidermal keratinocytes. Some cells did not respond to ATP before histamine stimulation but responded to ATP afterward, the phenomenon suppressed by chlorpheniramine maleate. These findings suggest that histamine enhances ATP-induced itching and that a potential mechanism could involve increased responsiveness to ATP in keratinocytes.

1. Introduction

Recent studies reported that the intradermal injection of adenosine triphosphate (ATP) induces itching in mice via $P2X_3$ receptors expressed on primary afferent sensory neurons.[1,2] In an atopic dermatitis mouse model of chronic pruritus, $P2X_3$ receptor mRNA increases in the dorsal root ganglion. The intradermal injection of the $P2X_3$ receptor antagonist partially suppresses scratching behavior (itching-related behavior).[2] These findings indicate that extracellular ATP could be partially involved in chronic pruritus, such as atopic dermatitis. Mechanical stimulation increases the intracellular calcium ion $[Ca^{2+}]$ concentrations ($[Ca^{2+}]_i$) in keratinocytes, causing the extracellular release of ATP. Keratinocytes surrounding the stimulated keratinocytes are responsible for the released ATP, and the newly responded keratinocytes increase $[Ca^{2+}]_i$, thereby propagating Ca^{2+} waves via the ATP.[3–5] Suramin, a P2 receptor antagonist abolishes the Ca^{2+} wave, and also apyrase, an ATP-degrading enzyme.[3,6] ATP is therefore involved in the increase in $[Ca^{2+}]_i$ and acts as a transmitter.[3] Sensory nerve endings attach to keratinocytes and receive various signals from them. In a co-culture system of epidermal keratinocytes and small-sized dorsal root ganglion neurons, communication between them occurs in an ATP/$P2Y_2$ receptor-dependent manner.[3] Therefore, a signaling is transmitted to dorsal root ganglion neurons via ATP that keratinocytes have received mechanical stimuli.[3] The keratinocytes' histamine-producing ability is low in healthy conditions. However, ultraviolet B light or surfactant stimuli enhance histamine production through increased expression of the protein histidine decarboxylase, which is a key enzyme of histamine biosynthesis, and process to a form with high enzyme activity.[7,8] Epidermal histamine is involved in itching induced by the topical application of

Abbreviations: ATP, Adenosine triphosphate; $[Ca^{2+}]_i$, Intracellular calcium ion concentration; DMSO, Dimethylsulfoxide; Fura-2AM, Fura-2 acetoxymethyl ester; NHEKs, Normal human epidermal keratinocytes; SEM, Standard error of the mean.

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surfactants to mouse skin[8,9] and affects intraepidermal nerve elongation.[10] An alloknesis reaction is observed when an external stimulus is applied to the skin with increased epidermal histamine at the timing spontaneous pruritus has not occurred.[11] Epidermal histidine decarboxylase positively correlates with the degree of idiopathic itching in monkeys and patients with atopic dermatitis.[12,13] In this study, we investigated the action of histamine at a concentration that does not cause itch on ATP-induced itching and the sensitivity to ATP of histamine-stimulated keratinocytes.

2. Materials and methods

2.1. Animals

Male Crl:ICR mice of age in 7–8 weeks were used. All animals were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). These animals were housed in a room with a controlled temperature (21–23 °C), humidity (45%–65%), and light (lights on from 7:00 AM to 7:00 PM). Food and water were provided *ad libitum*. The committee for animal experiments of the University of Toyama, Japan, approved the experimental animal procedures (Approval No.: A2019PHA-13).

2.2. Materials

Histamine dihydrochloride (Cat# 081-03551, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and ATP disodium salt (Cat# 45140000, Oriental Yeast Co., Ltd., Tokyo, Japan) were dissolved in saline (Otsuka Normal Saline, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for the *in vivo* experiments and culture medium (*vide infra*) for the *in vitro* experiments. A317491 (Cat# S8519, a $P2X_3/P2X_{2/3}$ receptor antagonist; Selleck Chemicals, Houston, TX, USA) was dissolved in dimethylsulfoxide (DMSO, Cat# 13445-45, Nacalai tesque, Inc., Kyoto, Japan) and diluted in 0.05% Tween 20-containing saline (final DMSO concentration of 5%); the resulting precipitation was suspended before use. Terfenadine (Cat# T9652, Sigma–Aldrich Co., St. Louis, MO, USA) was suspended in 0.5% sodium carboxymethyl cellulose (Cat# 039-01335, Fujifilm Wako Pure Chemical Corporation). In addition, (+)-chlorpheniramine maleate (Cat# 030-13271, Fujifilm Wako Pure Chemical Corporation) was dissolved in a culture medium (*vide infra*), and the ATP-containing solution was placed on ice immediately before use.

2.3. Behavioral experiments

The rostral back was shaved at least 1 day before starting the experiment. Scratching behavior was assessed as previously described.[8,9,14] Briefly, as acclimation, the mice were placed individually into an acrylic observation cage 1 h a day for a few consecutive days. Each mouse was placed individually into the cage immediately after an intradermal injection of a mixture of histamine and ATP (or their respective solutions) into the rostral back skin. Scratching behavior was video-recorded for 30 min, and the hind-paw scratching directed toward the injection site was counted. Terfenadine was administered orally at a dose of 30 mg/kg 30 min before starting the behavioral observations. A317491 was included in the mixture of histamine and ATP or the ATP solution and jointly it injected intradermally.

2.4. Cell culture

Normal human epidermal keratinocytes (NHEKs) (Cat# KK4009, Kurabo, Osaka, Japan) were cultured in CELLview™ glass-bottom cell culture dishes and divided into 4 compartments (Cat# 627870, Greiner Bio One GmbH, Frickenhausen, Germany). NHEKs were seeded at 4.5×10^4 cells/compartment and grown in Humedia-KG2 medium (Cat# KK2350S, Kurabo) supplemented with insulin (10 μg/mL) (Cat# KK6150, Kurabo), human recombinant epidermal growth factor (0.1 ng/mL) (Cat# KK6150, Kurabo), hydrocortisone (670 ng/mL) (Cat# KK6150, Kurabo), gentamicin (50 μg/mL) (Cat# KK6150, Kurabo), amphotericin B (50 ng/mL) (Cat# KK6150, Kurabo), and bovine pituitary extract (0.4% v/v) (Cat# KK6150, Kurabo) in 5% CO_2 and at 37 °C. The medium was replaced every 2–3 days. NHEKs were cultured until 30%–50% confluence before use in this study.

2.5. $[Ca^{2+}]_i$ image

We measured the changes in $[Ca^{2+}]_i$ with fura-2 acetoxymethyl ester (Fura-2AM) (Cat# CS23 Calcium Kit-Fura 2, Dojindo Laboratories Co., Ltd., Kumamoto, Japan), as previously described.[15] The NHEKs were incubated and loaded in KG2 medium, which did not include phenol red (Cat# KK2170S, Kurabo) but contained 5-μM Fura-2AM, 1.25 mM probenecid, and 0.05% Pluronic F-127. The loading was performed for 60 min in the dark, in 5% CO_2, and at 37 °C. After this loading period, the loading medium was replaced with the recording medium (the KG2 medium did not include phenol red but contained 1.25 mM probenecid). The cells were put on the stage of a fluorescence microscope (Nikon Eclipse Ti; Nikon Corporation, Tokyo, Japan) equipped with an electron multiplier CCD camera (C9100, Hamamatsu Photonics, Hamamatsu, Japan) and a high-speed scanning polychromatic light source (C7773, Hamamatsu Photonics). We performed the measurements at room temperature and estimated the $[Ca^{2+}]_i$ level by the fluorescence ratio at 340 nm and 380 nm using an image analyzer system (Aquacosmos; Hamamatsu Photonics). The drugs dissolved in the recording medium, and 2-fold concentrated solution was applied by pipetting. The cells were washed with the recording medium. The experimental protocol was as follows: 1) record the baseline for 1 min, then add the first ATP (3 μM) solution and record for 5 min; 2) wash and wait for 5 min; 3) record the baseline for 1 min, then add histamine (10 μM) solution and record for 5 min; 4) wash and wait for 5 min; and 5) record the baseline for 1 min, then add the second ATP (3 μM) solution and record for 5 min. Images were recorded at an interval of 2 s. In a separate series of experiments, (+)-chlorpheniramine maleate was included in the recording medium at a concentration of 100 μM, and this medium was used from the start of the histamine treatment. Approximately 45 cells were analyzed in each examination, and 4–7 examinations were performed. Regarding the data analysis, the recorded traces of the 340 nm/380 nm ratios were normalized at the average of each baseline value (calculated from 1 min before each drug application). A normalized peak ratio of 1.2 or higher was considered a positive reaction.

2.6. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) or scatter plot, with each circle representing 1 mouse. After confirming normality using the Shapiro–Wilk test and variance equivalency using the *F* test, the group means were compared using Student's *t*-test, Welch's *t*-test, or the Mann–Whitney *U* test. The multigroup comparison was analyzed with a one-way analysis of variance followed by Dunnett's test or Bonferroni's test. A P-value < 0.05 denoted statistical significance. The statistical analyses were performed using GraphPad Prism (version 8.0.1; GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Scratching behavior induced by the mixture of histamine and ATP

The intradermal injection of histamine increased scratching bouts in a concentration-dependent manner (Fig. 1A) (one-way ANOVA, $F_{3,\ 28} = 12.73$, $P < 0.0001$). This study aimed to use a concentration near the upper limit that did not induce statistically significant scratching (see Introduction section). Therefore, we used 1 nmol of histamine per site in the subsequent studies. The intradermal injection of ATP (400 nmol/50 μL) increased scratching bouts in the ICR mice (Fig. 1B) (one-way ANOVA, $F_{3,\ 60} = 25.04$, $P < 0.0001$). Interestingly, the mixture of histamine and ATP ((1 nmol + 400 nmol)/50 μL, intradermal) increased the number of scratching bouts more than when induced by ATP alone (Fig. 1B). The mean scratching events in the saline, histamine, ATP, and mixture groups were 17.4 (baseline value), 31.4, 65.8, and 97.0, respectively. The baseline value was subtracted from the mean scratching events in each drug-treated group. The mean number of scratching events induced by the histamine and ATP mixture was increased by 28% over the sum of that induced by histamine and ATP separately; the calculation was as follows: (97.0 − 17.4) / ((31.4−17.4) + (65.8−17.4)) = 1.28. Terfenadine (a histamine H1-receptor antagonist, 30 mg/kg, orally) did not affect the mixture-induced scratching (Fig. 1C) (F test, $F_{11,\ 11} = 1.16$, $P = 0.808$). By contrast, A317491 (a $P2X_3/P2X_{2/3}$ receptor antagonist, 400 nmol, intradermal) partially but significantly suppressed both the ATP-induced and the mixture-induced scratching; the reduced number of scratching events was 24 and 31 for the ATP-induced and mixture-induced scratching, respectively (Fig. 1D, Shapiro–Wilk test, non-normality; Fig. 1E, F test, $F_{11,\ 11} = 1.25$, $P = 0.720$).

3.2. Concentration-response curves for ATP-evoked and histamine-evoked increase in $[Ca^{2+}]_i$ in NHEKs

To investigate the excitability of histamine-sensitized keratinocytes to exogenous ATP, we first examined the EC_{50} values that cause $[Ca^{2+}]_i$ increase by histamine and ATP separately. Adding exogenous ATP or histamine increased the proportion of cells that increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 2A and B). We selected concentrations close to the EC50 of ATP and histamine, 3 μM ATP, and 10 μM histamine. Supplementary Figure 1 illustrates the representative traces of a single cell's Ca^{2+} responses evoked by 3 μM ATP and 10 μM histamine.

3.3. Histamine enhanced responsiveness to ATP in NHEKs

Eight trace patterns can be classified by reactivity (positive or negative reaction) to the first ATP, drugs (vehicle or histamine), and the second ATP. For example, the first ATP (−)–vehicle (−)–second ATP (+) reaction indicates that an analyzed cell showed a negative reaction to the first ATP. The cell also showed a negative reaction to the vehicle after washing out the first ATP, and the cell reacted to

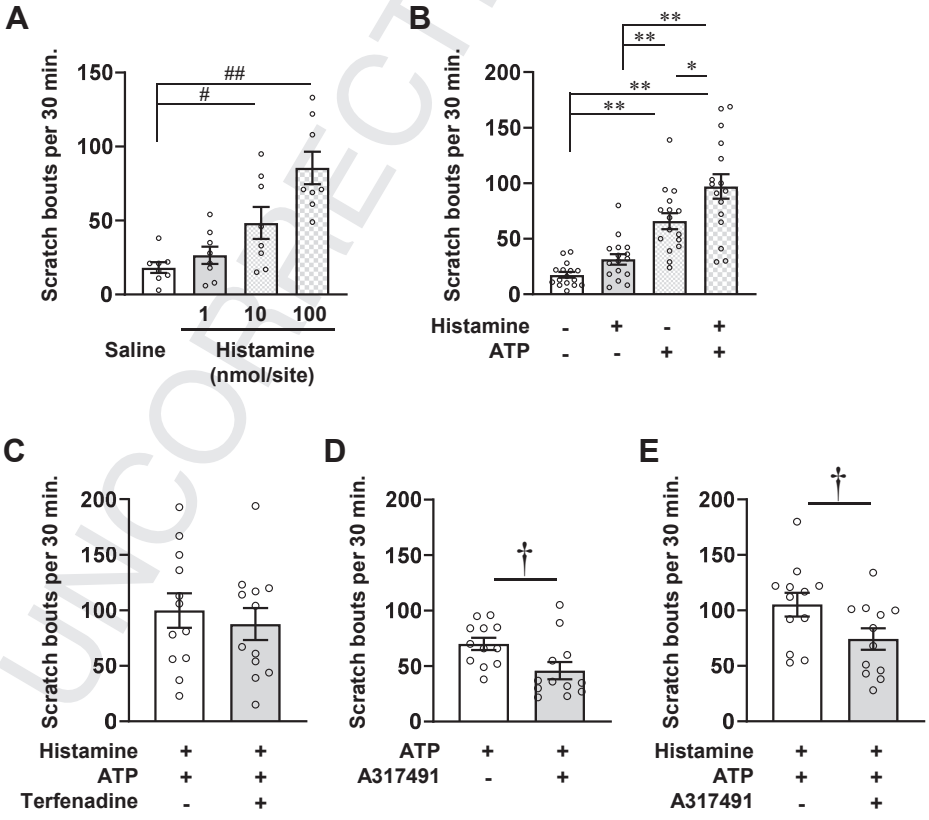

Fig. 1. Scratching behavior induced by a single or mixed solution of histamine and ATP and the effects of terfenadine and A317491 on this behavior. (A, B) ICR mice were intradermally injected with histamine (1–100 nmol/50 μL), ATP (400 nmol/50 μL), or a mixture of histamine and ATP ((1 nmol + 400 nmol)/50 μL) to the rostral back skin. (A) #$P < 0.05$, ##$P < 0.01$ (vs. Saline), Dunnett's test after one-way ANOVA ($F_{3,\ 28} = 12.73$, $P < 0.0001$). (B) *$P < 0.05$, **$P < 0.01$, Bonferroni's test after one-way ANOVA ($F_{3,\ 60} = 25.04$, $P < 0.0001$). (C) Terfenadine (30 mg/kg) was orally administered 30 min prior to the start of recording. $P = 0.573$, Welch's t-test after F test ($F_{11,\ 11} = 1.16$, $P = 0.808$). (D, E) A317491 (400 nmol) was injected intradermally together with a single solution of ATP or a mixed solution of histamine and ATP. (D) †$P < 0.05$ (Mann–Whitney U test). (E) †$P < 0.05$, Welch's t-test after F test ($F_{11,\ 11} = 1.25$, $P = 0.720$). Values represent the mean ± SEM ((A) n = 8, each; (B) n = 16, each; (C, D, E) n = 12, each).

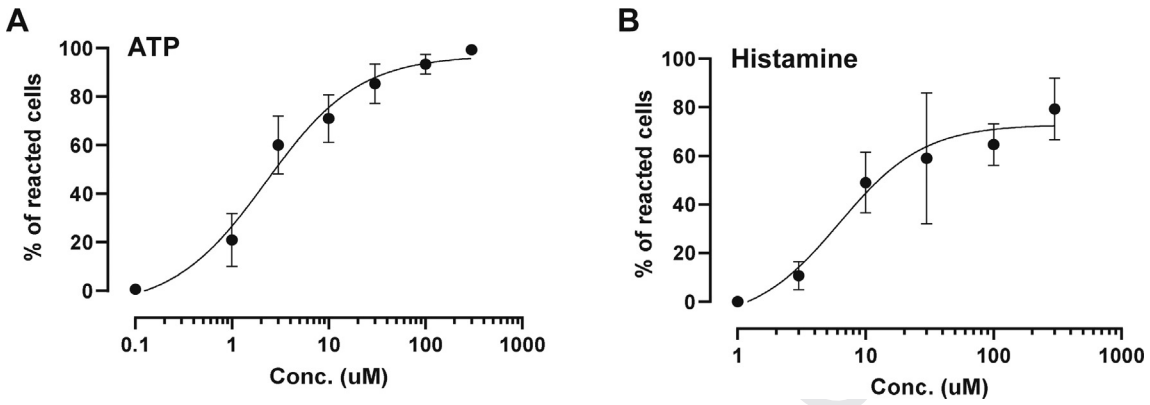

Fig. 2. Concentration-response curves for increased $[Ca^{2+}]_i$ evoked by ATP or histamine and representative traces of calcium response in NHEKs. Changes in $[Ca^{2+}]_i$ were measured with Fura-2AM. (A, B) Adding exogenous ATP or histamine increased the proportion of cells that increased the $[Ca^{2+}]_i$ in a concentration-dependent manner. Approximately 40–50 cells were analyzed in each examination, and 3–4 examinations were performed. The ED_{50} values of ATP and histamine were approximately 3 μM and approximately 10 μM, respectively. Results are expressed as the means ± SEM (n = 3–4 examinations). Fura-2AM, fura-2 acetoxymethyl ester.

the second ATP after washing out the vehicle. This study focused on cells that were unresponsive to the first ATP but responsive to the second ATP. The cells that did not respond to the first ATP but responded to the second ATP accounted for 0.5% and 15.9% of all analyzed cells in the first ATP–vehicle–second ATP assessment (Fig. 3A and B) and the first ATP–histamine–second ATP assessment (Fig. 4A and white arrows in Fig. 4E), respectively. As the breakdown, approximately 4.9% of all the analyzed cells responded to histamine and the second ATP (Fig. 4A and B), and 11.0% responded only to the second ATP (Fig. 4A and C). Supplementary Table 1 shows the number and percentage of cells that correspond to the six remaining trace patterns, and Supplementary Figures 2 and 3 display the representative traces of a single cell's Ca^{2+} responses of each pattern.

3.4. *Effects of (+)-chlorpheniramine maleate on histamine-induced and second ATP-induced $[Ca^{2+}]_i$ change*

We replaced the recording medium with a recording medium containing the antihistamine chlorpheniramine maleate after we finished recording the first ATP treatment. Terfenadine, used for evaluating scratching behavior, did not dissolve in the recording medium; therefore, we used chlorpheniramine maleate. In the presence of chlorpheniramine maleate, histamine-induced $[Ca^{2+}]_i$ elevation was almost completely suppressed (Supplementary Table 2). Some 5.5% of all the analyzed cells responded to only the second ATP (Fig. 5A and B). Supplementary Table 2 and Fig. 4 show the number of cells and representative traces of the remaining single cell's Ca^{2+} responses of each pattern.

4. Discussion

There are mechanisms in the skin that sense influences from outside the body. The mechanical stimulation of cultured keratinocytes, as well as living epidermis, increases $[Ca^{2+}]_i$ in the stimulated cell. It subsequently propagates a Ca^{2+} wave to neighboring keratinocytes via ATP/$P2Y_2$ receptors.[3–5] Recent behavioral, pharmacological evidence reveals that exogenous ATP induces itching via $P2X_3$ receptors in mice.[2] Accordingly, extracellular ATP is considered involved in external stimuli-induced itching. Alloknesis is a type of itching induced by external stimuli. Certain patients with chronic pruritus, such as atopic dermatitis, are hypersensitive to external stimuli to which healthy individuals do not respond and therefore do not experience itching. Lightly touching (involving innocuous mechanical stimuli) the normal skin surrounding a site of histamine-induced experimental itching (intradermal) elicits an alloknesis reaction.[16–18] An alloknesis reaction also occurs when an external stimulus is applied to the skin with increased epidermal histamine when spontaneous pruritus does not occur.[11] In other words, while the alloknesis reaction is recognized, it is assumed

A

Classification	Percentage of cells corresponding to each trace pattern
1st ATP (−) vehicle (+) 2nd ATP (+)	0% (0/190)
1st ATP (−) vehicle (−) 2nd ATP (+)	0.5% (1/190)
Total	0.5% (1/190)

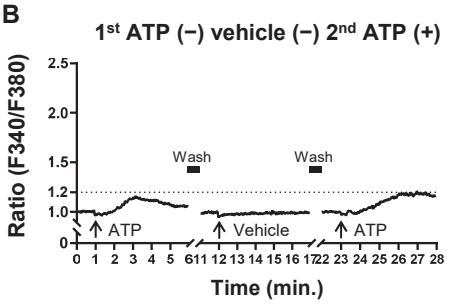

Fig. 3. Percentage of cells that did not respond to the first ATP but responsive to the second ATP, and the representative trace of a single cell's Ca^{2+} responses in the first ATP–vehicle–second ATP assessment. We first applied 3 μM ATP, followed by vehicle (medium) and then 3 μM ATP. NHEKs were washed before each reagent application. (A) A total of 190 cells were analyzed. The number and percentage of the responding cells in relation to the total number of analyzed cells are shown. (B) Representative trace of a single cell's Fura-2AM ratio (340 nm/380 nm) in the 1st ATP (−) vehicle (−) 2nd ATP (+) group. The representative trace was normalized at the mean baseline value (calculated 1 min before the first ATP application). The ratio was calculated using the 1-min baseline value before each drug application. A ratio of ≥1.2 means a positive reaction. (+) indicates a positive reaction, and (−) indicates a negative reaction. Fura-2AM, fura-2 acetoxymethyl ester. NHEKs, Normal human epidermal keratinocytes.

A

Classification	Percentage of cells corresponding to each trace pattern
1st ATP (−) histamine (+) 2nd ATP (+)	4.9% (13/263)
1st ATP (−) histamine (−) 2nd ATP (+)	11.0% (29/263)
Total	15.9% (42/263)

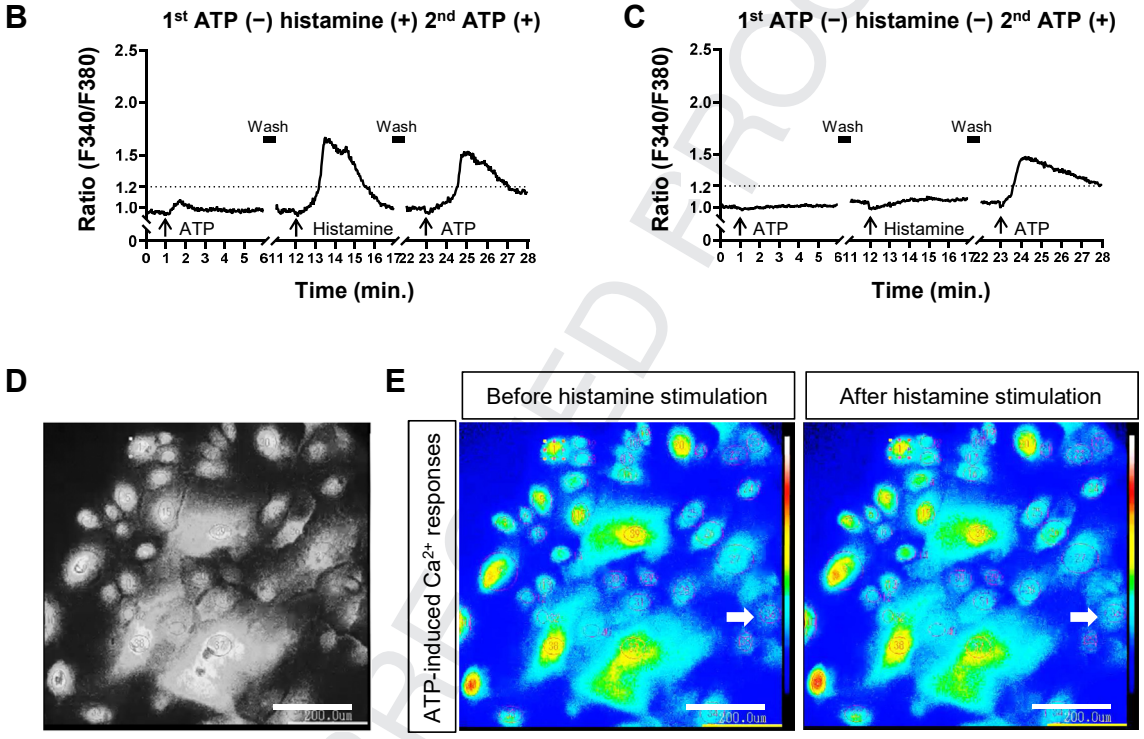

Fig. 4. Percentage of cells that were unresponsive to the first ATP but responsive to the second ATP, the representative traces of a single cell's Ca^{2+} responses, and typical pseudo $[Ca^{2+}]_i$ images in the first ATP–histamine–second ATP assessment. The effect of histamine on $[Ca^{2+}]_i$ evoked by ATP administration was assessed by Fura-2AM Ca^{2+} imaging. (A) A total of 263 cells were analyzed. The number and percentage of the responding cells in relation to the total number of analyzed cells are shown. (B, C) Representative traces of a single cell's Fura-2AM ratio (340 nm/380 nm) in the 1st ATP (−) histamine (+) 2nd ATP (+) and the 1st ATP (−) histamine (−) 2nd ATP (+) groups. (D) Pseudo black-and-white image. This image was obtained by fluorescence microscopy, showing cells that have taken up Fura-2AM. (E) Pseudo color images representing the intensity ratio (340 nm/380 nm), indicating ATP-induced Ca^{2+} responses before (left panel) and after (right panel) histamine stimulation in NHEKs. White arrows indicate cells that did not respond to the first ATP but responded to the second ATP. Bars = 200 μm $[Ca^{2+}]_i$, intracellular calcium ion concentrations. Fura-2AM, fura-2 acetoxymethyl ester. NHEKs, Normal human epidermal keratinocytes.

that skin without spontaneous pruritus has received an external stimulus. Therefore, we investigated the histamine concentrations that do not cause itching and the ATP concentrations that do cause itching. The ATP concentration we employed was similar to that reported by Shiratori-Hayashi et al. (2019).[2]

Histamine (1 nmol/site, intradermal) did not promote scratching behavior; however, ATP (400 nmol/site, intradermal) increased the scratching behavior significantly. Interestingly, the mixture of histamine and ATP induced the increase of the number of scratching events. Antihistamine terfenadine (30 mg/kg, orally) did not decrease the mixture-induced scratching behavior. Thus, the sensitized increase of the scratching behavior appears to be unrelated to increased histamine sensitivity. A317491 (a $P2X_3/P2X_{2/3}$ receptor antagonist) partially suppressed the ATP-induced and the mixture-induced scratching behavior. Shiratori-Hayashi et al. (2019)[2] revealed that ATP (intradermal) induces scratching behavior via $P2X_3$ receptors; however, the inhibitory effect of $P2X_3$ receptor inhibitor is partial, and the results between the report by Shiratori-Hayashi et al. (2019)[2] and ours were similar.

Interestingly, the number of scratching events reduced by A317491 was greater in the mixture group than in the ATP group, suggesting that the sensitized increase of the scratching behavior by the mixture is associated with increased sensitivity to ATP. The scratching behavior not suppressed by A317491 could be related to receptors other than the $P2X_3$ receptor; however, it is not easy to identify the receptors because there are no specific antagonists for each receptor. According to a previous report using airway smooth muscle tissue, stimulation of the tissue with histamine resulted in stronger ATP-induced smooth muscle contraction.[19] Thromboxane A_2 produced via histamine-induced cyclooxygenase-2 upregulation

A

Classification	Percentage of cells corresponding to each trace pattern
1st ATP (−) His-CPM (+) 2nd ATP-CPM (+)	0% (0/293)
1st ATP (−) His-CPM (−) 2nd ATP-CPM (+)	5.5% (16/293)
Total	5.5% (16/293)

B

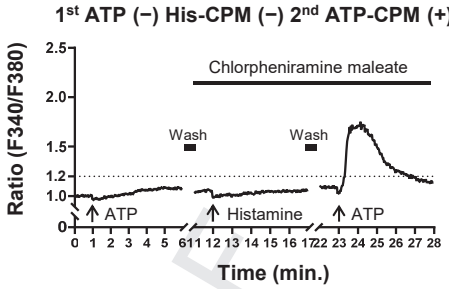

Fig. 5. Effects of (+)-chlorpheniramine maleate on histamine-induced and second ATP-induced $[Ca^{2+}]_i$ change. Chlorpheniramine maleate at 100 μM concentration was added after recording the first ATP (3 μM) treatment. In the presence of chlorpheniramine maleate, the 10 μM histamine-induced increase in $[Ca^{2+}]_i$ was almost 100% suppressed (see Supplementary Table 2). (A) A total of 293 cells were analyzed. The number and percentage of the responding cells in relation to the total number of analyzed cells are shown. (B) Representative traces of a single cell's Fura-2AM ratio (340 nm/380 nm) in the 1st ATP (−) His-CPM (−) 2nd ATP-CPM (+) group. The representative traces were normalized at the mean baseline value (calculated 1 min before the first ATP application). The ratio was calculated using the 1-min baseline value before each drug application. A ratio of ≥1.2 means a positive reaction. (+) indicates a positive reaction, and (−) indicates a negative reaction. $[Ca^{2+}]_i$, intracellular calcium ion concentrations. CPM, (+)-chlorpheniramine maleate. Fura-2AM, fura-2 acetoxymethyl ester. His, histamine. NHEKs, Normal human epidermal keratinocytes.

results in hyperresponsiveness to ATP.[19] The intradermal injection of thromboxane A_2 induced itching-associated responses in mice.[20] Prostaglandin E_2 has an important role in signal transmission between keratinocytes and cutaneous sensory neurons [21]; prostaglandin E_2 enhances experimentally evoked itching in humans.[22,23] Accordingly, bioactive substances such as thromboxane A_2 and prostaglandin E_2 are considered involved in mixture-induced scratching behavior.

Next, we examined ATP-induced $[Ca^{2+}]_i$ changes before and after histamine stimulation to assess whether keratinocytes' input with histamine signaling alters their responsiveness to ATP. Interestingly, we found several cells that did not respond to ATP before histamine stimulation but responded to ATP after histamine stimulation. The phenomenon occurred in cells with and without increased $[Ca^{2+}]_i$ due to histamine stimulation. Treatment with antihistamine chlorpheniramine maleate completely suppressed the response to the second ATP in the cells with increased $[Ca^{2+}]_i$ stimulated by histamine. However, the same effect did not occur in the cells that underwent the same histamine stimulation, whereas $[Ca^{2+}]$ remains unchanged. We found that histamine/H1 receptor signaling was primarily involved in increasing the response rate of keratinocytes to ATP, but we presume that there might be another histamine receptor-mediated signaling involvement.

H1, H2, and H4 receptors are expressed in keratinocytes[24,25] and, along with the $P2Y_2$ receptor, which is involved in ATP-induced $[Ca^{2+}]_i$ elevation,[3] are G protein-coupled receptors. H1 and $P2Y_2$ receptors are both classified as Gq/11. We first considered the first ATP (−)—histamine (−)—second ATP (+) reaction. It has been reported that the Gi signal enhances the Gq-mediated response.[26] For example, activating the serotonin receptor (Gi) enhances noradrenaline receptor (Gq)-mediated rabbit thoracic vasoconstriction.[27] Activating the adenosine A_1 receptor (Gi) enhances ATP receptor ($P2Y_2$ and $P2Y_4$) (Gq)-mediated phosphatidylinositol phospholipid turnover.[28] According to these results, we can speculate that the H4 receptor (Gi) signal might facilitate the $P2Y_2$ receptor (Gq)-mediated $[Ca^{2+}]_i$ increased reaction to the second ATP. H4 receptors are expressed in suprabasal keratinocytes.[25] Since we used undifferentiated keratinocytes in this study, the involvement of H4 receptors could be small. If the distribution of histamine receptors in cells that responded to the first ATP (−)—histamine (−)—second ATP (+) reaction is clarified, the reaction mechanism could be determined.

We then considered the first ATP (−)—histamine (+)—second ATP (+) reaction. Since treatment with chlorpheniramine maleate almost completely suppressed the histamine-induced $[Ca^{2+}]_i$ increase, we can consider that the histamine/H1 receptor signal was dominantly involved in the histamine-induced $[Ca^{2+}]_i$ increase. The H1 receptor can form a functional heterodimer complex with the αb1 adrenergic receptor and the $P2Y_2$ receptor with the adenosine A_1 receptor.[29,30] In line with these reports, activated H1 receptors might alter their location on the plasma membrane, forming heterodimers with $P2Y_2$ receptors and enhancing $P2Y_2$ receptor activation. The $P2Y_2$ receptor has not only Gq/11 activity as the main mechanism but also Gi/o activity as a secondary transduction mechanism [31]; after Gq/11 is activated, Gi/o activation causes an increase in $[Ca^{2+}]_i$ via Gβγ-phospholipase Cβ.[32] Thus, the first ATP (−)—histamine (+)—second ATP (+) reaction is the calcium signal due to ATP/$P2Y_2$ (Gi/o) may add. Further studies are needed to obtain more direct evidence.

A previous study reported that undifferentiated and differentiated keratinocytes propagate Ca^{2+} waves by mechanical stimulation and that Ca^{2+} waves are suppressed by the ATP-degrading enzyme. The inhibition was complete in undifferentiated keratinocytes but not in differentiated keratinocytes,[4] suggesting that ATP and other factors are involved in transmitting the Ca^{2+} wave to mechanical stimuli in the upper epidermis. Therefore, our results could indicate that histamine is involved in the propagation of Ca^{2+} waves.

In conclusion, we found that 1) the mixture of histamine and ATP induced the increase of the number of scratching events compared with ATP alone, 2) histamine-stimulated keratinocytes had an increased response rate to ATP, and 3) the H1 receptor-mediated signal was mainly involved in the phenomenon. Therefore, these results suggest that histamine acts as an enhancer in ATP-induced itching and is a potential mechanism that could increase the responsiveness of keratinocytes to ATP.

Declaration of competing interest

Inami and Fukushima are employed by Hoyu Co., Ltd. who financially supported the research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2021.12.004.

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