ARTICLE

TRPV4 channel is involved in HSV-2 infection in human vaginal epithelial cells through triggering Ca^{2+} oscillation

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Herpes simplex virus (HSV) infection induces a rapid and transient increase in intracellular calcium concentration ($[Ca^{2+}]_i$), which plays a critical role in facilitating viral entry. T-type calcium channel blockers and EGTA, a chelate of extracellular Ca^{2+}, suppress HSV-2 infection. But the cellular mechanisms mediating HSV infection-activated Ca^{2+} signaling have not been completely defined. In this study we investigated whether the TRPV4 channel was involved in HSV-2 infection in human vaginal epithelial cells. We showed that the TRPV4 channel was expressed in human vaginal epithelial cells (VK2/E6E7). Using distinct pharmacological tools, we demonstrated that activation of the TRPV4 channel induced Ca^{2+} influx, and the TRPV4 channel worked as a Ca^{2+}-permeable channel in VK2/E6E7 cells. We detected a direct interaction between the TRPV4 channel protein and HSV-2 glycoprotein D in the plasma membrane of VK2/E6E7 cells and the vaginal tissues of HSV-2–infected mice as well as in phallic biopsies from genital herpes patients. Pretreatment with specific TRPV4 channel inhibitors, GSK2193874 (1−4 μM) and HC067047 (100 nM), or gene silence of the TRPV4 channel not only suppressed HSV-2 infectivity but also reduced HSV-2-induced cytokine and chemokine generation in VK2/E6E7 cells by blocking Ca^{2+} influx through TRPV4 channel. These results reveal that the TRPV4 channel works as a Ca^{2+}-permeable channel to facilitate HSV-2 infection in host epithelial cells and suggest that the design and development of novel TRPV4 channel inhibitors may help to treat HSV-2 infections.

Keywords: Herpes simplex virus type 2; TRPV4 channel; Ca^{2+} signals; NF-κB; GSK2193874; HC067047

INTRODUCTION

Herpes simplex virus (HSV) infection induces a rapid and transient increase in the intracellular calcium concentration ($[Ca^{2+}]_i$), which plays a critical role in facilitating viral entry [1]. Studies have shown that the interaction between HSV-2 glycoprotein H and integrin alpha V beta 3 promotes the release of intracellular calcium stores and contributes to viral entry into human cervical and primary genital tract epithelial cells [2]. Additionally, HSV-1 glycoprotein D binds the TRPC1 channel to elicit intracellular Ca^{2+} release [1]. Deletion of glycoprotein D from HSV-1 does not induce an $[Ca^{2+}]_i$ response [3, 4]. Moreover, T-type calcium channel blockers and EGTA [Ethylene-bis (oxyethylenenitrilo) tetraacetic acid], a chelate of extracellular Ca^{2+}, suppress HSV-2 infection [5].

The transient receptor potential vanilloid (TRPV) channel family is part of the transient receptor potential (TRP) superfamily, which includes TRPV1-6 [6]. Among them, the TRPV1 and TRPV4 channels are thermosensitive, share 40%–50% sequence identity, are widely expressed and control various physiological functions [6, 7]. Accumulating evidence indicates that the function of the TRPV1 channel is closely related to several viral infections. TRPV1 channel activation triggers Ca^{2+} influx that regulates coxsackievirus B [8], HSV-1 [9], Chikungunya virus [10], human rhinovirus

[11] and respiratory syncytial virus infections [12–14]. Glycoprotein G, secreted by HSV-2, mediates the localization and activation of the TRPV1 channel to alter thermal pain sensitivity in the dermis [15]. However, little is known about the relationship between HSV-2 infection and the TRPV4 channel. In this study, we investigated the involvement of the TRPV4 channel in HSV-2 infection using human epithelial cells, mouse models, and phallic biopsies from HSV-2-infected patients.

We found that the TRPV4 channel is a Ca^{2+}-permeable channel in host epithelial cells. During HSV-2 infection, viral glycoprotein D binds to the TRPV4 channel and induces an increase in intracellular calcium concentration. Blocking TRPV4 channels exerts antiviral and anti-inflammatory effects via suppression of Ca^{2+} oscillations. These findings reveal a previously unidentified role of the TRPV4 channel in HSV-2 infection.

MATERIALS AND METHODS

Cells, viruses, and animals

The human vaginal epithelial cell line VK2/E6E7 (ATCC® CRL-2616™) was cultured in keratinocyte serum-free medium supplemented with 50 μg/mL bovine pituitary extract, 0.1 ng/ml recombinant epidermal growth factor (rEGF), 100 μg/ml streptomycin, 100 units/ml penicillin,

and 0.4 mM $CaCl_2$. Vero cells, CHO cells, and human HaCaT keratinocytes were cultured in DMEM supplemented with glucose (Gobio, USA), 10% fetal bovine serum (Gibco, Life Technologies), and 1% gentamicin (Sigma).

The HSV-2 strain 333 was provided by the Guangzhou Institute of Biomedicine and Health (Guangzhou, China). Female BALB/c mice at the age of five to seven weeks were purchased from Guangdong Medical Laboratory Animals Center in Guangzhou, China. The animals were provided with *ad libitum* access to food and water in a room maintained at a constant temperature of $24 \pm 0.5\,°C$ with a 12:12 h light/dark photoperiod.

Reagents and solutions
GSK1016790A (HY-19608), GSK2193874 (HY-100720), HC067047 (883031-03-6), thapsigargin (HY-13433), ionomycin (56092-82-1), and forskolin (66575-29-9) were purchased from MedChemExpress (MCE). Fluo-3 AM (ab145254) and antibodies against HSV-2 gD were purchased from Abcam. p-NF-κB-p65, IκBα, β-actin, and GAPDH were purchased from Cell Signaling Technology. HRP-conjugated secondary antibodies were obtained from Sigma Aldrich. A normal physiological saline solution (NPSS) was created containing 137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM Herpes, and 10 mM glucose; this solution had a pH of 7.4. A Ca^{2+}-free physiological saline solution (Ca^{2+}-free PSS) with a pH of 7.4 was prepared by omitting Ca^{2+} and adding 2 mM EGTA to the NPSS.

Measurement of intracellular calcium in uninfected cells
The human vaginal epithelial cell lines VK2/E6E7 were seeded on coverslips. Cells were washed with normal physiological saline solution and then incubated with Fluo-3 acetoxymethyl ester (10 μM) at room temperature for 45 min. Then, the coverslips were transferred to a special circular groove of the laser confocal fluorescence microscope. Drugs were added to the coverslips in the circular groove. The fluorescence signal was recorded by a laser scanning confocal imaging system (TCS SP2, Leica Microsystems, Mannheim, Germany) at a 488-nm excitation wavelength and a 520 nm absorption wavelength [1]. The data were further analyzed with Origin 8.0.

Quantitative PCR
Total RNA was extracted using TRlzol (Invitrogen, USA). RNA concentrations were measured using a NanoDrop 2000c (Thermo Scientific). cDNA was reverse transcribed using a PrimeScript™ RT Reagent Kit (Takara). Quantitative PCR was performed with ChamQ SYBR qPCR Master Mix (Vazyme, China) on a LightCycler 480 System (Roche, Switzerland).

The primers used in the quantitative PCR are listed in Table 1. The levels of the target genes were normalized to the levels of GAPDH mRNA, and relative expression was determined according to the ΔΔCt method [16].

Western blot
Total protein from epithelial cells or vaginal tissue was extracted with precooled RIPA buffer (Bio-Rad, CA, USA). The protein concentration was detected by a BCA protein kit. After separation with SDS-polyacrylamide gel electrophoresis (SDS–PAGE), the protein samples were transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). The membranes were blocked with 5% fat-free milk for one hour at room temperature, incubated with primary antibodies overnight at $4\,°C$, and incubated with secondary antibodies for one hour at room temperature. Super-Signal ECL reagent (Millipore, MA, USA) was used to observe the chemiluminescence signals using a multifunctional imaging system (ProteinSimple, CA, USA) [17].

Immunofluorescence
Immunofluorescent staining was performed as previously described [5]. The infected cells, vaginal tissues, and phallic biopsies were fixed with acetone at $4\,°C$ for 10 min.

After washing with phosphate-buffered saline (PBS) and treating with 1% BSA for 30 min at room temperature, the samples were incubated with the first primary antibody at $4\,°C$ overnight, followed by the secondary antibody, goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) or Texas red (Santa Cruz Biotechnology), at room temperature for one hour. VECTA-SHIELD medium (Vector Labs., Burlingame, CA, USA) was used to preserve the samples. Finally, images were captured via a fluorescence microscope (TE2000U, Tecan, USA).

Virus infection
VK2/E6E7 cells were inoculated in 6-well plates at a density of 1×10^6 cells per well. The next day, HSV-2 (MOI = 5) was administered to the cells in the presence or absence of drugs for 1 h. Subsequently, the unabsorbed virus was removed, and keratinocyte serum-free medium containing drugs was added [5]. The cells were collected at the specified time points for quantitative PCR and Western blotting. The culture supernatants were collected for plaque assays.

Plaque assay
The generation of progeny viruses was evaluated by the plaque assay [5]. Vero cells were inoculated in 12-well plates at a density of 1.2×10^5 cells per well. Diluted culture supernatants of infected cells were added to the Vero cells. After incubation for 1 h at $37\,°C$, the culture supernatants were discarded, and DMEM containing 2% fetal bovine serum and 1% methylcellulose was added. After incubation in this solution for 3, 4 days at $37\,°C$,

Table 1. The primers used in quantitative PCR.

Factor	(Forward)	(Reverse)
TRPV1	5′-CGTTTCATGTTTGTCTACATCGTC-3′	5′-TGTGCGACGTGGACTCAGAC-3′
TRPV2	5′-TCTCGTTCATAGACAGCTACTTTGA-3′	5′-GGAAACACAGCACCTGGGAC-3′
TRPV3	5′-ACACCACCACGGACAACTCA-3′	5′-ACTTCATATGCAGCAGCGTGT-3′
TRPV4	5′-AGGTGTGCAATGAGGACCAGA-3′	5′-TGCTGAAGGTCTCG CTGTCA-3′
TRPV5	5′-ATCGCTATCACTCTTGACCTTACTC-3′	5′-TGACCCTTTAGGGATTGTTCTG-3′
TRPV6	5′-GAAAGCGTGTGAGTGAGGGA-3′	5′-GAAGATGGAGTTGGCAAGACC-3′
CXCL-9	5′- GGAGATCACCAGTGTGTGGCTCXCL-3′	5′- AGGCACTGCATTGTGGTAGGA-3′
CXCL-10	5′-AATCGATGCAGTGCTTCCAAGG-3′	5′-GCAGCTGATTTGGTGACCATCATT-3′
IFN-β	5′-TGCCTGGACCATAGTCAGAGTG-3′	5′-CAGTTTCGGAGGTAACCTGTAAGTC-3′
TNF-α	5′-CCCAGGGACCTCTCTCTAATCA-3′	5′-GCTTGAGGGTTTGCTACAACATG-3′
IL-6	5′-GTAGCCGCCCCACACAGA-3′	5′-CATGTCTCCTTTCTCAGGGCTG-3′

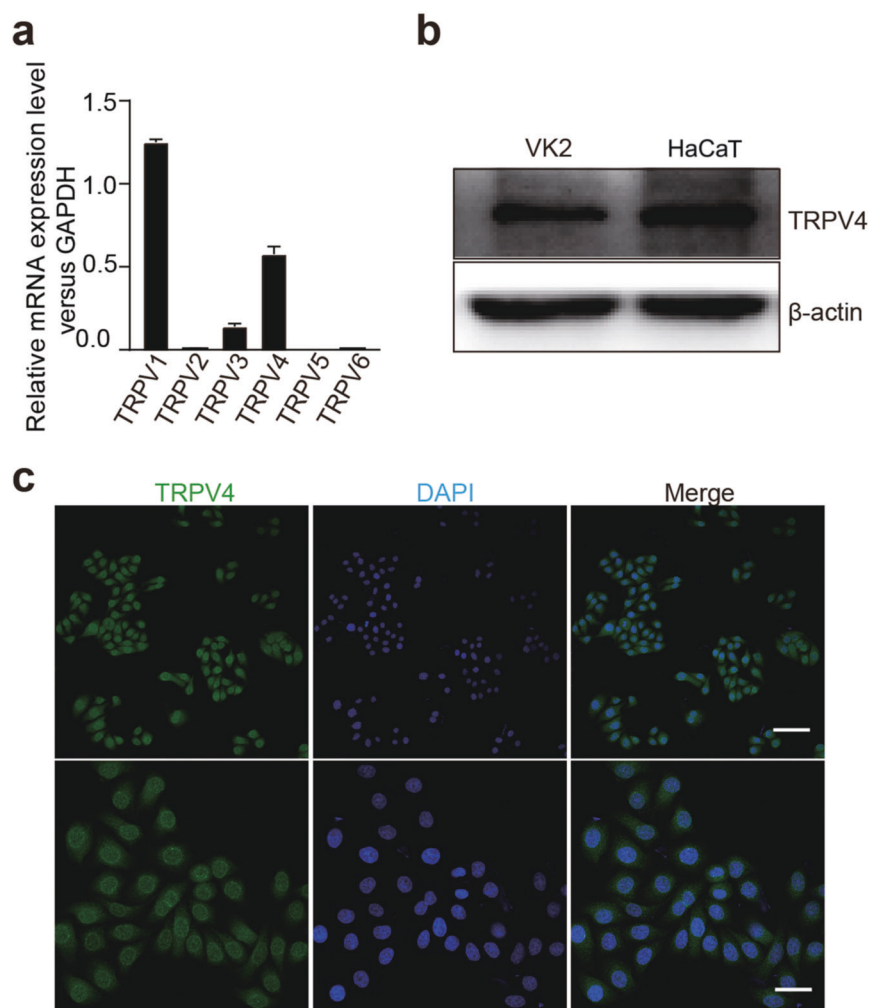The involvement of the TRPV4 channel in HSV-2 infection
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Fig. 1 The TRPV4 channel was expressed in host epithelial cells. a mRNA expression of TRPV1-6 in the human vaginal epithelial cell line VK2/E6E7 was measured with quantitative PCR. **b** TPRV4 channel expression in human HaCaT keratinocytes was detected by Western blot. **c** The localization of the TRPV4 channel (green) was observed by immunofluorescent staining in VK2/E6E7 cells. Upper scale bar: 10 μm; lower scale bar: 20 μm.

crystal violet was used to stain the cells. The yield of progeny viruses in the supernatant was determined by calculating the number of plaques.

Measurement of intracellular Ca^{2+} concentrations in infected cells
The intracellular Ca^{2+} concentrations of infected cells were measured with BB-cell probe F03 (BB-48112, BestBio, China) [18]. Briefly, cells were inoculated in 12-well plates at a density of 1.5×10^5 cells per well. After infection with HSV-2 (MOI = 5), the cells were washed three times with Hanks' balanced salt solution (HBSS) at the specified time points. The diluted F03 probe was loaded and incubated for 1 h at 37 °C. After three washes, the cells were incubated for another 40 min. The fluorescence values were detected with a fluorescence microplate reader. Data are expressed as fluorescence units.

Mouse model of vaginal HSV-2 infection
All animal experiments were conducted following protocols approved by the Animal Use and Care Committee of Southern Medical University. The vaginal cavity of mice was washed with PBS and rubbed with sterile cotton to increase mouse susceptibility to vaginal HSV-2 infection. The mice were then inoculated vaginally with 1×10^6 PFU HSV-2. After seven days, the phenotypes of the infected mice were scored as follows [19]:

"1", slight genital erythema and edema; "2", moderate genital inflammation; "3", severe exudative genital lesions; "4", hind limb paralysis; or "5", death. Vaginal tissues were collected for immunofluorescent analysis or immunoprecipitation.

Immunoprecipitation
The interaction between the TRPV4 channels and HSV-2 glycoprotein D was investigated by immunoprecipitation [1]. Epithelial cells were infected with HSV-2 (MOI = 5) for one hour. After the cells were washed with precooled PBS, IP lysis buffer (Beyotime, Shanghai, China) was added for 30 min on ice. Next, the lysates of infected cells or vaginal tissues were centrifuged at 12,000 rpm for 15 min at 4 °C. The 10% supernatant was removed for use as the input group. The other lysates were mixed with protein A/G beads (GE Healthcare, Sweden) and incubated overnight with anti-TRPV4 antibody at 4 °C. After the lysates were washed with precooled IP buffer, an appropriate amount of the solution was analyzed by Western blot.

Statistical analysis
Experimental data are expressed as the means ± SEMs. The Students' two-tailed t-tests were used for two sets of data analysis. Three or more groups were analyzed with one-way ANOVAs followed by Dunnett's post hoc test using GraphPad Prism 8.0 software
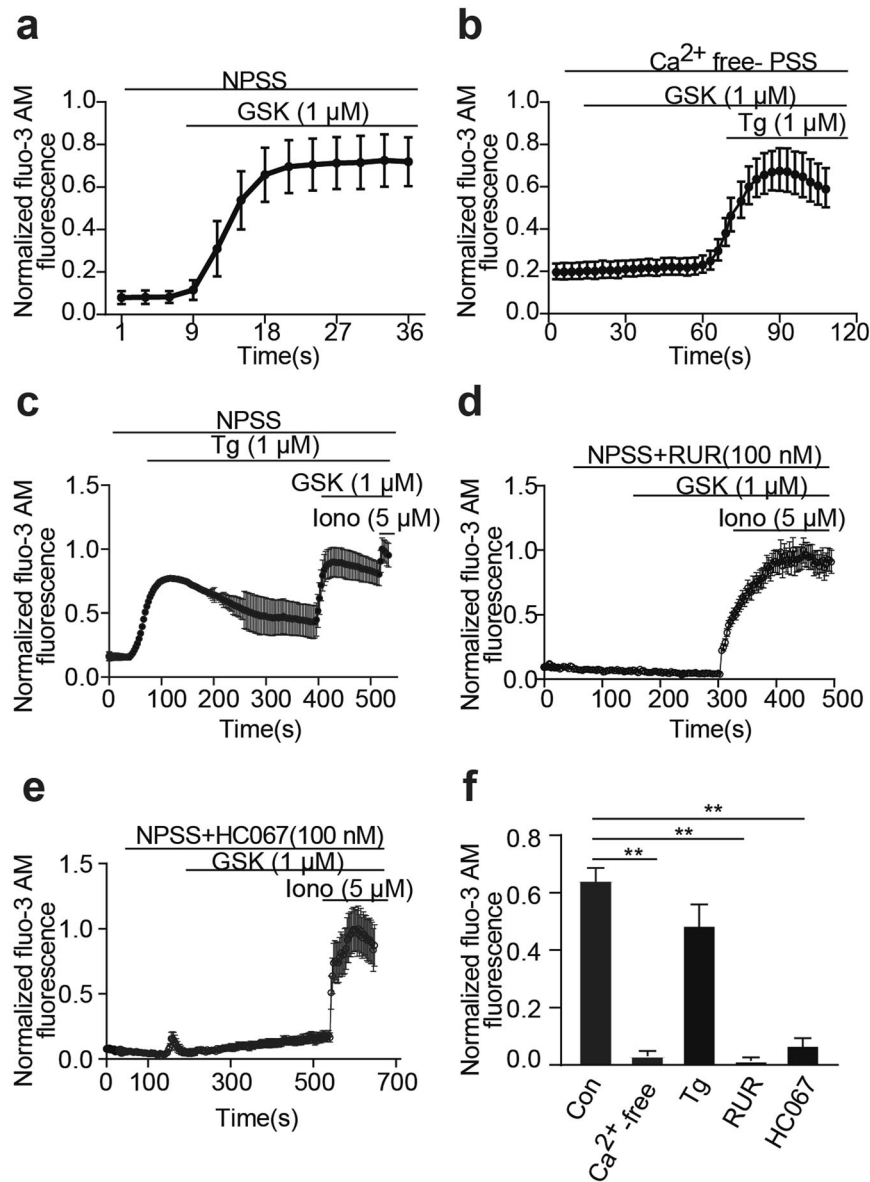
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Fig. 2 Changes in intracellular Ca^{2+} were induced by the activation of the TRPV4 channel. a GSK1016790A (GSK, 1 μM), a specific TRPV4 channel activator, significantly increased the intracellular Ca^{2+} concentration in normal physiological saline solution (NPSS). **b** GSK1016790A (GSK, 1 μM) did not increase the intracellular Ca^{2+} concentration in Ca^{2+}-free solution (Ca^{2+}-free-PSS). Thapsigargin (Tg, 1 μM) was used to indicate cell survival. **c** Thapsigargin (Tg, 1 μM), which induces the release of Ca^{2+} stores in the endoplasmic reticulum (ER) and prevents refilling by inhibiting the ER Ca^{2+}-ATPase, had no noticeable effect on the elevation of intracellular Ca^{2+} induced by GSK in NPSS. Ionomycin (Iono, 5 μM) was used to indicate cell survival. **d, e** Ruthenium red (RUR, 100 nM, a nonspecific TRPV4 inhibitor) or HC067047 (HC067, 100 nM, a specific TRPV4 inhibitor) obviously hindered GSK-induced elevation of intracellular Ca^{2+}. **f** The inhibitory effects of different conditions on the GSK-induced Ca^{2+} influx are summarized. Data are represented as the mean ± SD (**$P < 0.01$, compared with controls) and are from three independent experiments consisting of 30 cells each.

(*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Two-tailed P values < 0.05 were considered statistically significant.

RESULTS

The TRPV4 channel is expressed in HSV-2-infected epithelial cells
The TRPV channel family has six members, namely, TRPV1 through TRPV6 [20]. The expression of TRPV channels was measured by real-time quantitative PCR. The results showed that mRNA from the TRPV1, TRPV3, and TRPV4 channels was present in human vaginal epithelial cells (VK2/E6E7), as shown in Fig. 1a. The expression of the TRPV4 channel was detected by Western blot in human HaCaT keratinocytes (Fig. 1b) and was further confirmed

by immunofluorescent staining. Intense immunofluorescence from the TRPV4 channel (green) was observed in the plasma membrane (Fig. 1c). These results demonstrate the presence of the TRPV4 channel in HSV-2 host epithelium cells.

The TRPV4 channel is a Ca^{2+}-permeable channel in host epithelial cells
To assess the function of the TRPV4 channel in epithelial cells, we observed the dynamic changes in intracellular Ca^{2+} with confocal microscopy. The classic activator of the TRPV4 channel, GSK1016790A (GSK, 1 μM), induced a significant increase in $[Ca^{2+}]_i$ in epithelial cells in normal physiological saline solution (NPSS), as seen in Fig. 2a. Interestingly, GSK did not evoke this

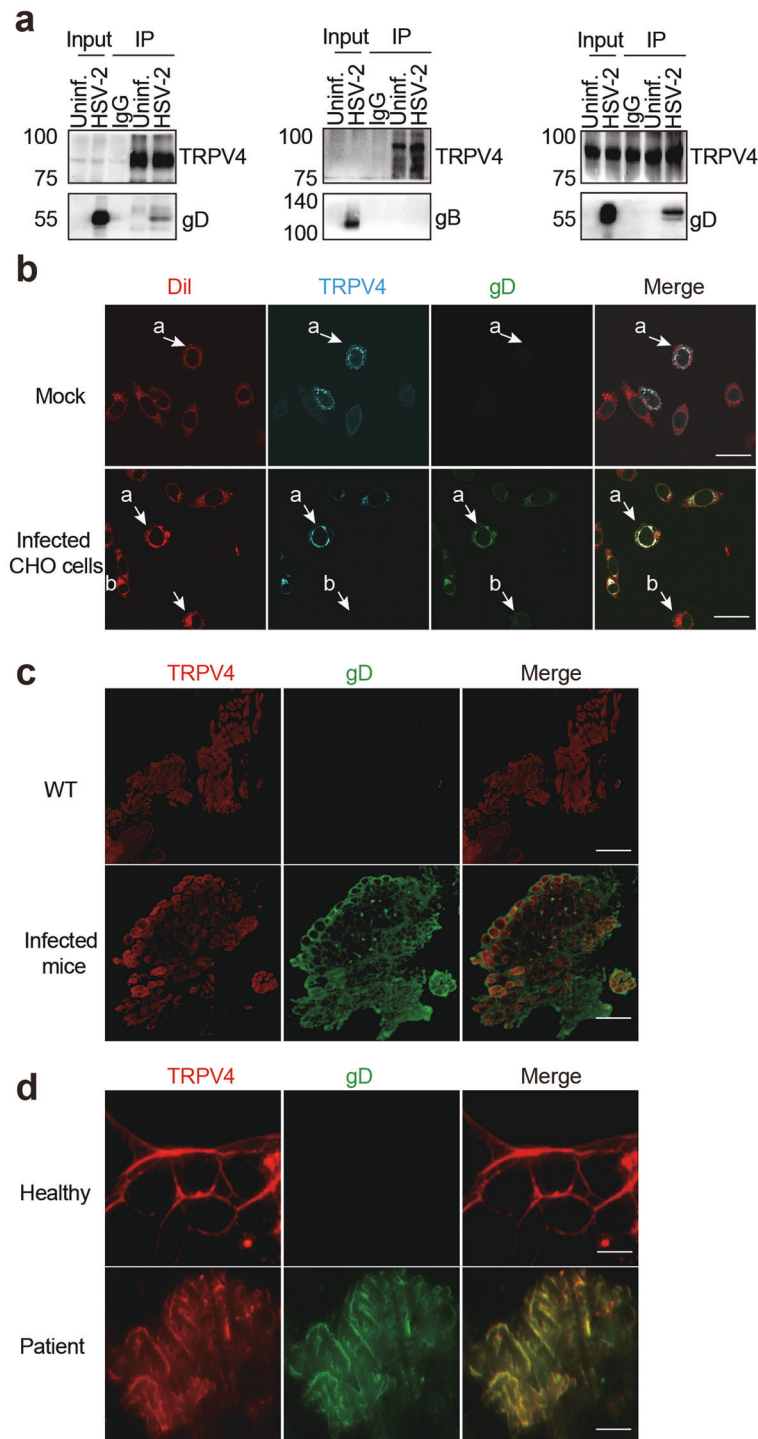The involvement of the TRPV4 channel in HSV-2 infection
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Fig. 3 The TRPV4 channel protein interacted with HSV-2 glycoprotein D. a The human vaginal epithelial cell line VK2/E6E7 was infected with HSV-2 (MOI = 5). After 24 h of incubation, the cells were collected for immunoprecipitation to evaluate the interaction between the TRPV4 channel and glycoprotein D (left) or glycoprotein B (middle). Vaginal tissues collected from infected mice underwent immunoprecipitation to observe the TRPV4-gD interaction (right). **b** After transfection with the TRPV4 channel, CHO cells were infected with HSV-2 (MOI = 5) for 24 h and then collected for immunofluorescence staining to determine TRPV4-glycoprotein D colocalization. In the Pearson correlation analysis, Image J calculated $r = 0.78 \pm 0.09$. Dil indicates the plasma membrane in red, the TRPV4 protein in blue and the HSV-2 glycoprotein D in green. "a" represents CHO cells successfully transfected with the TRPV4 channel; "b" represents CHO cells not successfully transfected with the TRPV4 channel. **c** A WT-mouse model of vaginal infection was established using HSV-2 strain 333; mouse vaginal tissues were collected for immunofluorescent staining. Here, red represents the TRPV4 protein, and green represents HSV-2 glycoprotein D. **d** The phallic biopsies from genital herpes patients were fixed and labeled with immunofluorescent staining, in which red indicates the TRPV4 protein and green indicates HSV-2 glycoprotein D.
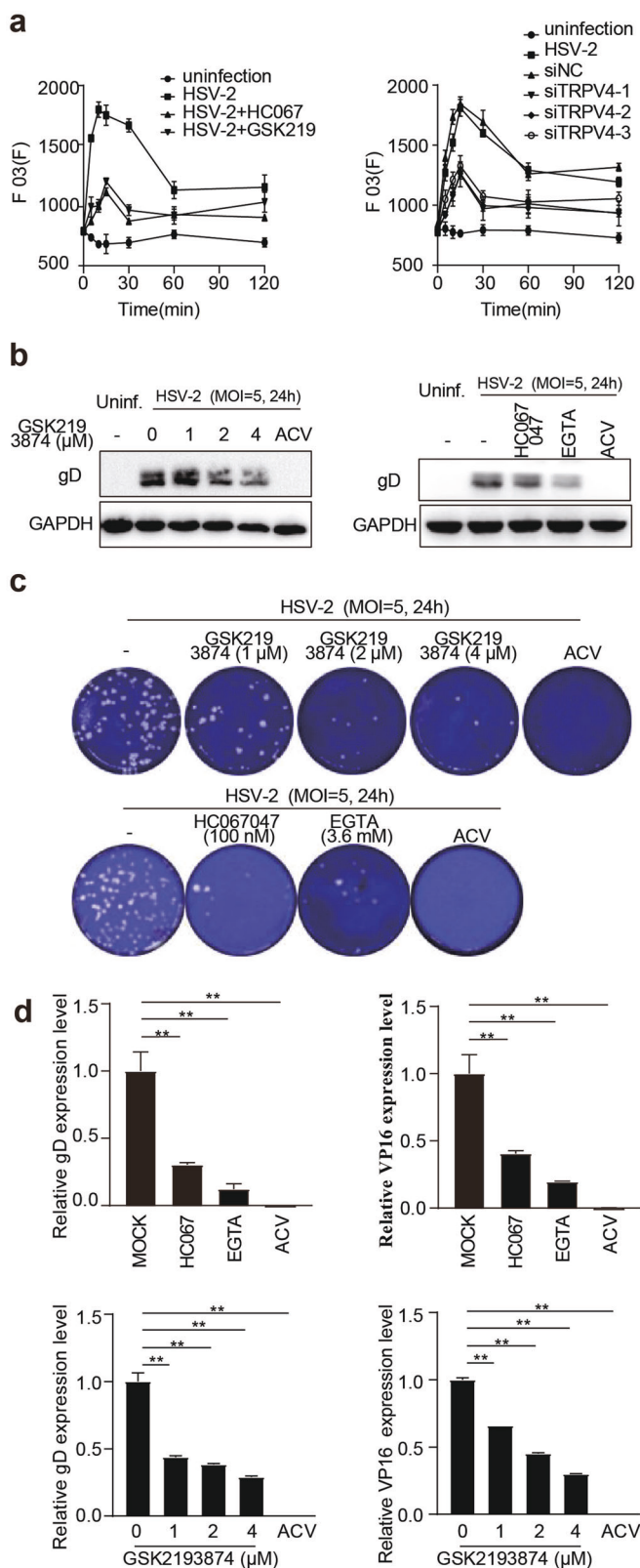
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Fig. 4 TRPV4 channel inhibitors suppressed HSV-2 infection. a Effects of TRPV4 channel inhibitors and siRNA on HSV-2-induced Ca^{2+} oscillations in epithelial cells. The cells were infected with HSV-2 (MOI = 5) in the presence or absence of the specific TRPV4-channel inhibitors HC067047 (HC067, 100 nM) and GSK2193874 (GSK219, 4 μM). The cells were collected at the time specified by the calcium determination kit, and the changes in Ca^{2+} oscillations are shown in the left image. After transfection with siRNA, the cells infected with HSV-2 (MOI = 5) were collected at the time specified by the kit. The changes in Ca^{2+} oscillations are shown in the right image. **b** Effects of TRPV4 channel inhibitors on viral protein expression. The cells were infected with HSV-2 (MOI = 5) in the presence of the indicated concentrations of GSK2193874, HC067047 (100 nM), EGTA (an extracellular calcium chelating agent; 3.6 mM), or acyclovir (ACV; 1 μg/ml) separately for 24 h. The cells were collected to analyze viral glycoprotein D expression by Western blot. **c** The supernatant was harvested for virus titration by plaque assay. **d** The expression of the viral genes glycoprotein D and VP16 was measured by real-time quantitative PCR. Acyclovir, the typical drug used to treat HSV infection, served as the positive control. Data represent the mean ± SD of three independent experiments. **$P < 0.01$ vs. the control.

the TRPV4 channel causes Ca^{2+} influx. In addition, the GSK-induced Ca^{2+} influx was eliminated by pretreating cells with ruthenium red (RUR, a nonspecific TRPV4-channel inhibitor) or HC067047 (a specific TRPV4-channel inhibitor) [21], as shown in Fig. 2d, e. These data suggest that the TRPV4 channel is a Ca^{2+}-permeable channel in host epithelial cells. Each experiment was independently repeated three times, and the data from 30 cells were selected for statistical analysis, as shown in Fig. 2f.

The TRPV4 channel interacts with HSV-2 glycoprotein D
During the early stages of viral entry, HSV glycoprotein D interacts with multiple cell surface receptors, including HVEM [22, 23], nectin-1 [24], and nectin-2 [25]. HSV-1 glycoprotein D also directly interacts with the TRPC1 channel, another member of the TRP superfamily [1]. This study aimed to examine whether HSV-2 glycoprotein D interacts with the TRPV4 channel. In coimmunoprecipitation, the TRPV4 channel and HSV-2 glycoprotein D were detected in the infected epithelial cells, as shown in Fig. 3a (left), but other viral glycoproteins, such as glycoprotein B, were not found, as shown in Fig. 3a (middle). To further explore the interaction of the TRPV4 channel and glycoprotein D under pathological conditions, we established a WT mouse model of vaginal infection using HSV-2 strain 333. Consistent with the results in infected cells, coimmunoprecipitation of the TRPV4 channel and glycoprotein D was observed in infected mice, as shown in Fig. 3a (right). These data suggest that the TRPV4 protein directly interacts with HSV-2 glycoprotein D.

To evaluate the colocalization of the TRPV4 protein and glycoprotein D, we transfected CHO cells lacking nectin-1 (a glycoprotein D receptor [26]) with the TRPV4 channel. The overlap of the fluorescence signal between the TRPV4 channel (blue) and glycoprotein D (green) was observed in the TRPV4-transfected cells, as shown in Fig. 3b. The CHO cells that were successfully transfected with the TRPV4 channel are indicated with "a"; those not successfully transfected with the TRPV4 channel are indicated with "b". The data show that "a" exhibit greater glycoprotein D fluorescence than "b" in the plasma membrane. Image J was used to perform Pearson correlation analysis on fifty images; r was calculated as 0.78 ± 0.09, indicating that the green fluorescence of glycoprotein D and the blue fluorescence of the TRPV4 channel had high colocalization.

To further examine the colocalization of TRPV4 and glycoprotein D under pathological conditions, vaginal tissues from infected mice and phallic biopsies from genital herpes patients were collected for immunofluorescence assays. The fluorescence analysis indicated colocalization of the TRPV4 channel (red) and

$[Ca^{2+}]_i$ increase in Ca^{2+}-free solution, as seen in Fig. 2b. Thapsigargin (Tg, 1 μM) induces the release of intracellular endoplasmic reticulum (ER) Ca^{2+} stores and prevents refilling by inhibiting ER Ca^{2+}-ATPase. However, thapsigargin pretreatment did not eliminate the GSK-induced Ca^{2+} influx (Fig. 2c), indicating that activation of

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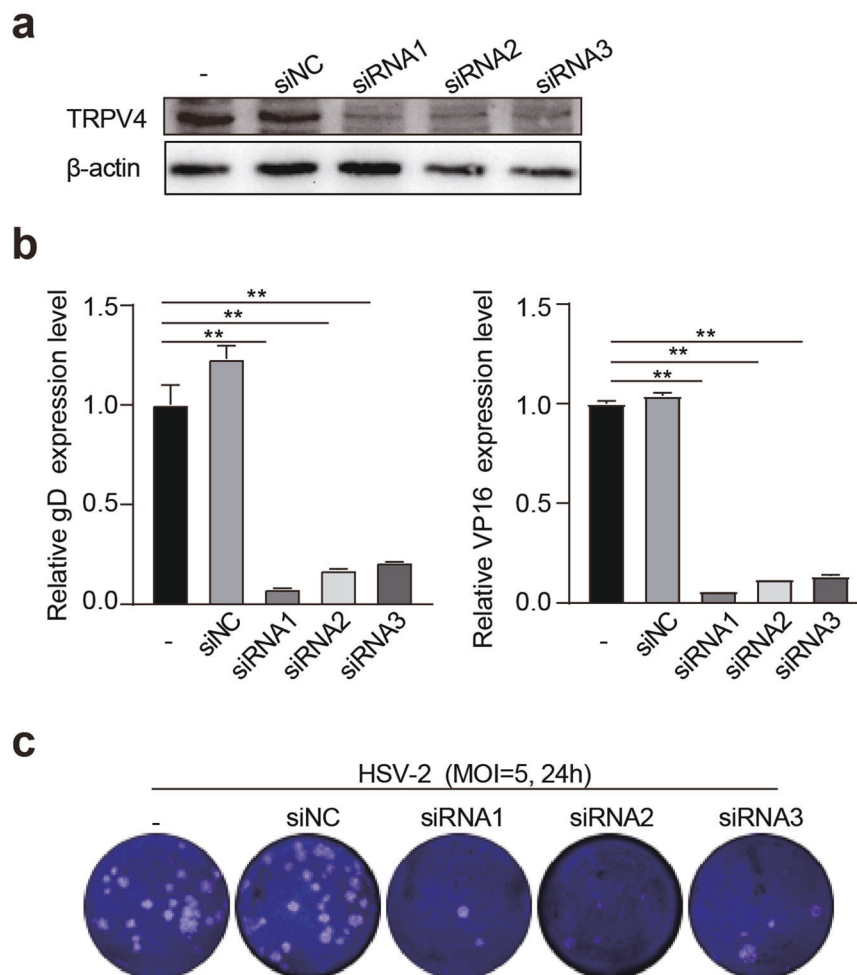P Jiang et al.

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Fig. 5 **Knockdown of the TRPV4 channel inhibited HSV-2 infection. a** Western blot analysis of TRPV4 channel expression in human epithelial cells transfected with siRNA against the TRPV4 channel. **b** The transfected cells were infected with HSV-2 (MOI = 5) for 24 h and collected for viral gene (glycoprotein D and VP16) expression analysis by real-time quantitative PCR. **c** The supernatant was harvested for virus titration by plaque assay. Data represent the mean ± SD of three independent experiments. **$P < 0.01$ vs. the control.

glycoprotein D (green) in the vaginal tissues of infected mice, as shown in Fig. 3c. Genital herpes and vaginitis are common symptoms of HSV-2 infection among humans [27]. Thus, some phallic biopsies from patients were selected for detection, and the same colocalization pattern of glycoprotein D and the TRPV4 channel was observed, as shown in Fig. 3d. These findings confirm that the TRPV4 channel and glycoprotein D colocalize in the plasma membrane of HSV-2-infected cells.

Blocking TRPV4 activity produces an anti-HSV-2 effect
The Ca^{2+} influx mediated by the TRPV4 channel controls RNA virus infectivity [28]. Here, we explored the possibility that Ca^{2+} influx through TRPV4 channels contributes to infection of HSV-2, a DNA virus. The specific TRPV4-channel inhibitors GSK2193874 and HC067047 markedly decreased the Ca^{2+} oscillations induced by HSV-2 infection (Fig. 4a; left). A reduction in Ca^{2+} oscillations was also observed in the cells with siRNA knockdown of the TRPV4 channel (Fig. 4a; right). The data show that the increase in the intracellular calcium concentration triggered by HSV-2 infection is partly dependent on the TRPV4 channel.

To determine the antiviral effect of specific TRPV4-channel inhibitors, we evaluated their effect on HSV-2 infection in host epithelial cells. The results showed that GSK2193874 and HC067047 visibly suppressed the synthesis of viral glycoprotein D (Fig. 4b), the production of progeny viruses (Fig. 4c), and the

transcription of viral genes (VP16 and gD) (Fig. 4d). Acyclovir, the typical drug used to treat HSV infections, served as the positive control [29]. EGTA, which depleted extracellular Ca^{2+}, as seen in Fig. 4b-d, had similar antiviral effects, indicating that the anti-HSV-2 effect of blocking the TRPV4 channel is closely related to Ca^{2+} oscillations. Further investigation showed that siRNA knockdown of the TRPV4 channel also inhibited viral gene transcription (Fig. 5b) and progeny virus production (Fig. 5c). These results suggest that blocking the TRPV4 channel inhibits HSV-2 infection by reducing the Ca^{2+} influx.

Blocking TRPV4 activity alleviates HSV-2-induced inflammation
Previous studies have shown that Ca^{2+} influx through TRPV4 channels creates a long-lasting inflammatory signal, and TLR-mediated inflammation is suppressed by blocking the TRPV4 channel [30]. Various pattern recognition receptors, such as TLR2, TLR9, and cGAS, can identify HSV-2 and then promote proin-flammatory cytokine generation [31, 32]. To explore the relation-ship between the TRPV4 channel, a Ca^{2+}-permeable channel, and the inflammatory signals in epithelial cells induced by HSV-2 infection, we investigated the anti-inflammatory effect of TRPV4-channel inhibitors in the infected cells. The specific TPRV4-channel inhibitor HC067047 (100 nM) reduced the HSV-2-induced expres-sion of cytokines (TNF-α and IL-6) and chemokines (CXCL-9 and CXCL-10) at three specific time points, as shown in Fig. 6a.
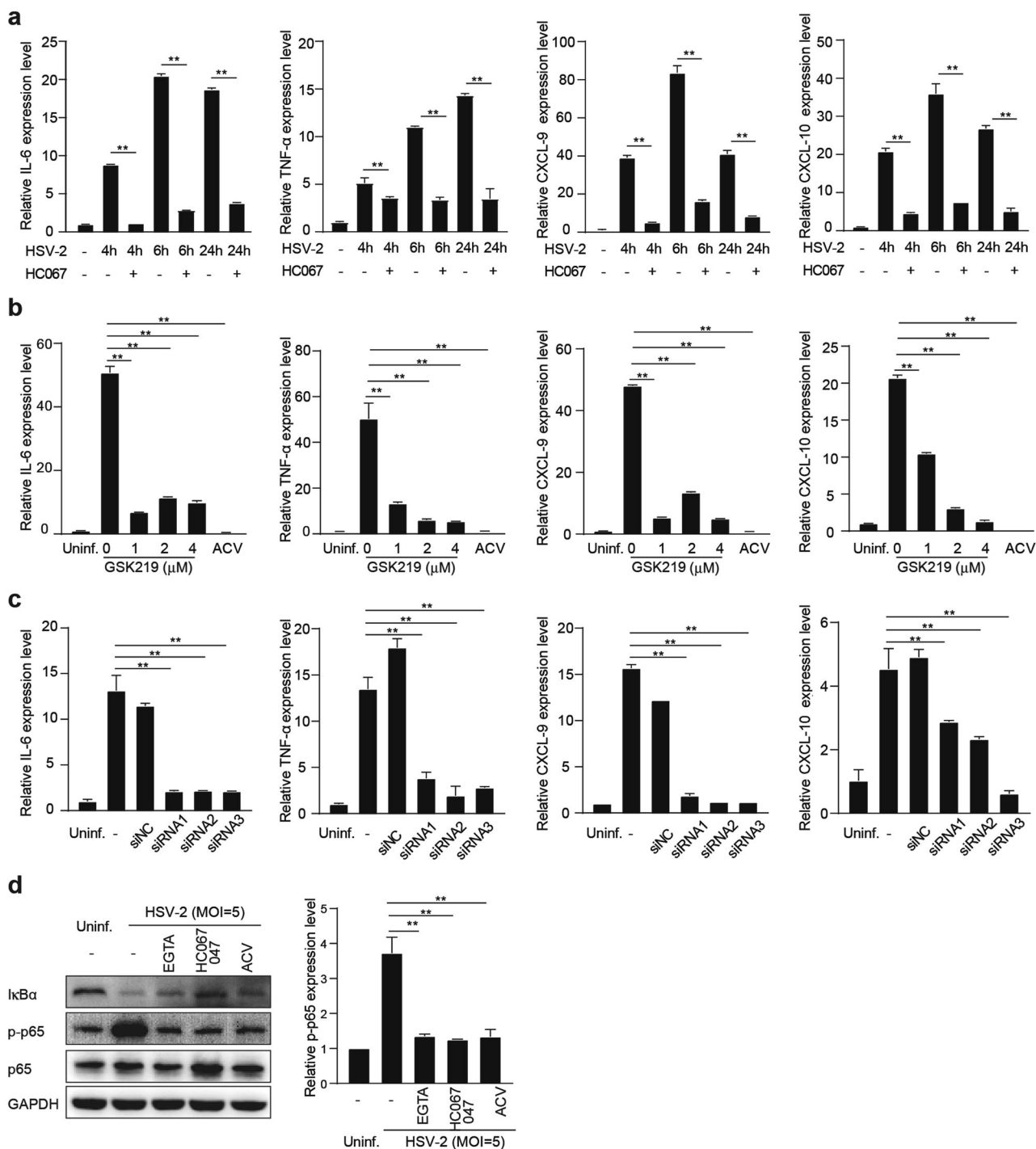
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Fig. 6 HSV-2-induced inflammation is inhibited by blocking TRPV4 activity. a Human epithelial cells were infected with HSV-2 (MOI = 5) in the presence or absence of HC067047 (100 nM) and then harvested at 2, 4, or 6 h to analyze the expression of cytokines (TNF-α, IL-6) and chemokines (CXCL-9 and CXCL-10) by real-time quantitative PCR. **b** The infected cells were treated with the indicated concentrations of GSK2193874 for 24 h and then collected for cytokine and chemokine expression analysis. **c** The transfected cells were infected with HSV-2 (MOI = 5) for 24 h and then digested for cytokine and chemokine expression analysis by real-time quantitative PCR. **d** The cells were infected with HSV-2 (MOI = 5) in the presence or absence of HC067047, EGTA, or acyclovir (ACV, 1 μg/ml) for 24 h and then IκBα and phosphorylated p65 protein expression was detected by Western blot. Data represent the mean ± SD of three independent experiments. **$P < 0.01$ vs. the control.

Three different concentrations of GSK2193874, another specific TPRV4-channel inhibitor, effectively decreased the expression of TNF-α, IL-6, CXCL-9, and CXCL-10 at 24 h, as shown in Fig. 6b. After transfection with siRNA against the TRPV4 channel, the inflammatory signals in infected cells markedly declined, as shown in Fig. 6c. These data indicate that the inflammatory signals induced by HSV-2 infection are closely tied to TRPV4 channel activity in epithelial cells.

An increase in intracellular Ca^{2+} has been shown to promote the transcriptional activity of NF-κB [33]. HSV-2 infection has also been shown to promote the activation of NF-κB and the secretion of interleukins and chemokines [34]. According to the above
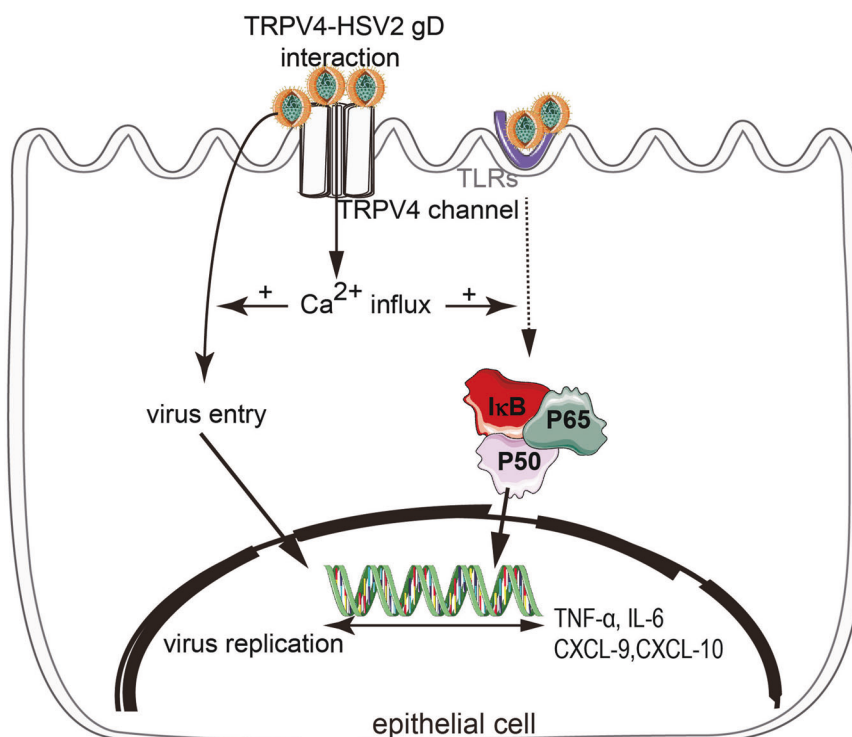
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Fig. 7 The proposed working model of the function and mechanism of the TRPV4 channel in HSV-2 infection. The TRPV4 channel is a calcium-permeable channel in epithelial cells. During HSV-2 infection, the interaction between glycoprotein D and the TRPV4 channel facilitates virus infection and exacerbates NF-κB activation by increasing the intracellular calcium concentration. Inhibition of TRPV4 channel activity diminishes viral infection and inflammation.

findings, we decided to explore whether NF-κB activation induced by HSV-2 infection was due to Ca^{2+} influx through TRPV4 channels. We found that inhibition of the TRPV4 channel by HC067047 (100 nM) or EGTA (3.6 mM) markedly reduced the expression of phosphorylated p65 and IκBα in infected cells, as shown in Fig. 6d. These data indicate that the TRPV4 channel participates in NF-κB activation in HSV-2-infected cells.

DISCUSSION

The transient receptor potential vanilloid (TRPV) channels have been implicated in various pathophysiological processes, including viral infections. The TRPV4 channel binds the DEAD-box RNA helicase DDX3X to regulate RNA virus infectivity [28]. Inhibition of the TRPV4 channel thus provides an antiviral effect against RNA viruses, such as the Zika virus [35]. This study found that TRPV1, TRPV3 and TRPV4 channels are expressed in human epithelial cells. A previous study reported a correlation between TRPV1 channel activity and HSV-2 infection and demonstrated that HSV-2 glycoprotein G modulates TRPV1 channel activation [15]. In infected epithelial cells, the expression of the TRPV3 channel is lower than that of the TRPV4 channel. In addition, the TRPV3 channel is mainly expressed in the endoplasmic reticulum (ER) [36]. The changes in $[Ca^{2+}]_i$ play a critical role in the entry of herpes simplex virus into cells [1], indicating that the Ca^{2+}-permeable channel expressed in the plasma membrane (TRPV4) is more important than the channel expressed in the ER (TRPV3) for viral entry. Thus, we investigate the effects of the TRPV4 channel on HSV-2 infection in this study. Our findings show that the TRPV4 channel, a Ca^{2+}-permeable channel, directly binds HSV-2 glycoprotein D to facilitate HSV-2 infection and inflammation in host epithelial cells.

The HSV-2 virus enters the body primarily through epithelial cells and eventually establishes latent infections in the sacral ganglion

[37]. The TRPV4 channel is abundantly expressed in nerve cells [38]. Therefore, we suspect that the interaction between the TRPV4 channel and glycoprotein D may be a potential mechanism underlying the establishment of latent infection in the sacral ganglion. Our study therefore implies that the TRPV4 channel plays a potential role in latent infection.

The TRPV4 channel protein contains 871 amino acids and four extracellular domains [6]. Which of these four extracellular domains is key to the interaction between the TRPV4 channel and glycoprotein D? We attempted to identify the specific binding site(s) of the TRPV4-gD interaction with virtual docking procedures. Multiple binding sites were predicted at the protein–protein interface (data not shown). These binding sites are mutated and individually expressed in CHO cells during HSV-2 infection. However, differences in the interactions between glycoprotein D and the mutant TRPV4s have not been detected in infected CHO cells. This outcome may be explained as follows. On the one hand, CHO cells transfected with mutant TRPV4 and infected with HSV-2 have low survival rates. On the other hand, the TRPV4 channel has five different splice variants (TRPV4A-E), all of which have different amino acid deletions [7, 38]. Therefore, it's reasonable for us to doubt that the effective interaction between glycoprotein D and the five different TRPV4 splice variants extensively differs; additionally, it would be complicated to analyze. The specific binding sites merit further evaluation.

Several pattern-recognition receptors (PRRs), such as TLRs and cyclic GMP-AMP synthase (cGAS), act as the sensors of HSV-2 [39–41]. These PRRs recognize the HSV-2 glycoprotein and then induce NF-κB activation and inflammatory secretion [32, 42], including TNF-α, IL-6, CXCL-9, and CXCL-10 [37, 43]. In addition, a rise in intracellular Ca^{2+} concentration can enhance transcription of NF-κB and thereby aggravate inflammatory signaling [44, 45]. Indeed, we found that blockade of the TRPV4 channel prevents the production of TNF-α, IL-6, CXCL-9, and CXCL-10 and reduces

the phosphorylation of p65 in HSV-2-infected cells, indicating that the TRPV4 channel regulates the inflammatory signals caused by HSV-2 infection.

In conclusion, we propose the model shown in Fig. 7. During HSV-2 infection, the interaction between glycoprotein D and the TRPV4 channel triggers intracellular Ca^{2+} oscillation, an essential factor in virus entry [2, 4] that facilitates virus infection and exacerbates the activation of NF-κB. Blockade of the TRPV4 channel decreases the production of progeny viruses and the activation of NF-κB. Our findings elucidate the previously unknown role of the TRPV4 channel in HSV-2 infection. Further investigations are urgently needed to confirm that the TRPV4 channel is an entry receptor for HSV-2.

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AUTHOR CONTRIBUTIONS

SWL and PZZ conceived the idea and designed the research. PJ, SSL, XFX, CY, CC and JSW performed the research. PJ, SSL, PZZ and SWL analyzed the results and wrote the paper. All authors read and approved the final manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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