



Activation of serotonin receptor 2 by glucosylsphingosine can be enhanced by TRPA1 but not TRPV1: Implication of a novel glucosylsphingosine-mediated itch pathway

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ABSTRACT

Glucosylsphingosine (GS) is an endogenous sphingolipid that specifically accumulates in the skin of patients with atopic dermatitis (AD). Notably, it was recently found that GS can induce itch sensation by activating serotonin receptor 2A and TRPV4 ion channels. However, it is still uncertain whether other molecules are involved in GS-induced itch sensation. Therefore, by using the calcium imaging technique, we investigated whether serotonin receptor 2 – specifically 2A and 2B – can interact with TRPV1 and TRPA1, because these are representative ion channels in the transmission of itch. As a result, it was found that GS did not activate TRPV1 or TRPA1 *per se*. Moreover, cells expressing both serotonin receptor 2 and TRPV1 did not show any changes in calcium responses. However, enhanced calcium responses were observed in cells expressing serotonin receptor 2 and TRPA1, suggesting a possible interaction between these two molecules. Similar synergistic effects were also observed in cells expressing serotonin receptor 2 and TRPA1, but not TRPV1. Furthermore, a phospholipase C inhibitor (U73122) and a store-operated calcium entry blocker (SKF96365) significantly reduced GS-induced responses in cells expressing both serotonin receptor 2 and TRPA1, but not with pre-treatment with a G $\beta\gamma$ -complex blocker (gal-1). Therefore, we propose a putative novel pathway for GS-induced itch sensation, such that serotonin receptor 2 could be coupled to TRPA1 but not TRPV1 in sensory neurons.

1. Introduction

Ceramides play an essential role in the barrier function of the human epidermis. Generally, ceramide levels in the normal stratum corneum are regulated by the dynamic balance of three sphingolipid hydrolysis enzymes: β -glucocerebrosidase, sphingomyelinase, and ceramidase [1]. However, studies have found that ceramide levels in the lesional stratum corneum of atopic dermatitis (AD) patients decreased significantly, along with impaired skin barrier function [2]. Interestingly, the activities of sphingolipid hydrolysis enzymes were mostly unchanged. Instead, sphingomyelin/glucosylceramide deacylase, a novel sphingolipid-metabolizing enzyme, has been identified in the skin lesions of patients with AD. This enzyme converts glucosylceramide to glucosylsphingosine (GS) at the acyl site [3]. The abnormal activity of

glucosylceramide deacylase catalyzes the conversion of ceramide-derived glucocerebroside into GS, leading to its high accumulation in the epidermis of patients with AD [4]. A study has revealed that sphingomyelin/glucosylceramide deacylase activity is elevated more than fivefold in the lesional stratum corneum of AD patients [5]. As a result, GS levels increase significantly in the lesional skin of patients with AD [5]. In general, these results confirm the fundamental characteristics of GS as a marker of altered skin barrier function in AD patients [6].

Interestingly, GS is not merely a byproduct of dysregulation of sphingolipid hydrolysis enzymes but also a pruritogen in AD. Indeed, GS induces typical itch-scratching responses in mice [7] and suggests that GS-induced molecular mechanisms may take advantage of the serotonin receptor family 2A and 2B, but not 2C [8]. Based on these previous

Abbreviations: AD, atopic dermatitis; GS, glucosylsphingosine; PLC, phospholipase C; Htr2a, serotonin receptor 2A; Htr2b, serotonin receptor 2B; SOCE, store-operated calcium entry; TRPV1, transient receptor potential vanilloid 1; TRPA1, transient receptor potential ankyrin 1.

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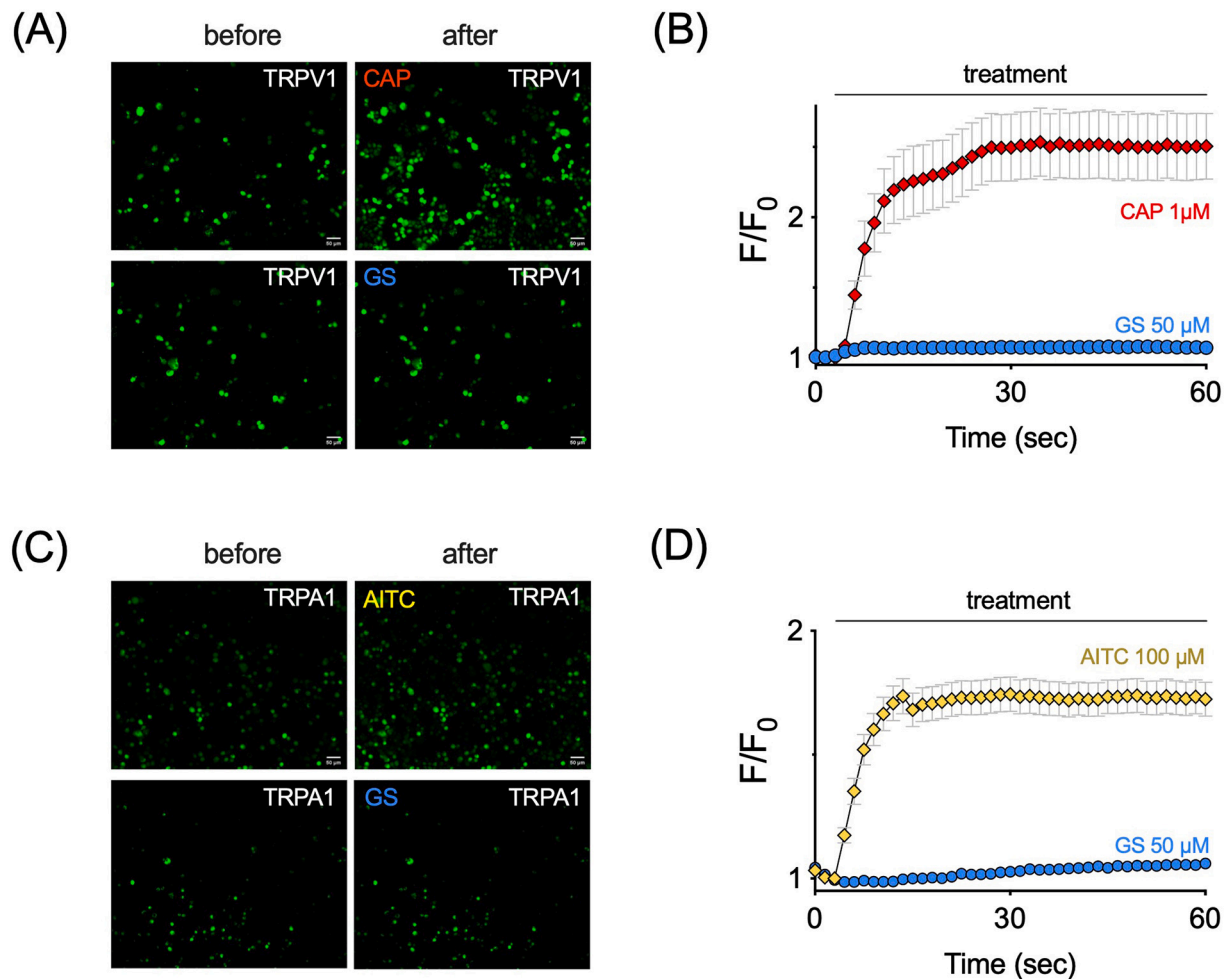


Fig. 1. Glucosylsphingosine (GS) does not activate TRPV1 nor TRPA1. (A) Representative microscopic fluorescent images of HEK293T cells transiently expressing TRPV1. Treatment of 1 μ M capsaicin (CAP) — a specific agonist of TRPV1 — significantly increased fluorescent intensity (top row). However, treatment of 50 μ M GS did not induce any noticeable changes in the fluorescent intensity (bottom row). (B) Time-course profiles of CAP and GS treatments on HEK293T cells expressing TRPV1. (C) Representative microscopic fluorescent images of HEK293T cells transiently expressing TRPA1. Treatment of 100 μ M AITC, a specific agonist of TRPA1, significantly increased fluorescent intensity (top row). Conversely, treatment of 50 μ M GS did not induce noticeable changes in the fluorescent intensity (bottom row). (D) Time-course profiles of CAP and GS treatments on HEK293T cells expressing TRPA1.

reports, our group has recently identified the detailed molecular pathways for GS-induced itch: GS not only utilizes serotonin receptor 2A but transient receptor potential vanilloid 4 (TRPV4) ion channels in sensory neurons as well [9].

The aforementioned finding raises the question of whether other TRP ion channels are involved in the GS-induced itch sensation. Because transient receptor potential vanilloid 1 (TRPV1) and Transient receptor potential ankyrin 1 (TRPA1) are representative ion channels that affect the transmission of itch in sensory neurons. TRPV1 is responsible for most histamine-dependent itch pathways, while TRPA1 represents other histamine-independent itch transmission mechanisms.

Therefore, we investigated whether the activation of serotonin receptors 2A and 2B by GS could be coupled with, or enhanced by, TRPV1 and TRPA1.

2. Materials and methods

2.1. Reagents

Glucosylsphingosine (CAS# 52050-17-6; also known as glucosyl(β) sphingosine (d18:1) or D-glucosyl- β 1-1-D-erythro-sphingosine) was purchased from Avanti Lipids (Alabaster, Alabama, US). Capsaicin (CAP), allyl isothiocyanate (AITC), ketanserin, HC030031, RS127445,

SKF96365, and gallein were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cloning

First-strand cDNA was reverse-transcribed from total RNA isolated from mouse dorsal root ganglia (DRG) using an easy-spin total RNA extraction kit (Intron Biotechnology Inc., Gyeonggi-do, Korea). Full-length coding sequence regions of mouse serotonin receptor 2A (Htr2a; NM_172812.2) were obtained by reverse transcription PCR using custom-synthesized oligomers, and cloning was done by using the “Single step ligation independent cloning (SLIC)” method [10]. Full-length serotonin receptor 2B was obtained from the Korea Human Gene Bank (KHC), and subcloned into pCMV sport6. TRPV1 and TRPA1 cDNAs were cloned from mouse DRG neurons. All cDNA genes were 100 % identical to those in the NCBI database.

2.3. Cell culture and transfection

HEK293T cells were cultured in DMEM (10 % FBS, 2 mM L-glutamine, penicillin/Zell shield). Grown cells were transfected using Fugene HD transfection reagent (Promega), according to the manufacturer's instructions. Between 24 and 48 h after transfection, calcium imaging

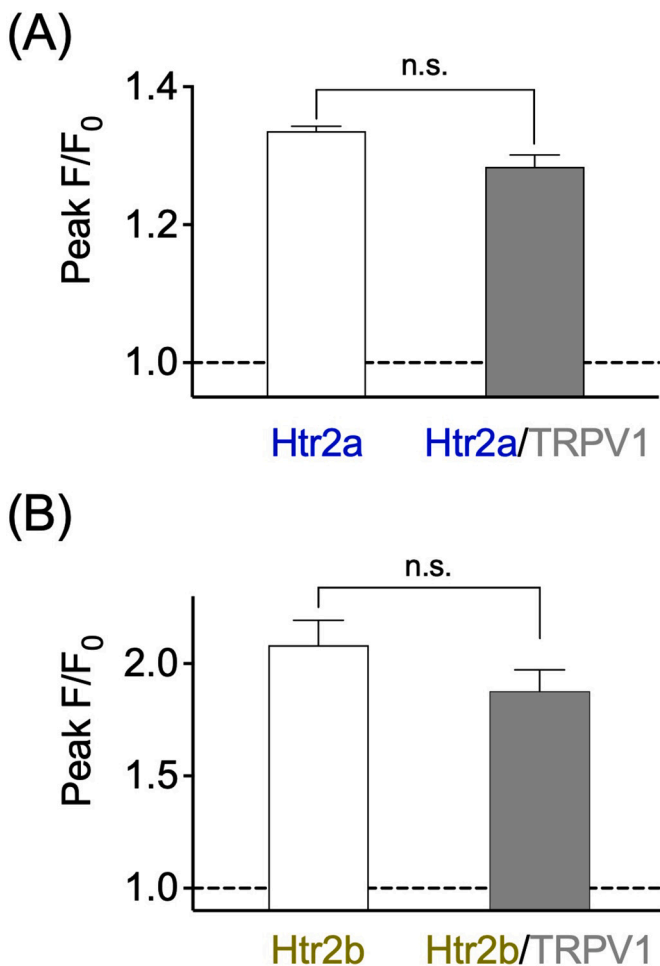


Fig. 2. Coexpression of serotonin receptor 2 and TRPV1 did not change calcium levels by GS. (A) Comparison of peak F/F_0 values between two groups, either HEK293T cells expressing serotonin receptor 2A alone (Htr2a; $n = 984$) or expressing both serotonin receptor 2A and TRPV1 (Htr2a/TRPV1; $n = 512$). (B) Comparison of peak F/F_0 values between two groups, either HEK293T cells expressing serotonin receptor 2B alone (Htr2b; $n = 1075$) or expressing both serotonin receptor 2B and TRPV1 (Htr2b/TRPV1; $n = 1230$). n.s. not significant.

experiments were performed.

2.4. Calcium imaging experiments

Intracellular calcium levels were measured using a fluorescence microscope (Nikon Eclipse Ti-U). Transfected HEK293T cells were loaded with 5 μ M Fluo-3/AM (Invitrogen) and incubated for 40 min at 37 $^{\circ}$ C. Fluo-3/AM, a calcium-specific fluorescent dye, was incubated with an NBS solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 0.5 mM MgCl_2 , 10 mM glucose, 5.5 mM HEPES, adjusted to pH 7.4). After incubation, the medium was washed, and the compounds were applied to elicit calcium influx. Fluorescence excitation was performed at 488 nm and the emitted fluorescence was measured at 515 nm. The microscopic fluorescence images were recorded for 2 min at time intervals of 1.5 s. Intracellular calcium changes were expressed as F/F_0 , where F_0 indicates the initial fluorescence intensity and F denotes the fluorescence intensity at the given time point. Image analysis was performed using ImageJ (NIH) with custom-made scripts for automatic cell counting and ratio metric image production. The n number in each group represents the total number of cells or 'regions of interest', which were automatically selected using ImageJ from three independent experiments.

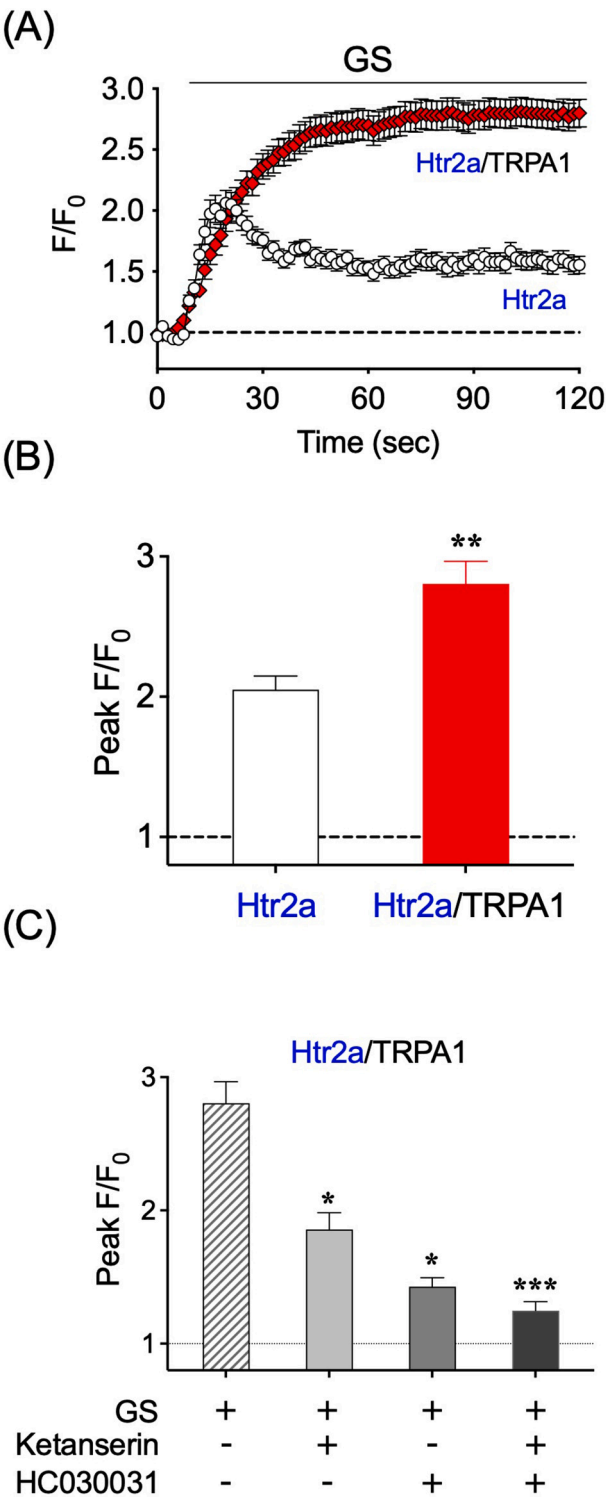


Fig. 3. Coexpression of serotonin receptor 2A and TRPA1 showed enhanced response to GS treatment. (A) Time-course profiles of GS treatment on HEK293T cells expressing serotonin receptor 2A alone (Htr2a) and expressing serotonin receptor 2A and TRPA1 (Htr2a/TRPA1). (B) Comparison of peak F/F_0 values between two groups, either HEK293T cells expressing serotonin receptor 2A alone (Htr2a; $n = 665$) or expressing both serotonin receptor 2A and TRPA1 (Htr2a/TRPA1; $n = 833$). (C) Effects of inhibitors of Htr2a and TRPA1 over GS-induced responses. Treatments of 500 nM ketanserin (a Htr2a inhibitor; $n = 253$) and 10 μ M HC030031 (a TRPA1 inhibitor; 281) and cotreatment of both ketanserin and HC030031 ($n = 260$) significantly inhibited GS-induced peak F/F_0 values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

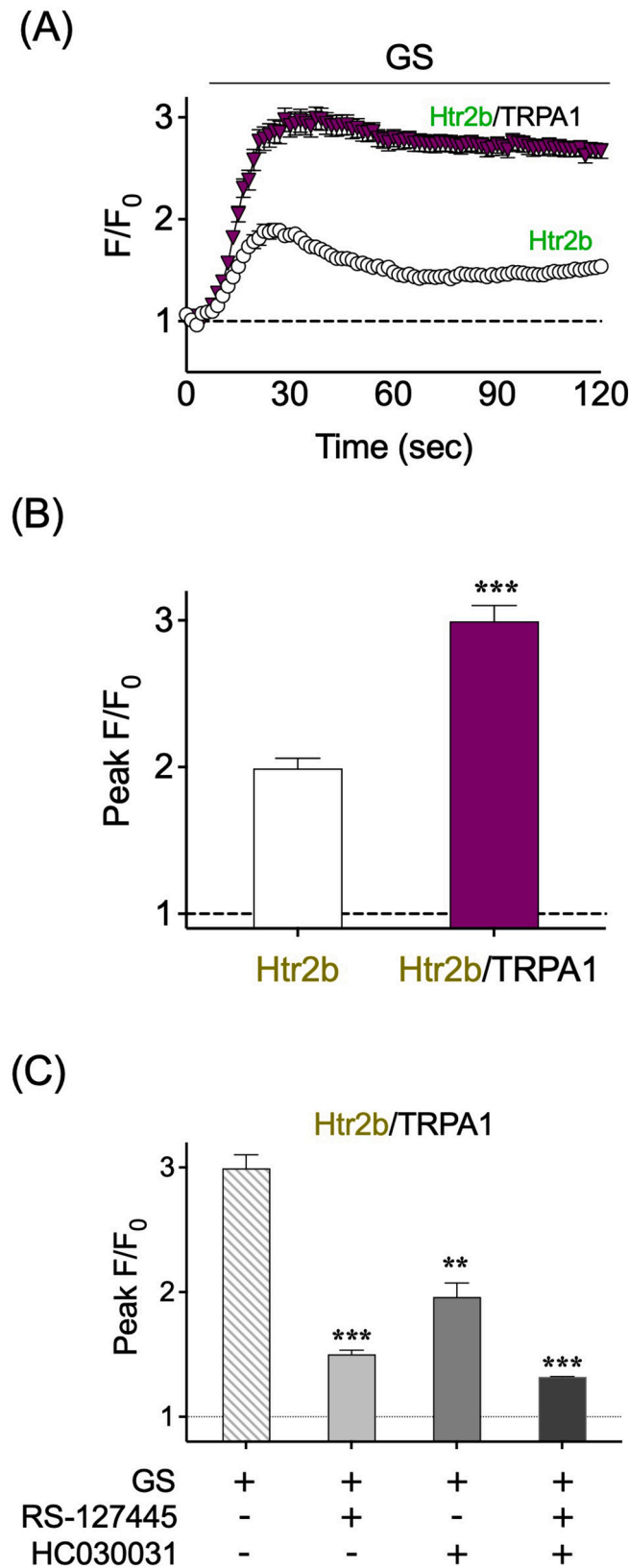


Fig. 4. Coexpression of serotonin receptor 2B and TRPA1 showed enhanced response to GS treatment. (A) Time-course profiles of GS treatment on HEK293T cells expressing serotonin receptor 2B alone (Htr2b) and expressing serotonin receptor 2B and TRPA1 (Htr2b/TRPA1). (B) Comparison of peak F/F_0 values between two groups: either HEK293T cells expressing serotonin receptor 2B alone (Htr2b; $n = 1286$) or expressing both serotonin receptor 2B and TRPA1 (Htr2b/TRPA1; $n = 1806$). (C) Effects of inhibitors of Htr2b and TRPA1 over GS-induced responses. Treatments of 500 nM RS-127445 (a Htr2b inhibitor; $n = 1343$) and 10 μ M HC030031 (a TRPA1 inhibitor; $n = 1274$), and cotreatment of both RS-127445 and HC030031 ($n = 2147$) significantly inhibited GS-induced peak F/F_0 values. ** $p < 0.01$, *** $p < 0.001$.

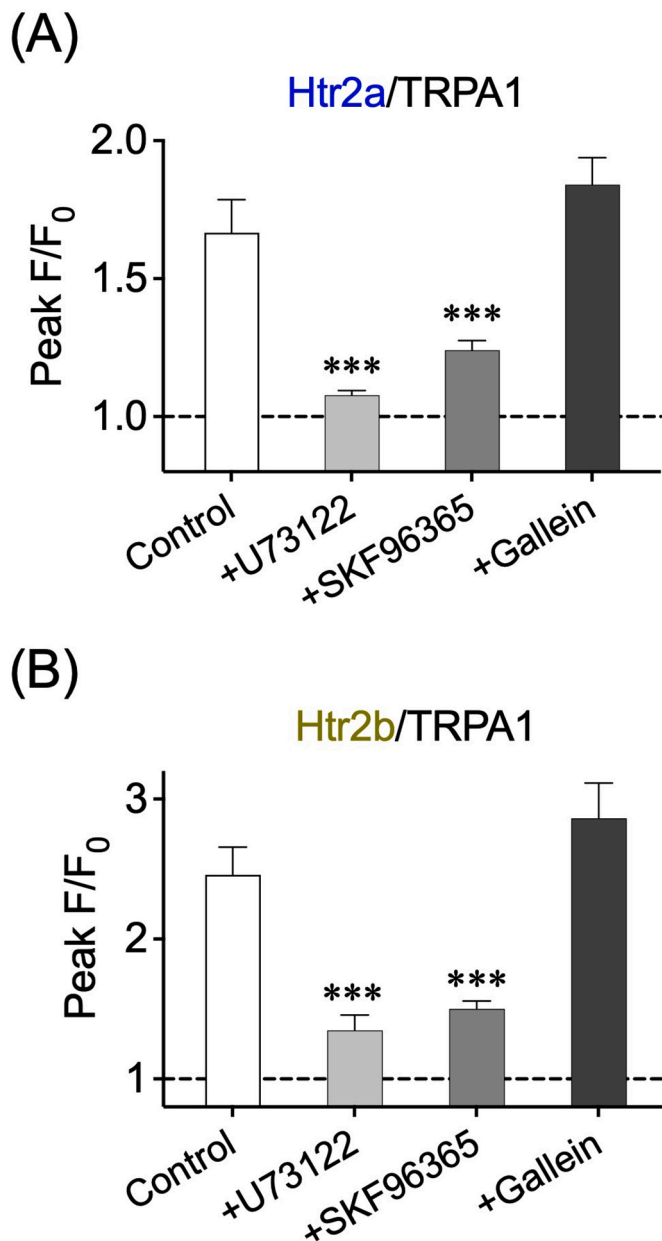


Fig. 5. Phospholipase C (PLC) and store-operated calcium entry (SOCE) are involved in the enhanced activation of serotonin receptor 2A/2B and TRPA1. (A) Comparison of peak F/F_0 values induced by 50 μ M GS treatment in HEK293T cells expressing both serotonin receptor 2A and TRPA1. When compared to the control ($n = 533$), pre-treatment of U73122 (a PLC inhibitor, $n = 889$) and SKF96365 (a SOCE blocker, $n = 648$) significantly inhibited the peak response, while gallein (an inhibitor of $G\beta\gamma$ complex, $n = 582$) did not induce noticeable changes. (B) Comparison of peak F/F_0 values induced by 50 μ M GS treatment in HEK293T cells expressing both serotonin receptor 2B and TRPA1. When compared to the control ($n = 539$), pre-treatment of U73122 ($n = 439$) and SKF96365 ($n = 501$), but not gallein ($n = 206$), significantly inhibited the peak response. *** $p < 0.001$.

2.5. Statistical analysis

All data are presented as mean $\pm$ S.E.M. Student's t -test was used for comparison between two groups. For comparisons among more than three groups, one-way analysis of variance (ANOVA) with Dennett's multiple comparison post-test was used. Statistical tests were performed using GraphPad Prism software.

3. Results

3.1. GS does not activate TRPV1 and TRPA1

We initially assessed whether the cloned TRPV1 and TRPA1 ion channels were functionally active in HEK293T cell lines. Cells transiently transfected with TRPV1 or TRPA1 were exposed to their specific agonists. As shown in Fig. 1A and B, 1 μ M capsaicin, a specific agonist for TRPV1, significantly elevated calcium-dependent fluorescence intensity, demonstrating that TRPV1 is functionally expressed in the cells. However, the application of 50 μ M GS did not increase fluorescence, suggesting that GS cannot activate TRPV1 *per se* (Fig. 1A and B). Similarly, 100 μ M AITC, a specific TRPA1 agonist, also caused an increase in intracellular calcium in HEK293T cells expressing TRPA1 (Fig. 1C and D), whereas GS did not show any notable increase. Therefore, these results demonstrate that both TRPV1 and TRPA1 were functionally active in HEK293T cells but are not activated by GS.

3.2. TRPV1 is not involved in the GS-induced serotonin receptor activation

Next, we investigated whether TRPV1 enhances GS-induced responses through the serotonin receptor 2 family. As it has been revealed that GS can activate both the serotonin receptor 2A (Htr2a) and 2B (Htr2b), TRPV1 is co-expressed with Htr2a or Htr2b in HEK293T cells. As shown in Fig. 2A, treatment with 50 μ M GS resulted in calcium influx in cells expressing only Htr2a. However, calcium responses did not change in cells expressing both Htr2a and TRPV1 ("Htr2a/TRPV1"). A similar approach was used in combination with Htr2b and TRPV1. As shown in Fig. 2B, there was no discernible difference between cells expressing only Htr2b and both Htr2b and TRPA1 ("Htr2b/TRPA1"). Taken together, it is assumed that TRPV1 is less likely to be involved in the GS-induced activation of the serotonin receptor 2 family.

3.3. TRPA1 enhanced Htr2a-mediated calcium influx induced by GS

We investigated the putative correlation between Htr2a and TRPA1 in a similar manner. Surprisingly, as shown in Fig. 3A and B, the expression of both Htr2a and TRPA1 in HEK293T cells ("Htr2a/TRPA1") significantly increased the calcium response induced by GS compared to that in cells expressing only Htr2a. These results imply that TRPA1 enhances GS-induced Htr2a activation. To further confirm this possibility, ketanserin (an Htr2a inhibitor) and HC030031 (a TRPA1 antagonist) were applied to Htr2a/TRPA1. As shown in Fig. 3C, the elevated GS-induced response was significantly reduced after ketanserin treatment. However, HC030031 treatment also significantly decreased the GS-induced response, and suppression was strongest when both ketanserin and HC030031 were co-treated before GS application. Taken together, these data suggest that TRPA1 enhances the elevated GS-induced response, implying a putative role in the transmission of the GS-induced Htr2a-mediated itch pathway.

3.4. TRPA1 also augmented Htr2b-mediated calcium influx induced by GS

We also evaluated the synergistic effects of Htr2b and TRPA1. As expected, HEK293T cells expressing both Htr2b and TRPA1 ("Htr2b/TRPA1") exhibited a significantly increased calcium response after GS application compared to cells expressing only Htr2b (Fig. 4A and B). Therefore, TRPA1 was speculated to also enhance GS-induced Htr2b activation, similar to Htr2a. This assumption was confirmed by applying RS-127445 (a Htr2b inhibitor) and HC030031 (an inhibitor of TRPA1). Calcium influx responses significantly decreased when these inhibitors were administered (Fig. 4C). Taken together, these data suggest that TRPA1 can augment GS-induced responses *via* Htr2b, implying the putative involvement of Htr2b and TRPA1 in the transmission of the GS-

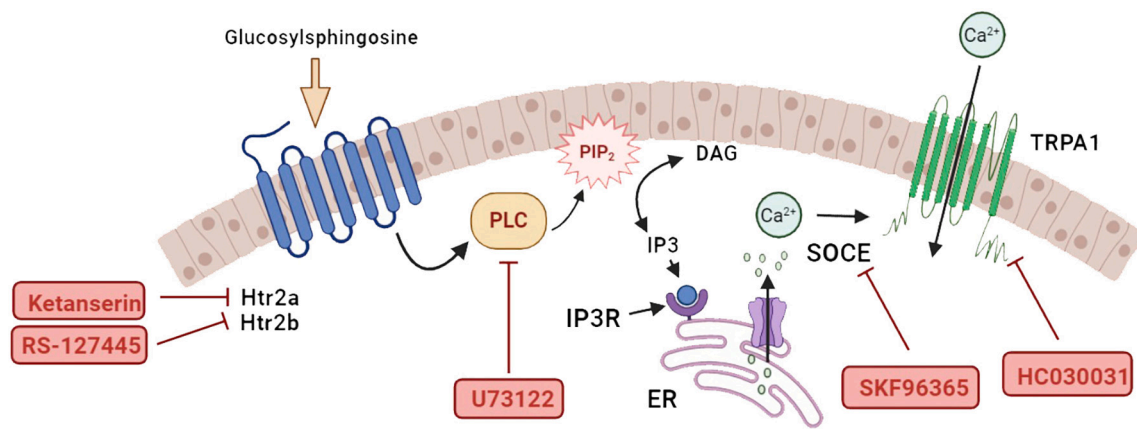


Fig. 6. A proposed signaling pathway for the GS-induced calcium influx via serotonin receptor 2A/2B and TRPA1.

induced itch pathway.

3.5. Phospholipase C and store-operated calcium entry are involved in the enhanced activation of serotonin receptor 2A/2B and TRPA1

To further investigate the molecular mechanism between Htr2a and TRPA1, various inhibitors were pretreated and the subsequent application of GS was followed. First, cells were pretreated with U73122, a phospholipase C (PLC) inhibitor, before applying GS to Htr2a/TRPA1. As shown in Fig. 5A, U73122 significantly suppressed the GS-induced response, indicating that PLC plays a role between Htr2a and TRPA1.

PLC activation results in the release of calcium from the endoplasmic reticulum, leading to the entry of stored calcium (SOCE). Thus, to further investigate the possible involvement of (SOCE), we pretreated cells with an SOCE inhibitor SKF96365, before applying GS. As shown in Fig. 5A, SKF96365 clearly inhibited the increase in intracellular calcium, implying that SOCE could be involved in Htr2a-mediated TRPA1 activation. On the other hand, we further pondered if G $\beta\gamma$ signaling could be affected in the pathway. Gallein - a G $\beta\gamma$ blocker - was administered before applying GS to Htr2a/TRPA1. However, gallein had no effect on the GS-induced response in Htr2a/TRPA1 cells, implying that G $\beta\gamma$ is not involved in the signaling between Htr2a and TRPA1.

Similar experiments were performed using the Htr2b/TRPA1 cells. As expected, treatment with U73122, SKF96365 and gallein showed results similar to those of Htr2a/TRPA1, as shown in Fig. 5B. Therefore, these data suggest that GS-induced activation of serotonin receptor 2A/2B may induce PLC activation and SOCE, but not G $\beta\gamma$ complex dissociation, to evoke TRPA1 activation.

4. Discussion

AD evokes severe pruritus that cannot be attenuated by antihistamine agents. This histamine-independent aspect of AD itching has led researchers to investigate AD-specific itch mechanisms. As mentioned in the introduction, altered metabolism of ceramide could be a factor that may explain the symptoms of AD-specific itch. Again, the high accumulation of GS in patients with AD is a pruritogen that does not involve the histamine-dependent itch pathway. In fact, a detailed molecular mechanism by which GS induces pruritus has been recently identified. In detail, serotonin receptors 2A and TRPV4 were shown to be key players in the transmission of GS-induced pruritus [9]. This led us to test another hypothesis that other TRP channels are involved in the GS-mediated signaling pathway. Based on the present data, we speculate that TRPA1, but not TRPV1, is affected by the GS-induced itch pathway. Nevertheless, further studies are mandatory, such as experiments with primary cultures of dorsal root ganglia and/or mouse behavior tests.

Our data clearly showed that neither TRPV1 nor TRPA1 could be

activated by GS treatment without serotonin receptors 2A and 2B (Fig. 1). This is in agreement with the previous report that neither TRPV1 nor TRPA1 are not activated by GS *per se* [9].

TRPV1 is a non-specific cationic channel that is involved in heat sensation, pain, and itching. TRPV1 is considered a representative ion channel for the histamine-dependent itch signaling pathway in sensory neurons. TRPV1 is functionally related to histamine receptor type 1 (H1R) in mouse sensory neurons, and TRPV1 knockout mice show significantly suppressed scratching behaviors evoked by histamine [11]. In this context, it is interesting to observe that GS-induced activation of serotonin receptor 2A/2B is not functionally related to histamine-related TRPV1 (Fig. 2). In fact, this could be interpreted as GS-induced itch sensation is essentially histamine-independent, which is a characteristic of AD-specific pruritus. Therefore, we speculate that GS-induced activation of sensory neurons is not likely to be related to TRPV1.

In contrast, TRPA1 is widely linked to various histamine-independent itch pathways in the sensory neurons. For example, chloroquine, an antimalarial agent that induces severe itch, binds to the unique receptor MrgprA3 and subsequently activates TRPA1 [12]. It has been reported that the activation of PAR2 results in further activation of TRPA1 [13]. Furthermore, it has been found that TRPA1 is multi-functionally involved in the transmission of itching induced by hydrogen peroxide [14], IL-31 [15], and TSLP [16]. Therefore, TRPA1 is considered a histamine-independent ion channel that is involved in various non-histamine pruritogenic signals. In this context, it is not surprising that serotonin receptor 2A/2B is linked to TRPA1 when GS is applied (Figs. 3 and 4).

However, whether TRPA1 is involved in the actual sensation of GS-induced itch remains to be verified. Previous studies have reported that serotonin-induced itch signals are conferred by serotonin receptor 2 and TRPV4 but not TRPA1 [17,18]. Indeed, TRPA1 knockout mice did not show any difference in scratching bouts when serotonin was administered [17], implying that TRPA1 may not be fully effective in conveying signals derived from serotonin receptor 2. However, it should be noted that these previous studies used serotonin as a pruritogen, but not GS. Therefore, further in-depth experiments are warranted to decipher the putative interactions between TRPA1 and serotonin receptor 2 in mouse sensory neurons. We are currently planning to perform such an investigation in the near future.

As shown in Fig. 5, inhibition of PLC activity resulted in a reduction in GS-induced responses between Htr2a/2b and TRPA1. In fact, Htr2a and Htr2b are typical G protein-coupled receptors that activate PLC via the G α_q subunit [19]. These data imply that GS-induced activation of serotonin receptor 2A/2B evoked G $\alpha_{q/11}$ -dependent activation of the PLC. In general, PLC catalyzes the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol triphosphate (IP₃). IP₃ is an important factor that allows release of

intracellular calcium from the calcium store present in the endoplasmic reticulum. Specifically, IP₃ induces an increase in intracellular calcium through a process called *store depletion* by acting on IP₃ R in the endoplasmic reticulum [20]. Simply put, IP₃ binds to IP₃R to transiently increase intracellular calcium. This increase further enables additional calcium entry from the extracellular region, a process known as store-operated calcium entry (SOCE). As SKF96365 inhibits the SOCE process, the data shown in Fig. 5 demonstrate that changes in intracellular calcium levels are required to exhibit an enhanced response to GS in Htr2a/TRPA1 and Htr2b/TRPA1. In fact, it has been reported that intracellular calcium can directly activate TRPA1 by binding to its EF-hand domain [21]. On the other hand, pretreatment with a Gβγ complex inhibitor gallein had no effect (Fig. 5). Although PLC can also activate certain channels via Gβγ signaling pathway [22], it was clearly found that the involvement of Gβγ complex is less likely between Htr2a/b and TRPA1.

Overall, we propose the following sequential event: when GS activates the serotonin receptor 2A/2B, it stimulates PLC to produce more IP₃, which in turn releases intracellular calcium from the calcium store. This transient rise in intracellular calcium then binds to the EF-hand domain of TRPA1, resulting in the opening of the channel, allowing the influx of cations, including calcium, from the extracellular regions. This large influx of calcium can be recognized as an enhanced response to GS (Fig. 6).

5. Conclusion

The present study has found that GS-induced activation of serotonin receptor 2A/2B can be enhanced by TRPA1 via stimulation of PLC and SOCE. Therefore, the present data suggest the implication of a novel alternative GS-mediated itch pathway in sensory neurons, especially in the case of AD patients.

CRedit authorship contribution statement

Afzal Ramsha: Investigation, Formal analysis, Writing – original draft.

Won-Sik Shim: Conceptualization, Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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