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Imiquimod induces a Toll-like receptor 7-independent increase in intracellular calcium via IP_3 receptor activation

Heehong Hwang [a], Hyunjung Min [a], Donghoon Kim [a], Seong-Woon Yu [b], Sung Jun Jung [c], Se-Young Choi [a,*], Sung Joong Lee [a,*]

[a] Department of Neuroscience and Physiology, Dental Research Institute, Seoul National University School of Dentistry, Seoul 110-749, Republic of Korea
[b] Department of Brain Science, Daegu Gyeongbuk Institute of Science and Technology, Daegu 711-873, Republic of Korea
[c] Department of Physiology, Hanyang University College of Medicine, Seoul 133-791, Republic of Korea

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ABSTRACT

Imiquimod is an itch-promoting, small, synthetic compound that is generally used to treat genital warts and basal cell carcinoma. The pruritogenic effect of imiquimod is considered to be due to TLR7 activation; however that idea has been challenged by our studies showing intact pruritogenic effects of imiquimod in TLR7 KO mice. Thus, the signaling pathways of imiquimod have not been completely elucidated. Here we investigated the novel effects of imiquimod on intracellular calcium ($[Ca^{2+}]_i$) signaling. We found that imiquimod induces $[Ca^{2+}]_i$ increases in PC12 and F11 cells, and even in NIH-3T3 and HEK293T cells, which do not express TLR7. This $[Ca^{2+}]_i$ increase was due to Ca^{2+} release from the internal store without extracellular Ca^{2+} influx. Neither FCCP, a mitochondrial Ca^{2+} reuptake inhibitor, nor dantrolene, a ryanodine receptor inhibitor, affected the imiquimod-induced $[Ca^{2+}]_i$ increase. However, 2APB, an IP_3 receptor blocker, inhibited the imiquimod-induced $[Ca^{2+}]_i$ increase. U73122, a PLCβ inhibitor, failed to block the imiquimod-induced $[Ca^{2+}]_i$ increase. These data indicate that imiquimod triggers IP_3 receptor-dependent Ca^{2+} signaling independently of TLR7.

1. Introduction

The most accepted definition of itch is "an unpleasant sensation that evokes a desire to scratch" [1]. Itch is generated by primary afferent neurons of which function is affected by many different factors related to skin diseases, systemic diseases, and neurological diseases [2]. Recent intensive studies have elucidated the cause, mechanism, and regulation of itch. Among these trials, the studies of itch-generating molecules and their cellular mechanisms have contributed to understanding the itch transduction mechanism [3].

One of most well-known pruritogens is imiquimod. Imiquimod is a synthetic nucleoside analogue of the imidazoquinoline family [4] that was discovered by compound screens for anti-herpes virus activity [5]. Topical application of the compound is currently approved for the treatment of genital and anal warts, a highly contagious sexually transmitted disease caused by human papillomavirus. It is also widely used to treat skin cancers such as basal cell carcinoma and squamous cell carcinoma, and infectious skin diseases such as molluscum contagiosum.

Interestingly imiquimod has also been shown to induce pruritic or itch responses in mice [6,7]. In a study by Liu et al., it was shown that imiquimod triggers inward cation currents and action potentials in a certain type of mouse sensory neurons through TLR7 activation [6]. However, in a later study, we have found that imiquimod also induces intracellular Ca^{2+} signaling and action potentials in TLR7-deficient mouse sensory neurons [7]. This suggested that imiquimod may induce TLR7-independent intracellular signals. Therefore, it is still controversial how imiquimod triggers an increase in $[Ca^{2+}]_i$.

In this study, we tried to elucidate the mechanism of imiquimod-induced intracellular Ca^{2+} signaling using TLR7-lacking cells. Our data conclusively confirmed that imiquimod is able to induce $[Ca^{2+}]_i$ increases independently of TLR7, rather it induces $[Ca^{2+}]_i$ via IP_3 receptor activation.

2. Materials and methods

2.1. Cell culture

PC12 and THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine

* Corresponding authors. Address: Department of Neuroscience and Physiology, Seoul National University School of Dentistry, 28 Yeongeon, Jongno, Seoul, Republic of Korea. Fax: +82 2 762 5107.
E-mail addresses: hhwang0101@hanmail.net (H. Hwang), showgi84@hotmail.com (H. Min), jikfrit@gmail.com (D. Kim), yusw@dgist.ac.kr (S.-W. Yu), eurijj@naver.com (S.J. Jung), sychoi@snu.ac.kr (S.-Y. Choi), sjlee87@snu.ac.kr (S.J. Lee).

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serum (FBS), 2 mM glutamine, and 1× antibiotic/antimycotic. F11, NIH-3T3 and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1× antibiotic/antimycotic. All cells were grown at 37 °C in the presence of 5% CO_2.

2.2. Intracellular Ca^{2+} measurement

The $[Ca^{2+}]_i$ was determined using the fluorescent Ca^{2+} indicator fura-2/AM (Invitrogen Life Technologies Co., Carlsbad, CA, USA) as previously described [8]. Briefly, cells were seeded onto poly-l-lysine-coated 12 mm coverslips and incubated for 12 h for cell attachment. The cells were stained with Fura-2 AM (2 μM) in a serum-free DMEM for 50 min at 37 °C. The loaded cells were then washed twice with a HEPES-buffered solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH). Intracellular Ca^{2+} concentrations were monitored with dual excitation at 340 and 380 nm and emission at 500 nm in Locke's solution (NaCl, 154 mM; KCl, 5.6 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 2.2 mM; HEPES, 5.0 mM; glucose, 10 mM, pH 7.4 with NaOH) or Ca^{2+}-free Locke's solution (156.2 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, and 10 mM glucose, pH 7.3). All drugs were applied through a bath perfusion at a flow rate of 2.5 ml/min. Changes in fluorescence ratio were measured by a Lambda DG-4 monochromator wavelength changer (Shutter Instrument, Novato, CA, USA) and digital video microfluorometry with an intensified charge-coupled-device camera (CasCade, Roper Scientific, Trenton, NJ, USA) paired with a microscope and a computer with Metafluor software (Universal Imaging, Downington, PA, USA). Experiments with suspended cells were performed with the spectrofluorophotometer (Shimadzu RF-5301-PC). In this case, fluorescence was measured in 1 ml aliquots of magnetically stirred cellular suspension (1×10^6 cells/ml) at 37 °C.

2.3. RT-PCR

Total RNA was extracted from HEK293T and THP-1 cells using TRIzol (Invitrogen Life Technologies). The cDNA was generated by a reverse transcription system (Invitrogen Life Technologies) according to the manufacturer's instructions. RT-PCR was performed using the cDNA as a template. The primers prepared for this RT-PCR were 5′-AAG AAA ACT GAA AAT GGT GTT TTC GA-3′ (TLR7 forward primer), 5′-ACA GGT ACA CAA TTG CAT CTT AAA TCG-3′ (TLR7 reverse primer), 5′-ACC ACA GTC CAT GCC ATC AC-3′ (GAPDH forward primer), and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (GAPDH reverse primer). The PCR products were resolved by 1% agarose gel electrophoresis and stained by ethidium bromide.

2.4. Electrophysiology

Whole-cell recording were made using patch electrodes (4–6 MΩ) pulled from borosilicate glass on a Brown-Flaming P-97 horizontal micropipette puller (Sutter Instrument). Voltage-clamp experiments were performed using an HEKA EPC9 amplifier and digitized using ITC16 and Pulse v8.54 software (both from HEKA, Pfalz, Germany). All recordings were done at room temperature in extracellular solution containing 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES, 10 mM D-glucose, adjusted to pH 7.4 with NaOH. Pipettes were filled with an intracellular solution containing 136 mM K-gluconate, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM EGTA, 2 mM Mg-ATP, 0.1 mM Na-GTP adjusted to pH 7.4 with KOH. Cells were continuously perfused with extracellular solution using a gravity-fed perfusion system.

2.5. Statistical analysis

Statistically significant differences were determined by the student's *t*-test. All of the data shown here are presented as the means ± SEMs, and the differences are considered significant when the *p*-values are less than 0.05.

3. Results

3.1. Imiquimod increases $[Ca^{2+}]_i$ in TLR7-deficient cells

We stimulated cells that were supposedly deficient in TLR7 with imiquimod and measured $[Ca^{2+}]_i$. Notably, not only in neuronal cells PC12 (Fig. 1A) and F11 (Fig. 1B), but also in non-neuronal NIH-3T3 (Fig. 1C) and HEK293T cells (Fig. 1D), we found an imiquimod-induced $[Ca^{2+}]_i$ increase at 10 μg/ml which is the same effective concentration in DRG cells [7]. To bypass the complex crosstalk in neuronal signaling, we further characterize the imiquimod-induced $[Ca^{2+}]_i$ increase in the HEK293T cells. In addition, pre-treatment with imiquimod reduced thapsigargin-triggered $[Ca^{2+}]_i$ increase in a dose dependent manner. Interestingly, loxoribin, a TLR7 agonist, failed to increase $[Ca^{2+}]_i$ (Fig. 1E). To confirm that the effect of imiquimod was independent of TLR7 activation, we tested TLR7 mRNA expression in HEK293T cells by RT-PCR. While we detected TLR7 transcripts in the THP-1 monocytic cell line, we could not detect any TLR7 transcripts in the HEK293T cells (Fig. 1F), demonstrating the TLR7-independent effect of imiquimod in HEK293T cells.

3.2. Imiquimod-induced increases in $[Ca^{2+}]_i$ are due to Ca^{2+} release from internal Ca^{2+} stores

The inhibitory effect of imiquimod on the thapsigargin-induced $[Ca^{2+}]_i$ increase suggested the release of Ca^{2+} from intracellular stores. To confirm this, we challenged the cells with imiquimod in extracellular Ca^{2+}-free conditions, and found that imiquimod still induced comparable increases in $[Ca^{2+}]_i$ in HEK293T cells (Fig 2A). In addition, imiquimod treatment failed to induce any inward current of HEK293T cells in whole cell patch-clamp recordings, while it inhibited voltage-dependent potassium channel (Fig. 2B). This result indicates that imiquimod does not induce calcium influx although it can play as an inhibitor of voltage dependent potassium channel.

3.3. Imiquimod releases Ca^{2+} by the IP_3 receptor activation

Mitochondria and the ER are two major intracellular organelles involved in Ca^{2+} storage. To determine the intracellular Ca^{2+} store responsible for the imiquimod-induced increase in $[Ca^{2+}]_i$, we utilized FCCP, a mitochondrial uncoupler that depletes mitochondrial Ca^{2+}. In the presence of FCCP, imiquimod still increased $[Ca^{2+}]_i$ (Fig. 3A), indicating that the imiquimod-induced $[Ca^{2+}]_i$ increase was not from the mitochondria. Likewise, pre-treatment with dantrolene, an inhibitor for the ryanodine receptor of the ER, did not affect the imiquimod-induced $[Ca^{2+}]_i$ increase in HEK293T cells (Fig 3B). However, when we pre-treated cells with 2-APB, an IP_3 receptor inhibitor, we observed a 30% reduction in imiquimod-induced $[Ca^{2+}]_i$ (Fig. 3C). However, the inhibition of PLCβ by U73122 treatment did not block the imiquimod-induced increase in $[Ca^{2+}]_i$ (Fig. 3D). The results suggest that imiquimod induces the Ca^{2+} release from the IP_3 receptor-sensitive Ca^{2+} pool without affecting upstream pathways, including PLCβ activation.

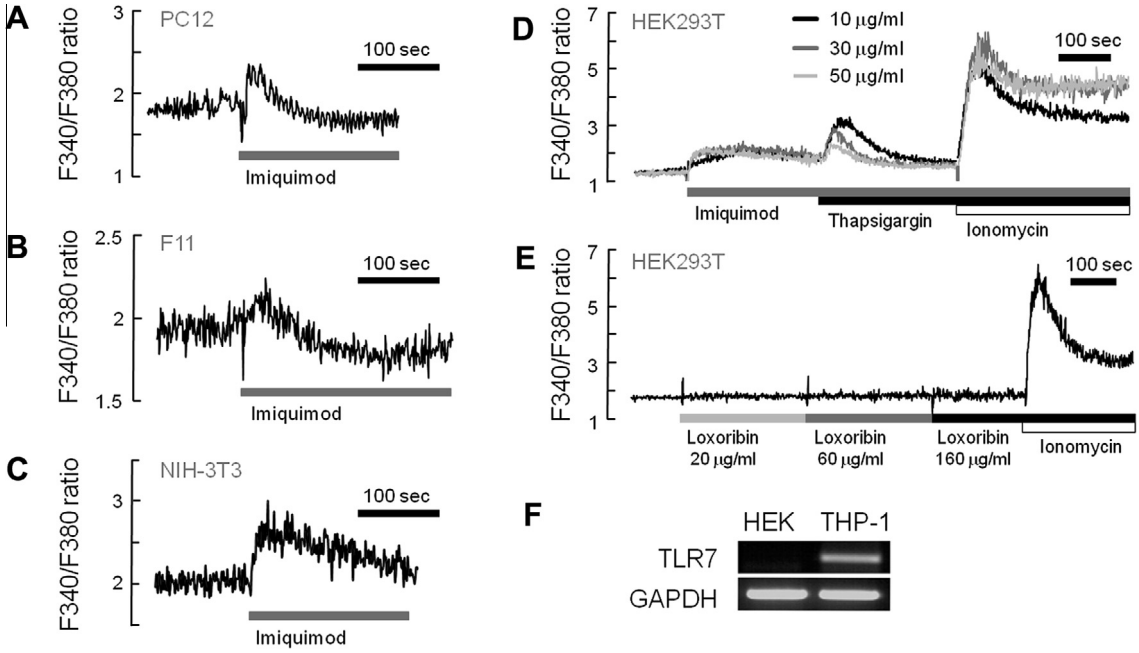

Fig. 1. Imiquimod induces $[Ca^{2+}]_i$ increases in TLR7-lacking cells. (A)–(C) Fura-2-loaded PC12 cells (A), F11 cells (B), and NIH-3T3 cells (C) were treated with 10 μg/ml imiquimod. (D) Fura-2-loaded HEK293T cells were challenged with various doses of imiquimod (10–50 μg/ml), followed by thapsigargin (1 μM) and ionomycin (0.3 μg/ml) treatment. (E) Fura-2-loaded HEK293T cells were challenged with indicated doses of loxoribin (20–160 μg/ml), followed by ionomycin (0.3 μg/ml) treatment. Changes in fluorescence ratio of F340/F380 were monitored and typical Ca^{2+} traces from more than five separate experiments are presented. (E) Total RNA was extracted from HEK293T cells and THP-1 cells and used for RT-PCR to measure TLR7 and GAPDH mRNA expressions.

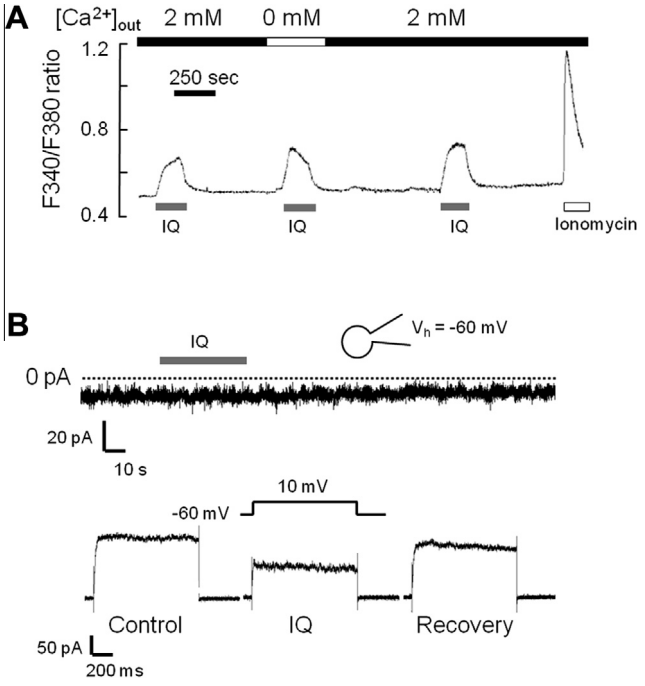

Fig. 2. Imiquimod induces Ca^{2+} release from internal Ca^{2+} stores without inward cation influx. (A) Fura-2-loaded HEK293T cells were challenged with imiquimod (IQ, 30 μg/ml) in extracellular Ca^{2+}-containing conditions (black bar) or extracellular Ca^{2+}-free conditions (white bar). Ionomycin (0.3 μg/ml) treatment was used for the positive control. The changes in the fluorescence ratio of F340/F380 were measured and a representative cell trace is depicted ($n = 38$). (B) Imiquimod (IQ, 30 μg/ml)-induced cation influx was measured in HEK293T cells at a holding potential of −60 mV with whole-cell patch clamp experiments ($n = 3$). Step pulses of membrane potentials from −60 to +10 mV were applied to monitor voltage-dependent cation influxes. Transient and leak currents were not canceled. Typical traces of voltage-dependent cation influxes before (Control), during (IQ), and after (Recovery) the imiquimod treatment are also depicted.

4. Discussion

In this study, we tried to resolve this discrepancy and found that imiquimod increases $[Ca^{2+}]_i$ not only in DRG sensory neurons, but also in HEK293T cells that are devoid of TLR7. These data conclusively demonstrate that imiquimod-induced intracellular Ca^{2+} signaling is independent of TLR7 and relies instead on the activation of the IP_3 receptor, a novel target for imiquimod.

TLR7 is a pattern recognition receptor that recognizes single-stranded RNA and triggers innate immune reactions [9]. In innate immune cells, TLR7 results in the activation of NF-κB and the production of pro-inflammatory cytokines including IFN-α and TNF-α [10]. In turn, these pro-inflammatory cytokines seem to be responsible for anti-viral responses and anti-tumor Th1 immunity [11]. Thus far, the anti-viral and anti-tumor effects of imiquimod have been mostly attributed to TLR7-dependent inflammatory/immune responses.

Even though itch is a known effect of imiquimod, the relationship between imiquimod and TLR7 activation in itch signaling is currently being debated. Liu et al. reported that imiquimod stimulation induces inward cation influx through TLR7 activation in DRG sensory neurons [6]. However, we found that imiquimod triggers Ca^{2+} signaling in TLR7-deficient DRG sensory neurons [7]. Both studies commonly agree that imiquimod can function as a pruritogen by directly activating a subtype of mouse sensory neuron, however the studies show differences in the imiquimod-mediated itch cellular mechanism. Thus, it is necessary to investigate the mechanisms by which imiquimod increases $[Ca^{2+}]_i$. We first confirmed that the 10 μg/ml imiquimod-induced $[Ca^{2+}]_i$ increase, which was found in DRG neurons, is also found in other neuronal PC12 and F11 cells. In addition, the imiquimod-induced $[Ca^{2+}]_i$ increase was also found in NIH-3T3 and HEK293T cells, which do not express TLR7. Most of all, loxoribin, a TLR7 agonist, did not induced $[Ca^{2+}]_i$ increase at all. The results strongly suggest that imiquimod induces Ca^{2+} signaling in a TLR7-independent manner.

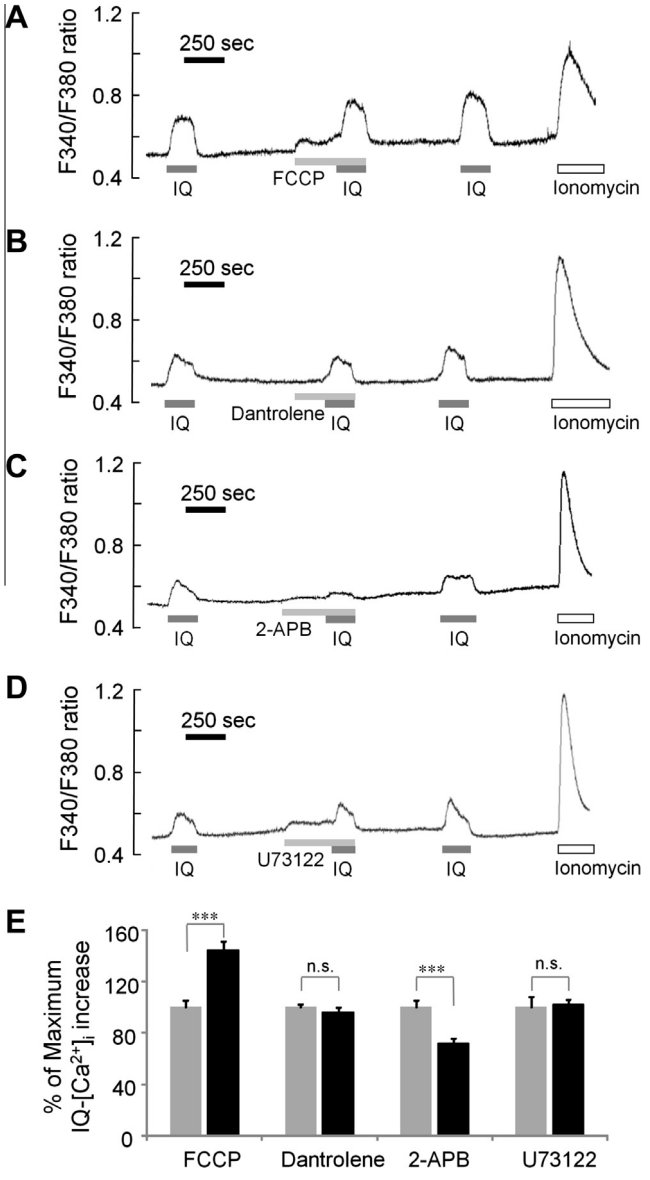

Fig. 3. Imiquimod-induced $[Ca^{2+}]_i$ increase is inhibited by blocking the IP_3 receptor. (A)–(D) Fura-2-loaded HEK293T cells were challenged with imiquimod (IQ, 30 μg/ml) with or without preincubation (5 min) of 5 μM FCCP, a mitochondrial Ca^{2+} reuptake inhibitor (A), 1 μM dantrolene, a ryanodine receptor inhibitor (B), 2-APB, an IP_3 receptor inhibitor (C), or 5 μM U73122, a PLC- inhibitor (D). Ionomycin (0.3 g/ml) treatment was used for the positive control in each experiment. The changes in the fluorescence ratio of F340/F380 were measured and a representative cell trace is depicted. (E) The percentages of the maximum IQ-induced $[Ca^{2+}]_i$ responses with FCCP, dantrolene, 2-APB, and U73122. The changes in the fluorescence ratio of F340/F380 were depicted with the mean ± SEM (FCCP, $n = 42$; dantrolene, $n = 42$; 2-APB, $n = 23$; U73122, $n = 39$; $^{***}p < 0.001$; n.s., not significant).

Our results show the need to modify the previously held belief that imiquimod induces itch through a TLR7-dependent pathway. We already found that imiquimod induces itch only, without any effect on pain behavior [7]. Although a large body of evidence supports that TLRs mediate itch signaling, it is necessary to consider TLR subtype specificity. In fact DRG neurons express various TLRs including TLR1, TLR3, TLR4, TLR5, TLR6, and TLR7 [12]. In addition, several studies have reported TLR7-independent effects of imiquimod [13–15]. Recently a study reported the role of TLR7 in pain behavior instead of itch [16]. Thus, the pruritogenic effect of imiquimod may not be derived from TLR7 activation, but from its role in increasing intracellular Ca^{2+}.

Then it is necessary to understand the imiquimod-mediated Ca^{2+} signaling. The activation of G-protein coupled receptors and phospholipase C produces IP_3 from PIP_2 and induces Ca^{2+} release from the intracellular Ca^{2+} pools [17,18]. The cytosolic Ca^{2+}-itch relationship is strongly supported by studies showing that the $[Ca^{2+}]_i$ increase is induced by many different pruritogens including histamine, chloroquine, and sphingosine phosphate [3]. We found for the first time that imiquimod increases $[Ca^{2+}]_i$ in DRG cells [7]. In an effort to elucidate the mechanisms of this effect, we found that extracellular Ca^{2+} is not required for the imiquimod-induced $[Ca^{2+}]_i$ increase in TLR7-lacking cells as well as TLR7-expressing cells. In addition, our experiments using different pharmacological inhibitors indicate that Ca^{2+} is released from the IP_3 receptor-dependent Ca^{2+} pool, without involvement of mitochondrial or ryanodine receptor-dependent Ca^{2+} pools. It was conceived that imiquimod induces $[Ca^{2+}]_i$ increases through the GPCR-PLCβ pathways, however, the inhibition of PLCβ did not block imiquimod-induced increases in $[Ca^{2+}]_i$. The results indicate that imiquimod-induced IP_3 receptor activation is independent of the GPCR-PLCβ pathway.

Our data also imply that imiquimod-containing drugs that are currently being used to boost TLR7-mediated immune responses may trigger unwanted off-target effects derived from its IP_3R activation and $[Ca^{2+}]_i$ increase. This is indeed what we are observing in Aldara, an imiquimod-containing topical cream for genital warts and basal cell carcinoma that often elicits severe pruritic side effects in some patients [19]. This also implies that this off-target increase in $[Ca^{2+}]_i$ may be involved in other side effects of this drug, including pain, burning, and thickening of the skin [19–22]. Therefore, future studies are warranted to distinguish between the TLR7-dependent and TLR7-independent effects of imiquimod-containing drugs. We believe our novel finding that imiquimod induces TLR7-independent Ca^{2+} signaling allows better understanding of not only its pruritogenic effect, but also other effects that were previously known side effects to the drug.

Taken together, we have demonstrated that imiquimod induces TLR7-independent $[Ca^{2+}]_i$ increases by directly activating IP_3 receptor. These data will be informative for understanding the mechanisms underlying the side effects of imiquimod-containing drugs and assist in the future development of anti-viral and anti-tumor skin drugs with fewer side effects.

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