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ORIGINAL PAPER

FK506 (tacrolimus) causes pain sensation through the activation of transient receptor potential ankyrin 1 (TRPA1) channels

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Abstract

FK506 (tacrolimus) is an immunosuppressant widely used as an ointment in the treatment of atopic dermatitis. However, local application of FK506 can evoke burning sensations in atopic dermatitis patients, and its mechanisms are unknown. In this study, we found that FK506 activates transient receptor potential ankyrin 1 (TRPA1) channels. In Ca^{2+}-imaging experiments, increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) by FK506 were observed in HEK293T cells expressing hTRPA1 or hTRPM8. FK506-induced currents were observed in HEK293T cells expressing hTRPA1 or mTRPA1, but less or not at all in cells expressing hTRPV1 or hTRPM8 using a patch-clamp technique. FK506 also evoked single-channel opening of hTRPA1 in an inside-out configuration. FK506-induced $[Ca^{2+}]_i$ increases were also observed in TRPA1-expressing mouse primary sensory neurons. Furthermore, injection of FK506 evoked licking or biting behaviors and these behaviors were almost abolished in TRPA1 knockout mice. These results indicate that FK506 might cause pain sensations through TRPA1 activation.

Keywords FK506 · Pain · TRPA1 · Sensory neuron · Adverse effect

Introduction

FK506 (also known as tacrolimus), a macrolide produced by *Streptomyces tsukubaensis*, is a calcineurin inhibitor and is widely used as an immunosuppressant after organ transplantation [1]. FK506 binds to FK506 binding protein 12 (FKBP-12), creating a complex [2, 3]. This complex further binds to calcium, calmodulin, and calcineurin, and inhibits calcineurin activation. This inhibition blocks the production of lymphokines and inflammatory cytokines such as interleukin-2, which is necessary for the proliferation and differentiation of lymphocytes. Calcineurin inhibitors, including FK506 and pimecrolimus, are also used as topical medications for the treatment of atopic dermatitis [4–6]. FK506 is reported to suppress cytokine production in T-cells in a fashion similar to steroids [7, 8], whereas the types of chemokines released from mast cells differ from those modulated by steroids [9]. FK506 ointment is available at concentrations of 0.1% for adults and 0.03% for children. It is frequently indicated for the face and neck where topical steroids are not recommended [10]. On the other hand, irritative symptoms such as transient burning pain or pruritus sensation often appear at the site of application, as adverse effects in atopic dermatitis patients, whereas most of these symptoms disappear with amelioration of the eruption [6, 11]. In particular, the most common local sensation is burning pain and its frequency is much higher than the sensation of itch [12–14]. However, the mechanisms underlying these adverse effects are largely unknown.

Most transient receptor potential (TRP) channels are Ca^{2+}-permeable non-selective cation channels, and they are activated by a wide variety of sensory stimuli, including nociceptive compounds, oxidants, protons, touch and

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changes in osmolarity and temperature [15–17]. TRPV1, TRPV2, TRPV3, TRPV4, TRPM2, TRPM3, TRPM8, and TRPA1 are expressed in sensory neurons and/or skin [18–20] and are involved in the perception of the information about the external and internal environment. TRPA1 (transient receptor potential ankyrin 1) channel is mainly expressed in primary sensory neurons [21] and is involved in perception of painful stimuli. TRPA1 is activated by various stimuli, including alkaline pH [22], Ca^{2+} and Zn^{2+} cations [23, 24], and pungent and/or noxious chemical compounds such as wasabi (AITC), pepper (piperin), garlic (allicin), cinnamon (cinnamaldehyde), Japanese pepper (sanshool), and olive (oleocanthal) [25–30]. Although its responsiveness to noxious cold and mechanical stimuli in mammals is still controversial [31–33], TRPA1 has attracted attention for its potential role in nociception [32, 34, 35]. In addition, TRPA1 is reported to be involved in a histamine-independent itch sensation. Many reports indicated that some drugs and cosmetics that cause irritation and painful sensations, such as parabens [36], cyclooxygenase inhibitors [37], menthol [38], propofol [39], and local anesthesia such as lidocaine [40], activate the TRPA1 channel. Activation of TRPA1 could cause irritation and pain sensations as adverse effects. Therefore, blockade of this channel might be a therapeutic approach in the treatment of pain syndromes.

In this study, to elucidate the mechanisms by which FK506 induces irritant sensations, we analyzed the effect of FK506 on TRP channels that are dominantly expressed in sensory neurons and the skin. We found that FK506 activates both mouse and human TRPA1. In addition, FK506 showed pain-related behaviors rather than itch-related behaviors through TRPA1 activation.

Materials and methods

Cell culture

Human embryonic kidney-derived 293T (HEK293T) cells were cultured in DMEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% FBS (Thermo Fisher Scientific Inc., MA, USA), 2 mM GlutaMAX (Thermo Fisher Scientific Inc.), 100 units/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific Inc.) at 37 °C in 5% CO_2. For calcium imaging, 1 µg of human TRPV1 (hTRPV1), TRPV2 (hTRPV2), TRPV3 (hTRPV3), TRPV4 (hTRPV4), TRPA1 (hTRPA1) or TRPM8 (hTRPM8) in pcDNA3.1 (Thermo Fisher Scientific Inc.), and 0.1 µg of pCMV-DsRed Express (Takara Bio Inc., Shiga, Japan) were transfected into HEK293T cells using Lipofectamine and Plus Reagent (Thermo Fisher Scientific Inc.) in OPTI-MEM (Thermo Fisher Scientific Inc.). For whole-cell patch-clamp recordings, 1 µg of hTRPV1, hTRPA1, hTRPM8, or mouse TRPA1 (mTRPA1) in pcDNA3.1, and 0.1 µg of pGreen Lantern 1 (Thermo Fisher Scientific Inc.) were transfected into HEK293T cells. Mock cells were transfected with 1 µg of empty vector with 0.1 µg of pCMV-DsRed or pGreen Lantern 1. Transfected-HEK293T cells were incubated for 2–3 h at 37 °C in a 5% CO_2 atmosphere. After incubation, cells were reseeded on coverslips in DMEM and further incubated under the same conditions. After an additional day of incubation, transfected-HEK293T cells were used for Ca^{2+}-imaging and whole-cell patch-clamp recording.

Animals

C57BL/6 N mice (males, 5–6 weeks old, Japan SLC Inc., Fukuoka, Japan) were used for extraction of dorsal root ganglion cells and pain behavior assays. TRPA1 knockout (TRPA1KO) mice were generously provided by Prof. D. Julius (UCSF, San Francisco, CA, USA). TRPM8 knockout (TRPM8KO) mice were graciously provided by Prof. A. Patapoutian (Scripps Research Institute, La Jolla, CA, USA). Both were used for behavioral experiments. Animals were housed in a controlled environment (12-h light/dark cycle, room temperature 22–24 °C, 50–60% relative humidity) with free access to food and water. All procedures involving the care and use of animals were approved by The Institutional Animal Care and Use Committee of National Institutes of Natural Sciences, and Animal Research Committee of Fukuoka Dental College, and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication number 85-23, revised 1985).

Primary culture of mouse dorsal root ganglion neurons

Dorsal root ganglia (DRG) were rapidly dissected from adult male wild-type (WT) C57BL/6 N mice and treated with 2.5 mg/ml collagenase (Sigma–Aldrich, MO, USA) in MEM complete for 20 min at 37 °C. MEM complete consisted of Earle's Balanced Salt Solution (Sigma–Aldrich) containing 10% FBS, 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM GlutaMAX and MEM Vitamin Solution (Sigma–Aldrich). After incubation, cells were resuspended and mechanically dissociated in MEM complete. Dissociated cells were centrifuged and plated on coverslips coated by 1 mg/ml poly-D-lysine (Sigma–Aldrich). After incubation for 10–15 h in 5% CO_2 at 37 °C, cells were used for Ca^{2+}-imaging experiment.

Chemicals

FK506, tacrolimus monohydrate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in dimethyl

sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd.) at a concentration of 10 mM. A967079 (Sigma–Aldrich), 2-aminoethoxydiphenylborane (2-APB, Sigma–Aldrich), GSK1016790A (Sigma–Aldrich), ionomycin (Wako Pure Chemical Industries, Ltd.), Fura-2AM (Molecular Probes, Thermo Fisher Scientific Inc.) and Fluo-4AM (Molecular Probes, Thermo Fisher Scientific Inc.) were dissolved in DMSO. Capsaicin (Wako Pure Chemical Industries, Ltd.), menthol (Wako Pure Chemical Industries, Ltd.) and carvacrol (Sigma–Aldrich) were dissolved in ethanol (Wako Pure Chemical Industries, Ltd.). Allyl isothiocyanate (AITC, Wako Pure Chemical Industries, Ltd.) was dissolved in methanol (Wako Pure Chemical Industries, Ltd.). The concentration of all solvents was no more than 0.1% in the bath solution except FK506. 55 μM FK506 bath solution contained 0.55% DMSO, and 30 μM FK506 bath solution contained 0.3% DMSO.

Ca^{2+}-imaging

HEK293T cells and neurons were loaded with 5 μM Fura-2AM or 5 μM Fluo-4AM for more than 40 min. After incubation, coverslips were put in an open chamber (Warner Instruments LLC, CT, USA) and were superfused with a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose and 10 mM HEPES (pH7.4 with NaOH) at room temperature. Images of Fura-2-loaded or Fluo-4-loaded cells were captured every 3 s with a CoolSNAP ES CCD camera (Photometrics, AZ, USA). Intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in HEK293T cells were measured by dual-wavelength Fura-2 microfluorometry with excitation at 340/380 nm and emission at 510 nm. The ratio was calculated and acquired using an imaging processing system (IP-Lab, Scanalytic Inc., VA, USA). For mouse DRG neurons, $[Ca^{2+}]_i$ was measured with Fluo-4 with excitation at 488 nm and emission at 510 nm. Images were acquired using NIS elements software (NIKON Corp., Tokyo, Japan). The viability of HEK293T cells and mouse DRG neurons was confirmed by responses to 5 μM ionomycin. Mouse DRG neurons were identified by the response to 50 mM KCl. For HEK293T cells transfected with each of the TRP channel genes, cells showing an increase in the ratio of more than 0.3 from basal values in response to agonists were defined as TRP channel-expressing cells. The ΔRatio value was normalized to the peak response induced by 5 μM ionomycin. The ratio of the fluorescence intensities of the two wavelengths of Fura-2 and fluorescence intensity of Fluo-4 was analyzed for changes in the fluorescence intensity within regions of interests (ROIs) using ImageJ (National Institutes of Health, Bethesda, MD, USA). Cells showing an increase in the fluorescence intensity (arbitrary units) of more than 30 from basal values in response to FK506 were defined as FK506-sensitive cells. Similarly, cells showing an increase in the fluorescence intensity of more than 50 from basal values in response to AITC or capsaicin were defined as AITC- or capsaicin-positive cells. All experiments were performed at room temperature.

Electrophysiology

For whole-cell patch-clamp recordings, HEK293T cells were transfected with hTRPV1, hTRPA1, hTRPM8, or mTRPA1 on coverslips that were put in a chamber and superfused with standard bath solution (the same as that described for Ca^{2+}-imaging experiments). The pipette solution contained 140 mM KCl, 5 mM EGTA, and 10 mM HEPES (pH 7.4 with KOH). Data were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP10 software, Axon Instruments, Union City, CA, USA). Membrane potential was clamped at − 60 mV and voltage ramp-pulses from − 100 to + 100 mV (300 ms) were applied every 3 s. For inside-out single channel recordings, HEK293T cells transfected with hTRPA1 gene on coverslips were put in a chamber. The bath solution and the pipette solution contained 140 mM KCl, 5 mM EGTA, and 10 mM HEPES (pH 7.4 with KOH). Data were sampled at 10 kHz and filtered at 2 kHz for analysis (Axon 200B amplifier with pCLAMP10 software). The membrane potential was clamped at + 30 mV. All experiments were performed at room temperature.

Pain-related and itch-related behavior tests

WT C57BL/6 N, TRPA1KO and TRPM8KO mice were used in these experiments. These mice were kept individually in transparent cages (15 × 23 × 14 cm) for more than 30 min before experiments. Licking or biting behaviors were observed by using the same method as we performed previously [22, 41]. In detail, 20 μl of 0.3 or 1 mM FK506 (6.7 or 20 nmol injection/site) dissolved in saline containing 20% DMSO or vehicle were injected into the planta of mouse hind-paws, and we immediately counted the time spent licking or biting for 10 min. Itch-related behavior was assessed using the model reported by Kuraishi and his colleagues [42]. In detail, the backs of mice were shaved before itch-related experiments; 50 μl of 400 μM FK506 (20 nmol injection/site) dissolved in saline containing 20% DMSO or vehicle was intradermally injected into the skin on the back. The mice generally scratched themselves several times for about 1 s and a series of such movements was counted as one incidence of scratching. After injection, the number of scratching incidents at the administration site by the hind paw was counted for 15 min.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed by the unpaired two-tailed Student's *t* test for comparison between two groups. For comparison of multiple groups, statistical analysis was performed by the one-way ANOVA followed by the post hoc Bonferroni's test using OriginPro 9 (OriginLab Corp., Northampton, MA, USA). $P < 0.05$ was defined as statistically significant.

Results

FK506 activated human TRPA1 in a heterologous expression system

We used a Ca^{2+}-imaging experiment to determine whether FK506 activated hTRPV1, hTRPV2, hTRPV3, hTRPV4, hTRPA1, and hTRPM8 channels. Application of 30 μM FK506 caused very little $[Ca^{2+}]_i$ increases in HEK293T cells expressing hTRPV1 (Fig. 1b), and large $[Ca^{2+}]_i$ increases in HEK293T cells expressing hTRPA1 or hTRPM8 (Fig. 1f, g). In contrast, $[Ca^{2+}]_i$ increases were not observed in HEK293T cells expressing hTRPV2, hTRPV3, or hTRPV4 (Fig. 1c–e). The summary of ΔRatio values also indicated that the cells expressing hTRPA1 or hTRPM8 responded to 30 μM and 55 μM FK506. The cells expressing hTRPV1 tended to respond to 30 μM FK506 (Fig. 1h).

We next examined the effect of FK506 on hTRPV1, hTRPM8, and hTRPA1 channels by whole-cell patch-clamp recordings. No inward currents (at − 60 mV) were observed in HEK293T cells expressing hTRPV1 or hTRPM8 after treatment with 55 μM FK506 alone. In contrast, in HEK293T cells expressing hTRPM8, we observed small outward currents at positive potentials during pulses ramped from − 100 to + 100 mV every 3 s (Fig. 2). We did not pursue concentrations higher than 55 μM because of the solubility of FK506. On the other hand, application of 55 μM FK506 caused both outward and inward currents in HEK293T cells expressing hTRPA1 (Fig. 3a). A current–voltage (*I–V*) curve from − 100 to + 100 mV in the presence of 55 μM FK506 crossed the origin and showed outward rectification, which is consistent with the *I–V* relationship of TRPA1 [21] (Fig. 3a). The currents evoked by 55 μM FK506 were inhibited by 3 μM A967079, a TRPA1 antagonist (Fig. 3b), whereas the current recovery by FK506 was not observed once the TRPA1 antagonist was applied. This irreversibility was also observed in a Ca^{2+}-imaging experiment using HEK293T cells expressing hTRPA1 (Supplemental Fig. 1). We then analyzed the dose–response profile. As shown in Fig. 3c, d, current densities at − 60 mV (C) and + 100 mV (D) in the presence of 30 μM FK506 were not significantly different between mock-transfected and hTRPA1-transfected HEK293T cells. Application of 55 μM FK506 showed significantly greater current densities at both − 60 mV (C) and + 100 mV (D) in HEK293T cells expressing hTRPA1 compared to those in mock-transfected HEK293T cells, whereas current densities at 100 mV were greater than those at 60 mV (Fig. 3c, d).

In order to examine whether FK506 activates TRPA1 in a membrane-delimited manner, we performed single-channel recordings with an inside-out configuration in HEK293T cells expressing hTRPA1. Figure 3e shows representative current traces of single-channel openings in HEK293T cells expressing hTRPA1. Single-channel currents were observed not only by application of 55 μM FK506, but also by application of 20 μM AITC. On the other hand, the currents were not induced by 55 μM FK506 in mock-transfected HEK293T cells (data not shown). The calculated unitary conductance of FK506-activated current was 104.1 ± 13.4 pS ($n = 4$), and a similar value was obtained for AITC-activated currents (98.8 ± 12.3 pS, $n = 4$). These properties at the single-channel level support the idea that TRPA1 can be activated by FK506 directly or through structures retained even in a small excised membrane patch. We then analyzed the NPo (channel number x open probability) to assess TRPA1 activation by FK506. The NPo during application of 55 μM FK506 was significantly higher than the basal level, and tended to be lower than that of 20 μM AITC (Fig. 3f).

FK506 activated mouse TRPA1 in both heterologous expression systems and primary sensory neurons

We confirmed the effect of FK506 on mTRPA1 by whole-cell patch-clamp recordings. Application of 55 μM FK506 caused both inward and outward currents in HEK293T cells expressing mTRPA1 (Fig. 4a). Current densities at − 60 mV in 55 μM FK506 in HEK293T cells expressing mTRPA1 were significantly larger than those in mock-transfected HEK293T cells (Fig. 4b).

We next examined the effect of FK506 on native mTRPA1 by using mouse DRG neurons. Figure 5a shows representative traces of $[Ca^{2+}]_i$ changes in each mouse DRG neuron. Application of 30 μM FK506 increased $[Ca^{2+}]_i$ in some mouse DRG neurons (Fig. 5a). We then classified the mouse DRG neurons by the response to AITC and capsaicin to determine TRPA1-expressing and TRPV1-expressing neurons. In a total of 186 cells, 24.2% of the neurons responded to 30 μM FK506 (Fig. 5b). In FK506-responding neurons, 42.2% of the cells (19 in 45 neurons) were positive for AITC. Furthermore, 29.7% of the AITC-positive neurons responded to FK506 (19 in 64 neurons). Increases in $[Ca^{2+}]_i$ by 30 μM FK506 were not observed in the absence of extracellular Ca^{2+} (Fig. 5c), indicating that increases in $[Ca^{2+}]_i$ by 30 μM FK506

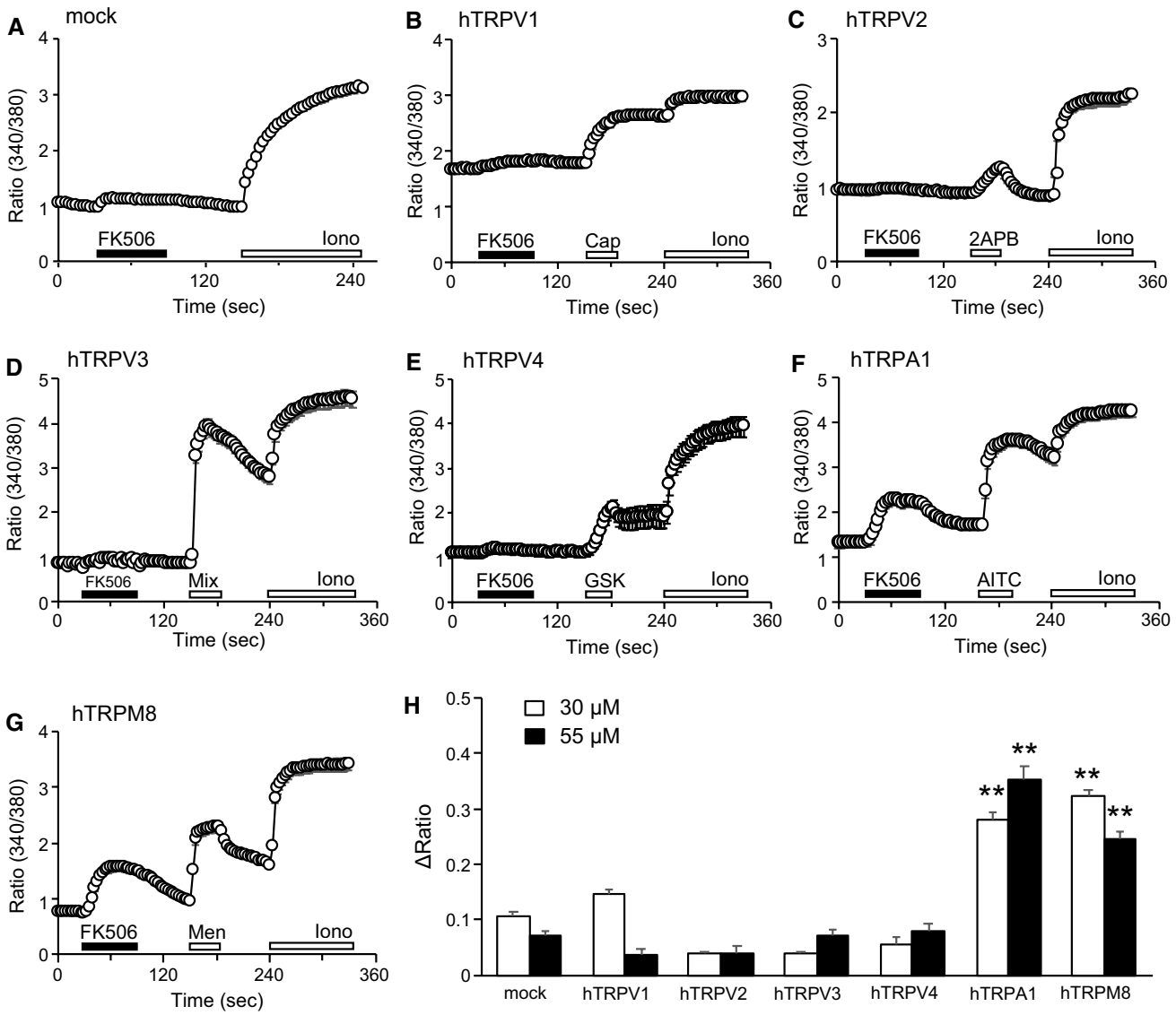

Fig. 1 Increased intracellular Ca^{2+} concentrations were observed after application of FK506 in HEK293T cells expressing human TRPA1 and TRPM8. **a**–**g** Representative changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) stimulated by 30 μM FK506 in mock-transfected HEK293T cells (**a**), or HEK293T cells expressing human TRPV1 (hTRPV1, **b**), TRPV2 (hTRPV2, **c**), TRPV3 (hTRPV3, **d**), TRPV4 (hTRPV4, **e**), TRPA1(hTRPA1, **f**) or TRPM8 (hTRPM8, **g**). To confirm the expression of each TRP channel, 1 μM capsaicin (Cap), 500 μM 2-aminoethyl diphenylborinate (2APB), a mixture of 250 μM 2APB and 500 μM carvacrol (Mix), 300 nM GSK1016790A (GSK), 100 μM allyl isothiocyanate (AITC), or 500 μM menthol (Men) was used. Cell viability was checked by application of 5 μM ionomycin (Iono). Y-axis: Fura-2 ratio (340 nm/380 nm). Each symbol represents mean ± SEM from 33 to 105 cells. **h** Summary of $[Ca^{2+}]_i$ increases following application of 30 μM (white column) or 55 μM (black column) of FK506 to HEK293T cells expressing hTRPV1, hTRPV2, hTRPV3, hTRPV4, hTRPA1 or hTRPM8. Y axis: ΔRatio calculated by the normalization of peak Fura-2 ratio (340 nm/380 nm) of FK506 to that of ionomycin. "Mock" indicates the $[Ca^{2+}]_i$ increases in HEK293T cells transfected with empty vector. Each column represents the mean + SEM from 22 to 184 cells. Statistical significance was assessed using ANOVA followed by a two-tailed multiple *t* test with Bonferroni correction. **, $P < 0.01$ vs. mock

occurred through Ca^{2+} influx from the extracellular space. Increases in $[Ca^{2+}]_i$ by 30 μM FK506 were almost abolished in AITC-positive neurons by application of 3 μM A967079 (Fig. 5d), although inhibition by A967079 was irreversible (also described above).

FK506 causes licking or biting behaviors but not scratching behaviors in mice

To clarify whether FK506 caused pain and itching sensations, we performed in vivo experiments. We observed pain-related behaviors (licking or biting) induced by intraplantar

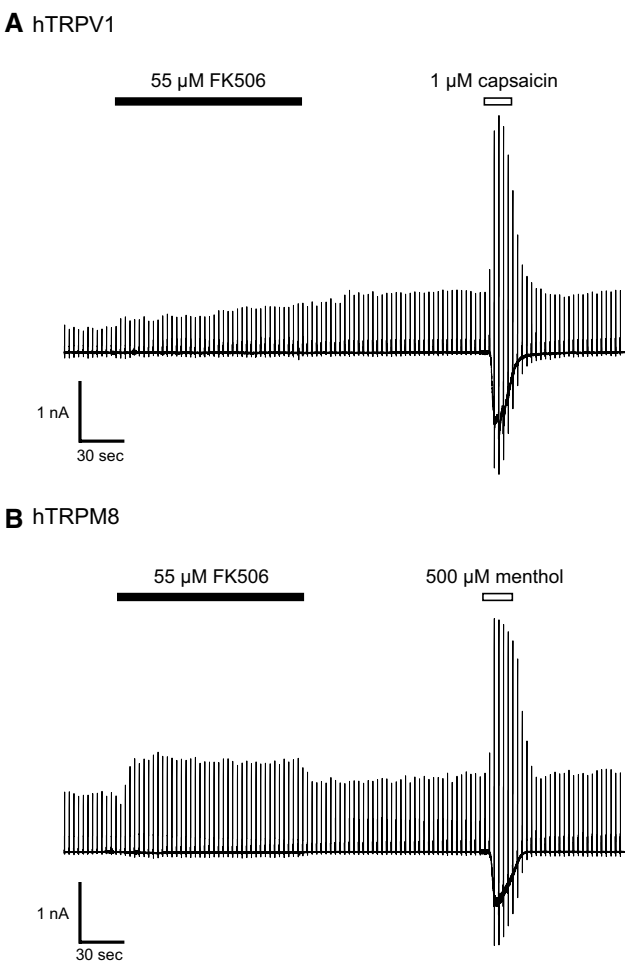

Fig. 2 FK506-induced inward currents were not observed in HEK293T cells expressing human TRPV1 or TRPM8. **a** A representative trace of whole-cell patch-clamp recording in HEK293T cells expressing human TRPV1 (hTRPV1) exposed to 55 μM FK506 (black bar) and 1 μM capsaicin (white bar). Membrane potential was held at −60 mV and ramp-pulses from −100 to +100 mV were applied every 3 s. **b** A representative trace of whole-cell patch-clamp recording in HEK293T cells expressing human TRPM8 (hTRPM8) exposed to 55 μM FK506 (black bar) and 500 μM menthol (white bar). Membrane potential was held at −60 mV and ramp pulses from −100 to +100 mV were applied every 3 s

injection of FK506 in mice. First, we checked the dose-dependency. Although injection of 10 μl of 1 mM FK506 (10 nmol) solution did not cause significant licking or biting behaviors, injection of 20 μl of 1 mM FK506 (20 nmol) solution induced licking or biting behaviors compared to that of 10 μl of 20% DMSO-saline (vehicle) or 10 μl of 1 mM FK506 (10 nmol) (Supplemental Fig. 2). The time spent licking or biting was significantly longer in WT mice injected with 20 μl of 1 mM FK506 (20 nmol) than those injected with 20 μl of vehicle (Fig. 6a and Supplemental Fig. 2). On the other hand, 20 μl of 0.3 mM FK506 (6.7 nmol) did not cause significant licking or biting behaviors in WT mice (Fig. 6a). Unfortunately, high doses of FK506 could not be used because of its low solubility. Licking or biting induced by 20 nmol FK506 was significantly impaired in TRPA1KO mice (Fig. 6b). On the other hand, intraplantar injection of 20 nmol FK506 induced licking or biting behaviors in TRPM8KO mice to the extent similar to WT mice (Fig. 6c), whereas FK506 weakly activated hTRPM8 as shown in the whole-cell patch-clump recording (Fig. 2b), indicating that TRPM8 might not be involved in FK506-induced licking or biting behaviors. We next examined itching-related behaviors induced by intradermal injection of FK506 into the back skin. As shown in Fig. 6d, FK506 did not induce scratching behaviors in WT mice.

Discussion

In this study, we demonstrated that FK506 activates both human and mouse TRPA1. In mouse DRG neurons, some of the neurons including both AITC-sensitive and -insensitive neurons showed $[Ca^{2+}]_i$ increases stimulated by FK506. In addition, FK506-induced licking and biting behaviors were impaired in TRPA1KO mice, suggesting that FK506 causes pain sensations through TRPA1 activation.

Within TRP channels, which are expressed in sensory neurons and skin keratinocytes, FK506 induced $[Ca^{2+}]_i$ increases in HEK293T cells expressing hTRPA1 or hTRPM8 (Fig. 1). On the other hand, in whole-cell patch-clamp recordings, FK506-induced currents were observed in HEK293T cells expressing hTRPA1, indicating that hTRPA1 can be activated by FK506 (Fig. 3a–d). In HEK293T cells expressing hTRPM8, application of FK506 exhibited only an outward current at +100 mV and no inward currents at −60 mV, indicating that its activity on TRPM8 is weak (Fig. 2b). Although some reports suggested the involvement of TRPV1 in the actions of FK506 [43, 44], FK506-induced TRPV1 currents were not observed in our experiments (Fig. 2a). Since one report showed that FK506 enhanced substance P release through phosphorylation of TRPV1 in primary sensory neurons [45], FK506 could modulate TRPV1 activity but not directly activate it. Thus, our data demonstrate that FK506 acts on TRPA1 more effectively than on other TRP channels. It is well known that the chemical sensitivities of TRPA1 differ between species. For example, caffeine activates mTRPA1 and inhibits hTRPA1 [46], and menthol activates hTRPA1 and inhibits mTRPA1 at high concentrations [38]. In this study, we observed that FK506 activates not only hTRPA1 but also mTRPA1 (Figs. 3, 4), suggesting that the effects of FK506 on TRPA1 are shared in between human and mouse.

We observed that FK506 caused $[Ca^{2+}]_i$ increases in 24.2% of mouse primary sensory neurons, and that 42.2% of FK506-positive neurons were sensitive to AITC (Fig. 5a,

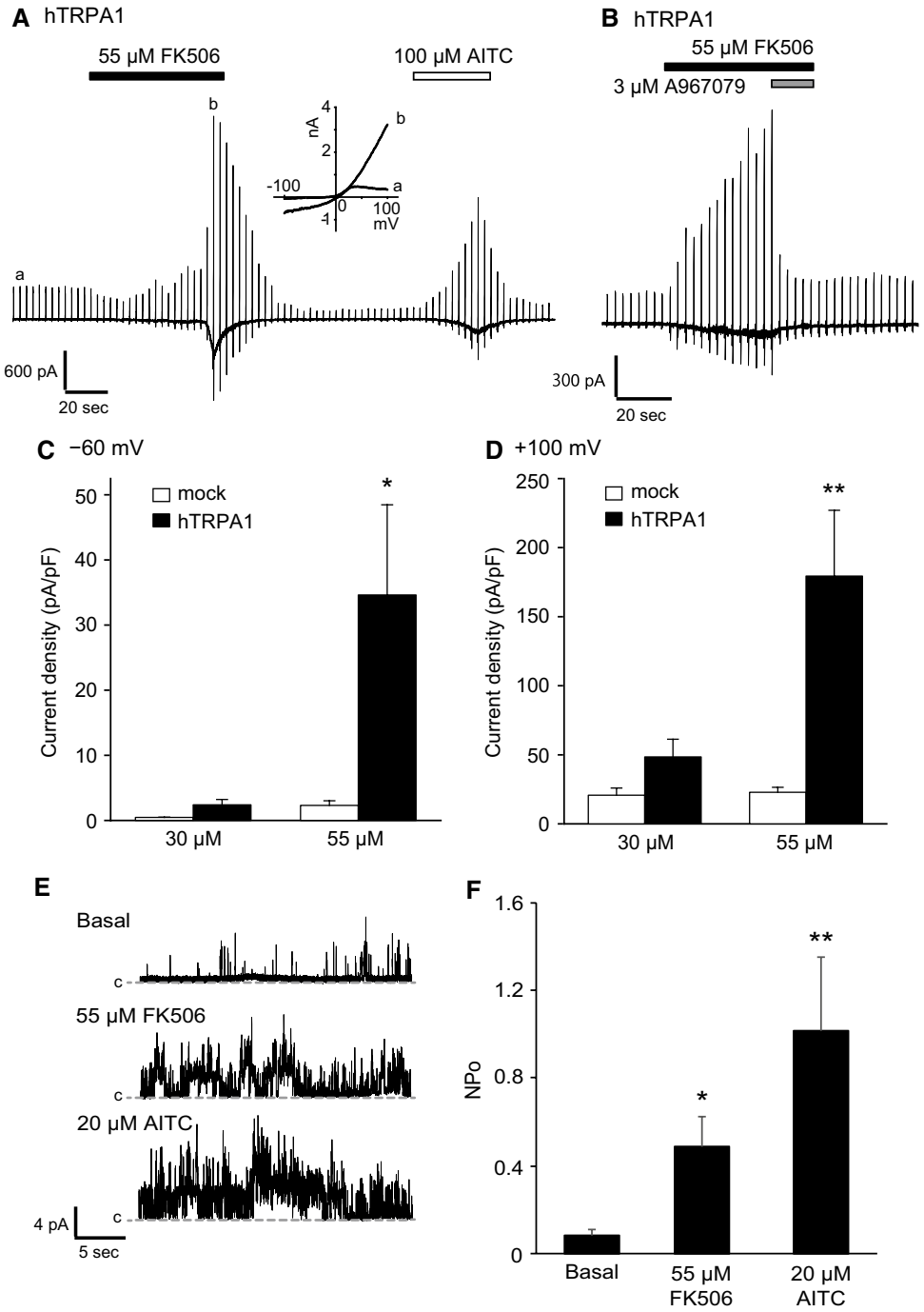

Fig. 3 FK506 activates human TRPA1. **a** A representative trace of a whole-cell patch-clamp recording in a HEK293T cell expressing human TRPA1 (hTRPA1) exposed to 55 μM FK506 (black bar) and 100 μM AITC (white bar). Membrane potential was held at −60 mV and ramp pulses from −100 to +100 mV were applied every 3 s. Insets indicate the representative *I–V* curves of the hTRPA1 currents in the absence (**a**) or presence (**b**) of 55 μM FK506. Allyl isothiocyanate (AITC) was used as a positive control for TRPA1. **b** A TRPA1 antagonist (3 μM A967079) inhibited a 55 μM FK506-induced current in a HEK293T cell expressing hTRPA1. **c**, **d** Summary of the peak currents at −60 mV (**c**) or +100 mV (**d**) induced by 30 μM or 55 μM FK506 in hTRPA1- or mock-transfected HEK293T cells. Each column represents the mean + SEM from 8–9 cells. Statistical significance was assessed using ANOVA followed by a two-tailed multiple *t* test with Bonferroni correction. *, $P < 0.05$; **, $P < 0.01$ vs. mock. **e** Representative traces of inside-out single channel recordings in HEK293T cells expressing hTRPA1 in the absence (basal) or presence of 55 μM FK506, or 20 μM AITC. Dotted lines indicate 0 current levels (**c**). Membrane potential was held at +30 mV. **f** NPo values were calculated in the single-channel currents. Ordinates: NPo (channel number × open probability). Each column represents the mean + SEM from four experiments. Statistical significance was assessed using ANOVA followed by a two-tailed multiple *t* test with Bonferroni correction. *; $P < 0.05$, **; $P < 0.01$ vs. basal

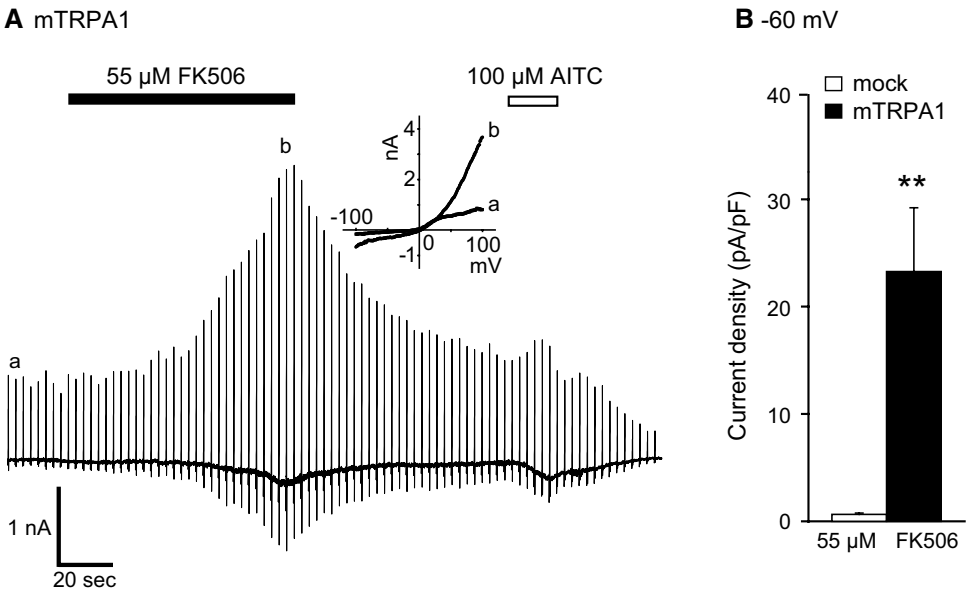

Fig. 4 FK506 activates mouse TRPA1. **a** A representative trace of whole-cell patch-clamp recording in a HEK293T cell expressing mouse TRPA1 (mTRPA1) exposed to 55 μM FK506 (black bar) and 100 μM AITC (white bar). Membrane potential was held at −60 mV and ramp pulses from −100 to +100 mV were applied every 3 s. Insets indicate the representative *I–V* curves of the mTRPA1 currents in the absence (**a**) or presence (**b**) of 55 μM FK506. **b** Summary of the peak currents at −60 mV induced by 55 μM FK506 in mTRPA1- or mock-transfected HEK293T cells. Each column represents the mean + SEM from 8 to 9 cells. Statistical significance was assessed using Student's *t* test. **; $P < 0.01$ vs. mock

b). In contrast to the results from HEK293T cells expressing mTRPA1, not all AITC-sensitive neurons responded to FK506 (Fig. 5a, b). One possibility is differences in the expression levels of the TRPA1 channel. Alternatively, the modulation levels of TRPA1 (e.g., hydroxylation by prolyl hydroxylases [47]) could be different among neurons. Another possibility is that other molecules expressed in some TRPA1-expressing neurons are necessary for activation of TRPA1-expressing primary sensory neurons, since FKBP is reported to interact with some TRP channels [48]. In this study, we found that application of a TRPA1 antagonist inhibited $[Ca^{2+}]_i$ increases by FK506 in AITC-sensitive neurons (Fig. 5d). Although we cannot discount the possibility of involvement of molecules that interact with FK506 in TRPA1-expressing sensory neurons, FK506 could directly activate native TRPA1 in mouse sensory neurons, which is consistent with the results from inside-out single-channel recordings (Fig. 3e, f). In contrast, FK506 increased $[Ca^{2+}]_i$ in AITC-insensitive primary sensory neurons (57.8% of FK506-sensitive neurons, Fig. 5a, b), indicating the existence of other target molecules of FK506. TRPM8, which is not colocalized with TRPA1 in peripheral neurons [21, 49], is a candidate because we found that FK506 increased $[Ca^{2+}]_i$ in HEK293T cells expressing TRPM8 (Fig. 1). TRPC channels are also candidates for causing $[Ca^{2+}]_i$ influx upon FK506 application based upon the observations that FKBP12 interacts with TRPC3, TRPC6, or TRPC7, and FKBP52 associates with TRPC1, TRPC4, or TRPC5 [48]. Moreover, TRPC1, TRPC3, TRPC5, and TRPC6 were reported to be expressed in sensory neurons [50, 51]. Furthermore, TRP channels as well as other channels could underlie $[Ca^{2+}]_i$ changes induced by FK506, since some reports indicated that calcineurin inhibitors might modulate $[Ca^{2+}]_i$ changes through the interaction with voltage-gated calcium channels, ryanodine receptors and IP_3 receptors (50, 51, 52, 53, 54). In this study, we found that FK506-induced $[Ca^{2+}]_i$ increases were completely abolished when there was no extracellular Ca^{2+} (Fig. 5c), indicating that $[Ca^{2+}]_i$ increases are due to Ca^{2+} influx from extracellular spaces, but not through Ca^{2+} release from intracellular Ca^{2+} stores. Thus, it is unlikely that ryanodine and IP_3 receptors are involved in $[Ca^{2+}]_i$ increases during FK506 treatment of mouse sensory neurons. Taking these observations into consideration, it is possible that some of the neurons expressing TRPM8, TRPC3, TRPC5, or TRPC6 respond to FK506, or, alternatively, that other ion channels could be involved in FK506-induced $[Ca^{2+}]_i$ increases. Additional experiments will be necessary to clarify this point.

Many reports have demonstrated that FK506 induces chronic pain in humans, and its interacting proteins (FKBP and calcineurin) could be involved in chronic pain. Chronic pain was observed in transplant patients treated with calcineurin inhibitors [52, 53]. Knockdown of FKBP51 reduced pro-inflammatory cytokines (TNF-α,

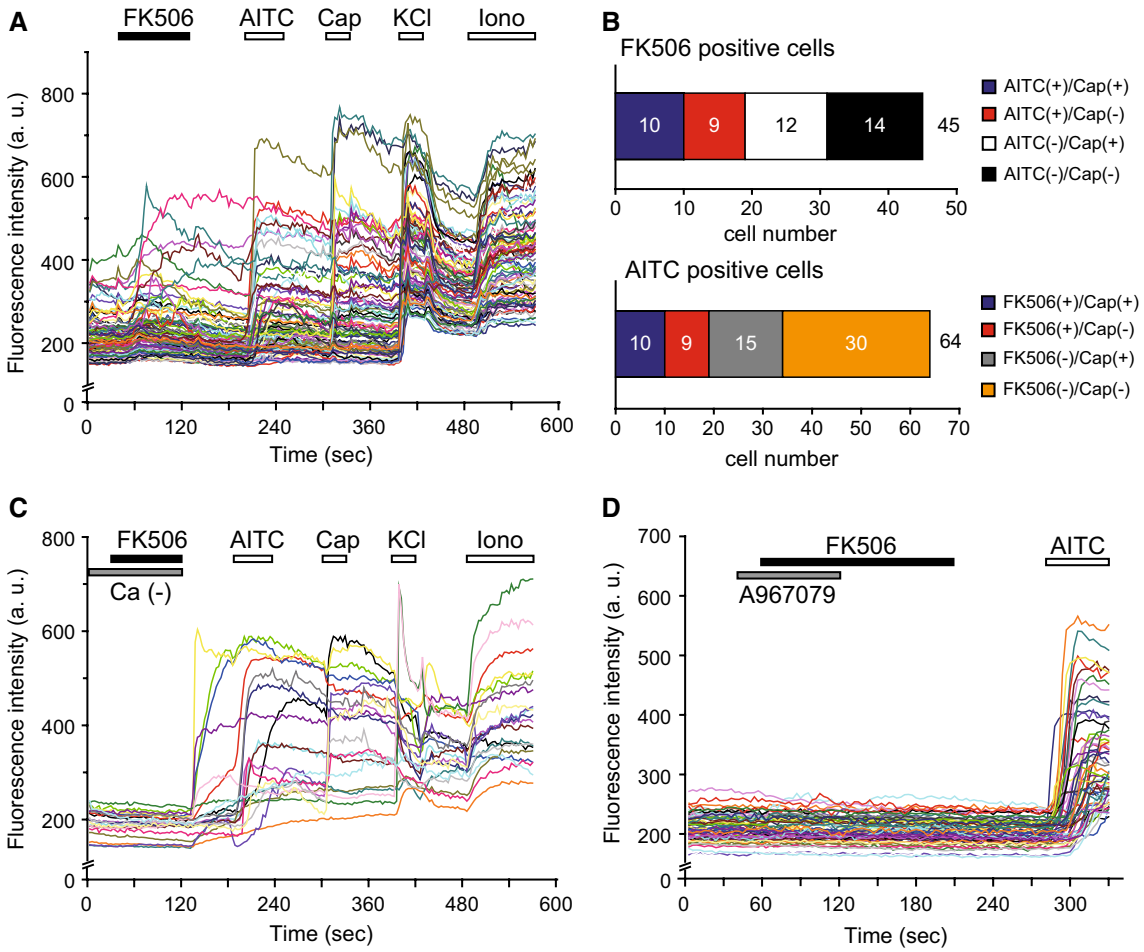

Fig. 5 Increases in intracellular Ca^{2+} concentration induced by FK506 in mouse dorsal root ganglion neurons. **a** Representative traces of changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) induced by 30 μM FK506 in mouse dorsal root ganglion (DRG) neurons. Mouse DRG neurons were categorized by 100 μM AITC and 1 μM capsaicin (Cap). Neurons were determined by 50 mM KCl and cell viability was confirmed by 5 μM ionomycin (Iono). Y-axis: fluorescence intensity of Fluo-4. **b** Classification of mouse DRG neurons depending on the response to 30 μM FK506 and 100 μM AITC. A total of 186 neurons were obtained from three mice. The X-axis indicates the number of cells. **c** $[Ca^{2+}]_i$ increases induced by 30 μM FK506 were almost abolished in the absence of extracellular Ca^{2+} (Ca (−)). **d** $[Ca^{2+}]_i$ increases induced by 30 μM FK506 were inhibited by 3 μM A967079, a TRPA1 antagonist, in the mouse DRG neurons responded to 100 μM AITC

IL-1β, IL-6) and NGF in DRG in a chronic constriction injury (CCI) model of rats [54], but it did not affect acute pain [55]. Inhibition of calcineurin and NFAT2 by A kinase anchor protein 150 (AKAP150) downregulated IL-4 in DRG during paclitaxel-induced neuropathic pain, but AKAP150 was not involved in acute pain in rodents [56]. In this study, we found that local injection of FK506 induced acute licking or biting behaviors and these behaviors were impaired in TRPA1KO mice (Fig. 6b). Furthermore, FK506 increased $[Ca^{2+}]_i$ in TRPA1-expressing primary sensory neurons (Fig. 5a, b). Taken together, we conclude that FK506-induced licking or biting behaviors are caused by the activation of TRPA1 expressed in primary sensory neurons and that the mechanism of acute irritation is different from that of chronic pain.

Some reports demonstrated the involvement of TRP channels in FK506-related sensations of pain and itch. FK506-induced pancreatitis-related pain was attenuated by an antagonist of TRPV1, but not by voltage-gated Ca^{2+} channels in mice [44]. FK506 enhanced TRPA1 expression in a murine model of oxazolone-induced chronic hypersensitivity and FK506-induced scratching behaviors were reduced by a TRPA1 antagonist [57]. In this study, we found that local injection of FK506 induced acute licking or biting behaviors through TRPA1 activation (Fig. 6a, b). Licking and biting behaviors are usually interpreted as chemical-induced acute pain-related behaviors. However, some reports showed that biting behaviors are rather related to itch sensation [58, 59]. In patients with atopic dermatitis, it is known that FK506 ointment causes

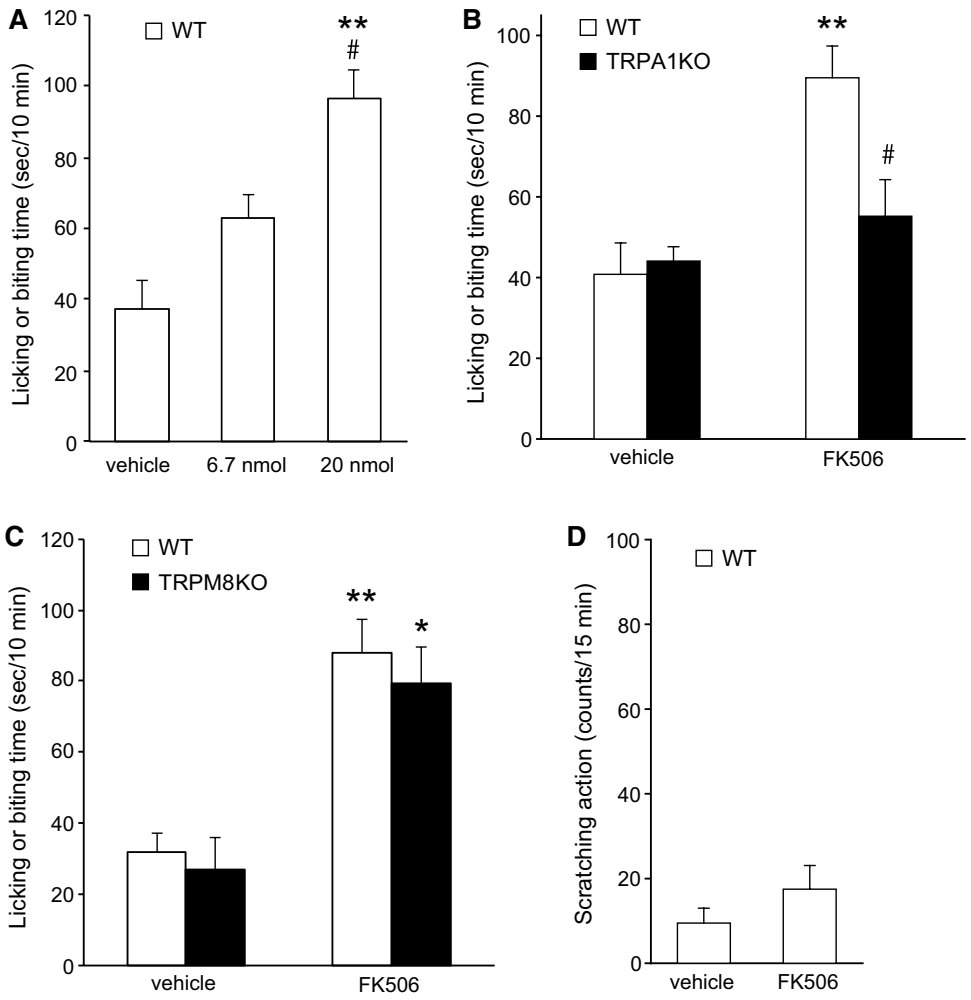

Fig. 6 Pain-related and itch-related behaviors induced by FK506 in mice. **a** Time spent by wild-type (WT) mice licking or biting in the 10 min following intraplantar injection of vehicle, 6.7 nmol FK506 or 20 nmol FK506. Each column represents the mean + SEM from 7 to 9 mice. Statistical analysis was performed using ANOVA followed by a two-tailed multiple *t* test with Bonferroni correction. **, $P < 0.01$ vs. vehicle; #, $P < 0.05$ vs. 6.7 nmol. **b** Time spent by WT or TRPA1 knockout (TRPA1KO) mice licking or biting in the 10 min following intraplantar injection of vehicle or 20 nmol FK506. Each column represents the mean + SEM from 8 or 9 mice. Statistical analysis was performed using ANOVA followed by a two-tailed multiple *t* test with Bonferroni correction. **, $P < 0.01$ vs. vehicle; #, $P < 0.01$ vs. WT. **c** Time spent by WT or TRPM8 knockout (TRPM8KO) mice licking or biting in the 10 min following intraplantar injection of vehicle or 20 nmol FK506. Each column represents the mean + SEM from 6 to 9 mice. Statistical analysis was performed using ANOVA followed by a two-tailed multiple *t* test with Bonferroni correction. *, $P < 0.05$, **, $P < 0.01$ vs. vehicle. **d** The number of incidents of scratching following intradermal injection of vehicle or 20 nmol FK506 into the back skin over a 15-min period. Each column represents the mean + SEM from 6 mice

transient burning pain or itch sensations at the site of application [12, 60]. The most common expression of local irritative symptoms is a burning sensation [12–14], and pruritus seemed to be related to burning sensations [12]. While it is difficult to distinguish between pain and itch sensation in mouse models, our data suggest that FK506 causes pain rather than itching sensations. Our finding that scratching behaviors were not induced by FK506 could support this idea (Fig. 6d). Thus, skin irritation by FK506 ointment could be caused by TRPA1 activation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Statement on the welfare of animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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