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TRPV4-Mediated Anti-nociceptive Effect of Suberanilohydroxamic Acid on Mechanical Pain

Geunyeol Choi[1] · Tae-Jin Yang[1] · Sungjae Yoo[1] · Seung-In Choi[1] · Ji Yeon Lim[1] · Pyung Sun Cho[1] · Sun Wook Hwang[1,2]

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Abstract

Biological effects of suberanilohydroxamic acid (SAHA) have mainly been observed in the context of tumor suppression via epigenetic mechanisms, but other potential outcomes from its use have also been proposed in different fields such as pain modulation. Here, we tried to understand whether SAHA modulates specific pain modalities by a non-epigenetic unknown mechanism. From 24 h Complete Freund's Adjuvant (CFA)-inflamed hind paws of mice, mechanical and thermal inflammatory pain indices were collected with or without immediate intraplantar injection of SAHA. To examine the action of SAHA on sensory receptor-specific pain, transient receptor potential (TRP) ion channel-mediated pain indices were collected in the same manner of intraplantar treatment. Activities of primarily cultured sensory neurons and heterologous cells transfected with TRP channels were monitored to determine the molecular mechanism underlying the pain-modulating effect of SAHA. As a result, immediate and localized pretreatment with SAHA, avoiding an epigenetic intervention, acutely attenuated mechanical inflammatory pain and receptor-specific pain evoked by injection of a TRP channel agonist in animal models. We show that a component of the mechanisms involves TRPV4 inhibition based on in vitro intracellular Ca^{2+} imaging and electrophysiological assessments with heterologous expression systems and cultured sensory neurons. Taken together, the present study provides evidence of a novel off-target action and its mechanism of SAHA in its modality-specific anti-nociceptive effect and suggests the utility of this compound for pharmacological modulation of pain.

Keywords SAHA · Pain · TRPV4 · Non-epigenetic mechanism

Introduction

Suberanilohydroxamic acid (SAHA, also known as vorinostat) is the first histone deacetylase (HDAC) inhibitor approved by the US Food and Drug Administration [1, 2]. It directly binds to and inhibits HDACs, whose main function is to promote transcription of genes by releasing part of the histone protein and allowing transcription factors to access DNA regions [3, 4]. Promotion of this transcriptional process appears to be associated with favorable prognosis through elevating of tumor suppressor gene expression in cancer [3, 4]. Currently, non-epigenetic effects have been suggested because deacetylation also occurs in cytosolic proteins [5]. Other histone-unrelated effects might also exist, but few have been demonstrated.

Differential effects of SAHA in pain modulation have been reported. Repeated systemic injection of SAHA attenuated pain levels in a formalin-induced inflammation model but exacerbated those in an incision model [6, 7]. In a neuropathic pain model, SAHA improved the excitability of nociceptive C-fiber by normalizing the expression level of Nav1.8 sodium channels and sensory neuronal TRP channels [8]. Despite such diffuse outcomes from its treatment, all of these studies have suggested transcriptional modulation of neuronal genes in the primary nociceptors and/or the spinal cord such as metabotropic glutamate receptor type 2, chemokine CC motif receptor 2, Nav1.8, or TRP channels as the underlying mechanisms. We hypothesized that a factor affecting such

Geunyeol Choi and Tae-Jin Yang contributed equally to this work.

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✉ Sun Wook Hwang
sunhwang@korea.ac.kr

[1] Department of Biomedical Sciences, Korea University College of Medicine, Seoul 02841, South Korea

[2] Department of Physiology, Korea University College of Medicine, Seoul 02841, South Korea

differential effects of SAHA on pain is unrelated to its known inhibitory effect on histone or other protein acetylation. In particular, different from previous studies based on systemic treatments, we focused on localized administration and immediate observation of receptor-specific phenotypes to look for a non-epigenetic molecular mechanism.

Materials and Methods

Nociceptive Behavioral Studies

The study was approved by the Committee on the Ethics of Laboratory Animal Experiments of Korea University. Six-week-old male ICR mice were used. For CFA-induced inflammation, 50 μl CFA was injected into a hind paw 24 h before SAHA injection. For 4-α-phorbol-12,13-didecanoate (4αPDD)-induced hypersensitivity, 4αPDD in 10 μl vehicle was injected into a hind paw 1 h before SAHA injection [9]. Assays for changes in mechanical or thermal behaviors were performed as described previously [10–12]. Briefly, animals were acclimated to the test environment for 1 h before performing assay experiments. The assays were started immediately after SAHA injection. For quantifying the extent of thermal hyperalgesia, we collected and averaged the withdrawal latency of hind paws to radiant heat stimulation by using Hargreaves apparatus (Plantar Analgesia meter, UGO Basile, Italy). For evaluating mechanical hyperalgesia, we measured the mechanical threshold for hind paw flexion reflex by conducting Randall-Selitto test (Analgesy-meter, UGO Basile). For assaying mechanical allodynia, we measured the hind paw withdrawal threshold by conducting von-Frey test (Dynamic Plantar Aesthesiometer, UGO Basile).

Time engaged in hind paw licking and flicking behavior was quantitated for ~ 20 min as previously described [13, 14]. For conventional TRPV4-mediated flinch assays, we used intraplantar injection of 10 μl deionized water for hypotonic stimulation 30 min after prostaglandin E2 (PGE2) priming (intraplantar pretreatment with 10 μl saline containing 100 ng PGE2) [14]. TRPV4-mediated nociceptive behaviors were observed by counting the number of hind paw flinching events for 10 min immediately after the hypotonic stimulus. Dimethylallyl diphosphate (DMAPP)-induced flinch assays were carried out as described previously [12]. Briefly, DMAPP in 10 μl vehicle was used instead of deionized water. Drugs were injected in 10 μl vehicle (phosphate-buffered saline containing 0.5% Tween 80) into hind paws intraplantarly at the doses detailed in the "Results" section.

Cell Cultures

HEK293T cells were maintained as previously described [12, 14]. Cells were transiently transfected with 0.55 μg of individual TRP channel plasmid DNA (mTRPA1, rTRPV1, or mTRPV4 in pcDNA3.1; hTRPV3 or mTRPM8 in PCDNA5/FRT) using Fugene HD (Roche Diagnostics Corp., Indianapolis, IN). Primary cultures of ICR mouse dorsal root ganglion (DRG) neurons were prepared as described previously [12, 14]. All cells were grown at 37 °C and 5% CO_2.

Ca^{2+} Imaging Experiments

Ca^{2+} imaging experiments were carried out as previously described [15]. Briefly, cells were loaded with 5 μM Fura-2AM dye and 0.02% pluronic F127 for 30 min. The cells were resuspended in (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES (titrated to pH 7.4 with NaOH). Images of dye-loaded cells were recorded with a cooled CCD camera (Retiga-SRV, Q-imaging Corp., Burnaby, BC, Canada). The ratio of fluorescence intensity at 340 nm/380 nm wavelengths in each experiment was analyzed using MetaFluor (Molecular Devices, Sunnyvale, CA).

Patch-Clamp Electrophysiology

Whole-cell voltage clamp recordings were performed using the same bath solution as in the Ca^{2+} imaging experiments. The pipette solution contained (in mM) 140 CsCl, 5 EGTA, 10 HEPES, 2 MgATP, and 0.2 NaGTP (titrated to pH 7.2 with CsOH). The holding potential was − 60 mV. For the current-voltage analysis, 800 ms voltage-ramp pulses from − 80 to + 80 mV were used.

Compounds

All chemicals were purchased from Sigma-Aldrich unless otherwise described. DMAPP was purchased from Echelon Research Laboratories (Salt Lake City, UT). Cinnamaldehyde was purchased from MP Biomedicals (Solon, OH). Stock solutions were prepared using water or ethanol and diluted with test solutions before use.

Statistical Analysis

Data were analyzed using the two-tailed Student's *t* test (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$) and shown as means ± S.E.M. For comparison of the accumulated licking/lifting time, one-way analysis of variance with Bonferroni post hoc test was performed.

Results

SAHA Suppresses Inflammatory Mechanical Pain

We asked whether SAHA modulates pathologic pain in an acetylation-unrelated manner. Local intraplantar injection can situate the compound only near the peripheral sensory terminals but does not deliver it to ganglionic cell bodies where histone modification occurs. In addition, immediate observation can exclude effects potentially mediated via intracellular signal transductions including deacetylase inhibition. From 24 h CFA-inflamed hind paws of mice, we collected mechanical and thermal inflammatory pain indices with or without immediate and local SAHA injection. When Randall-Selitto test was carried out to examine pressure-type mechanical pain, hind paw withdrawal latency was shortened in mice with inflammation, indicating development of mechanical inflammatory pain (Fig. 1a). The heightened sensitivity was significantly reversed by 1-min treatment with SAHA compared with mice treated with vehicle (Fig. 1a). The von-Frey threshold was also decreased in mice with CFA-induced inflammation, and the same SAHA treatment significantly blunted this hypersensitivity (Fig. 1b). Hypersensitivity in terms of thermal pain also occurred in the CFA-inflammation group, but intraplantarly, SAHA injection failed to alter the reduced heat threshold (Fig. 1c). Therefore, localized and immediate treatment of SAHA was specifically effective in alleviation of mechanical pain under inflammatory conditions.

SAHA Suppresses TRPV4-Mediated Nociception

As such modality-specific analgesic effects might be associated with a receptor-dependent mechanism, we examined the action of SAHA on sensory agonist-evoked acute pain. TRPV4 plays sensory roles as mechanical pain transducers [16–19]. Specific activation of TRP channels by intraplantar agonist injection is known to evoke immediate pain behaviors, and we reproduced this paradigm [12, 19–22]. When vehicle or SAHA alone was intraplantarly injected into a hind paw, no significant change in behavior was observed in naïve mice (data not shown; for SAHA, Fig. 2). To examine TRPV4-mediated pain, we used two in vivo test models: the hypotonicity-induced hind paw-flinching test and the DMAPP-induced flinching test [12, 19]. Intraplantar injection of deionized water in PGE2-primed mice (Fig. 2a, b) or that of DMAPP (Fig. 2c, d) immediately induced flinching behaviors that lasted ~ 5 min. These acute nociceptive responses to TRPV4 stimulation were significantly reduced after intraplantar pretreatment with SAHA, suggesting that SAHA is effective for alleviating the TRPV4-mediated pain. However, other agonist-specific nociceptive behaviors such as TRPV1-mediated licking/flicking induced by capsaicin, TRPA1-mediated licking/flicking induced by cinnamaldehyde, and depolarization-mediated licking/flicking induced by high doses of KCl-evoked depolarization were unaltered by SAHA treatment (Fig. 3a–f). TRPV1-mediated licking/flicking responses induced by capsaicin were enhanced by PGE2 priming, but unlike TRPV4-mediated responses, those were not significantly suppressed by SAHA treatment, indicating that SAHA may not affect PGE2-induced signaling (Supplementary Fig. 1a, b). The data from the acute pain response-based examination suggest that SAHA attenuates behavioral nociception in a receptor-associated manner.

We further examined TRPV4 relevance in the action of SAHA. In previous studies, priming by intraplantar TRPV4 agonist injection increased peripheral sensitivity, resulting in decreases in Randall-Selitto and von-Frey mechanical thresholds [9, 12]. In our study, these heightened mechanical sensitivities were reproduced using 4αPDD

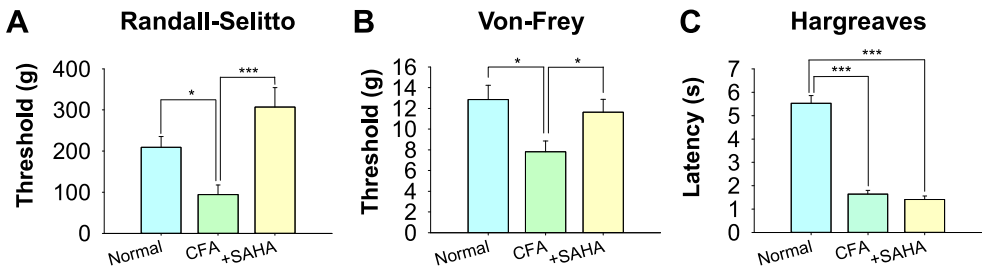

Fig. 1 SAHA reverses lowered mechanical thresholds but not heat thresholds in CFA-inflamed animals. **a** Summary of changes in the hind paw withdrawal thresholds from Randall-Selitto tests following SAHA treatment. The Randall-Selitto threshold was decreased by CFA inflammation ($n = 5$) to 45% compared with the control threshold ($n = 5$). Immediately after intraplantar administration of SAHA in the hind paw (100 μM, $n = 5$), the threshold decreases were reversed (147% compared to the control, $n = 5$). **b** Summary of changes in the mechanical thresholds from von-Frey tests by SAHA treatment. The von-Frey threshold was decreased by CFA inflammation ($n = 5$) to 61% compared with the control threshold ($n = 5$). Immediately after hind paw intraplantar administration of SAHA (100 μM), the threshold decreases were reversed (91% compared to the control, $n = 5$). **c** Summary of changes in paw withdrawal latencies induced by SAHA treatment from Hargreaves tests. The Hargreaves latency was decreased by CFA inflammation ($n = 5$) to 30% compared with control ($n = 5$). No significant change in the latency was observed upon intraplantar SAHA administration (100 μM, $n = 5$)

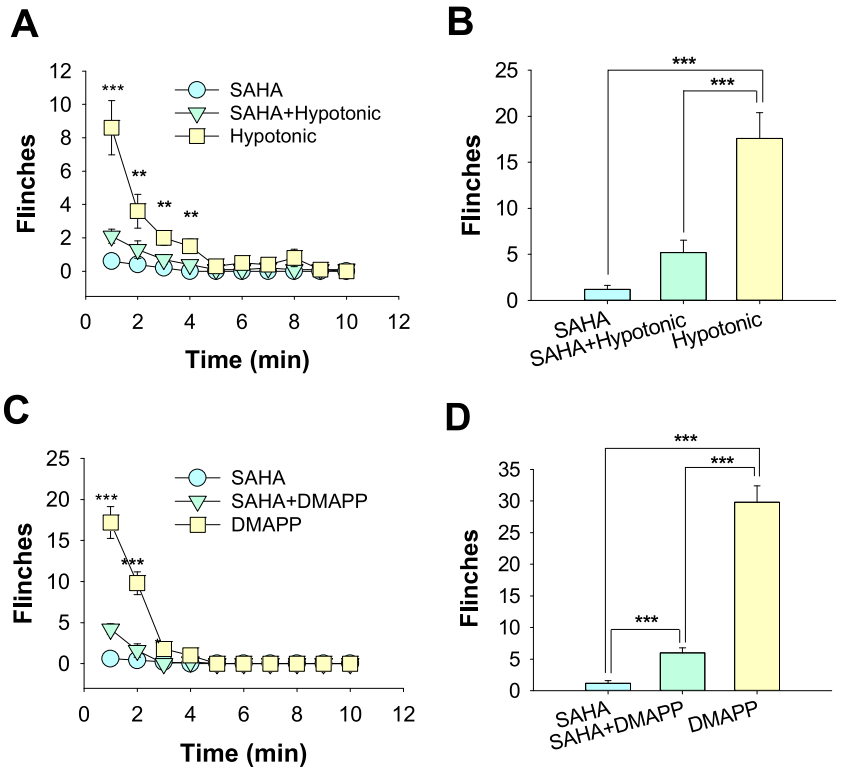

Fig. 2 SAHA suppresses TRPV4-mediated acute behavioral nociception. **a** Summary of the time course of flinching behaviors in mice injected intraplantarly with 10 μl deionized water over a 10-min period immediately after the injection. The hind paws were primed with 100 ng PGE2 5 min before injection of the deionized water, and the mice showed flinching behaviors in response to injection of deionized water ($n = 10$). Average flinching data from mice pretreated with SAHA (100 μM in 10 μl, 1 min before deionized water injection) are displayed using gray circles ($n = 10$). Mice with a hind paw treated with vehicle alone showed no flinch or lick/flicks in either injected or non-injected hind paws ($n = 10$, data not shown). **b** Summary histograms of the accumulated flinches in **a**. **c** Summary of the time course of flinching behaviors in mice injected intraplantarly with 10 μl of 3 mM DMAPP over a 10-min period immediately after the injection ($n = 5$). The hind paws were primed with 100 ng PGE2 5 min before the injection of DMAPP. Average flinching data from the mice pretreated with SAHA (100 μM in 10 μl, 1 min prior to deionized water injection) are displayed using gray circles ($n = 5$). Mice with a hind paw treated with SAHA alone showed no flinch or lick/flicks for either injected or non-injected hind paws ($n = 5$). **d** Summary histograms of the accumulating flinches in **c**

(Supplementary Fig. 2a, b). Heightened sensitivities in both Randall-Selitto and von-Frey tests were improved by SAHA treatment although the improvement was statistically marginal as to the shift of von-Frey threshold ($p = 0.07$), indicating that TRPV4 is inhibited by SAHA in vivo (Supplementary Fig. 2a, b). Interestingly, this local 4αPDD pretreatment also evoked a decrease in heat threshold in the Hargreaves assay (Supplementary Fig. 2c). SAHA failed to reverse this heat threshold decrease, suggesting that, as shown in the "Results" section from CFA inflammation, behavioral outcomes from SAHA injection are more readily associated with the contribution of TRPV4 mechanosensitivity.

SAHA Inhibits TRPV4 In Vitro

SAHA shares structural similarity of the chemical backbone with vanilloid agonists such as capsaicin which is a canonical binding ligand for TRPV1 (Fig. 4a). Accordingly, we hypothesized that SAHA exerts such anti-nociceptive effects through direct actions on TRPV4 and therefore examined the effects of SAHA and structurally related compounds on TRP channel activities in vitro. Compared with SAHA, m-carboxycinnamic acid bishydroxamide (CBHA) has a shorter carbon chain and an additional hydroxamide on the aromatic ring at the meta-position (Fig. 4a). Fura-2-based intracellular Ca^{2+} imaging with TRP channel-transfected heterologous cells was conducted to determine whether the Ca^{2+} influx via TRP channel openings was affected by CBHA and revealed less selective actions: CBHA suppressed TRPV4-mediated influx but activated TRPA1 and TRPV1 (Fig. 4b, c). Suberohydroxamic acid (SBHA) is composed of two hydroxamides linked by a simple carbon chain without an aromatic ring component. This compound was inert to the sensory TRP-mediated influx both in terms of activation and inhibition (Fig. 4b, c).

In vitro outcomes from SAHA treatment were more similar to those obtained with SBHA than with CBHA. SAHA exposure did not change the resting intracellular Ca^{2+} level. SAHA largely failed to significantly alter the elevation of Ca^{2+} level evoked by agonist-induced sensory TRP channel activations

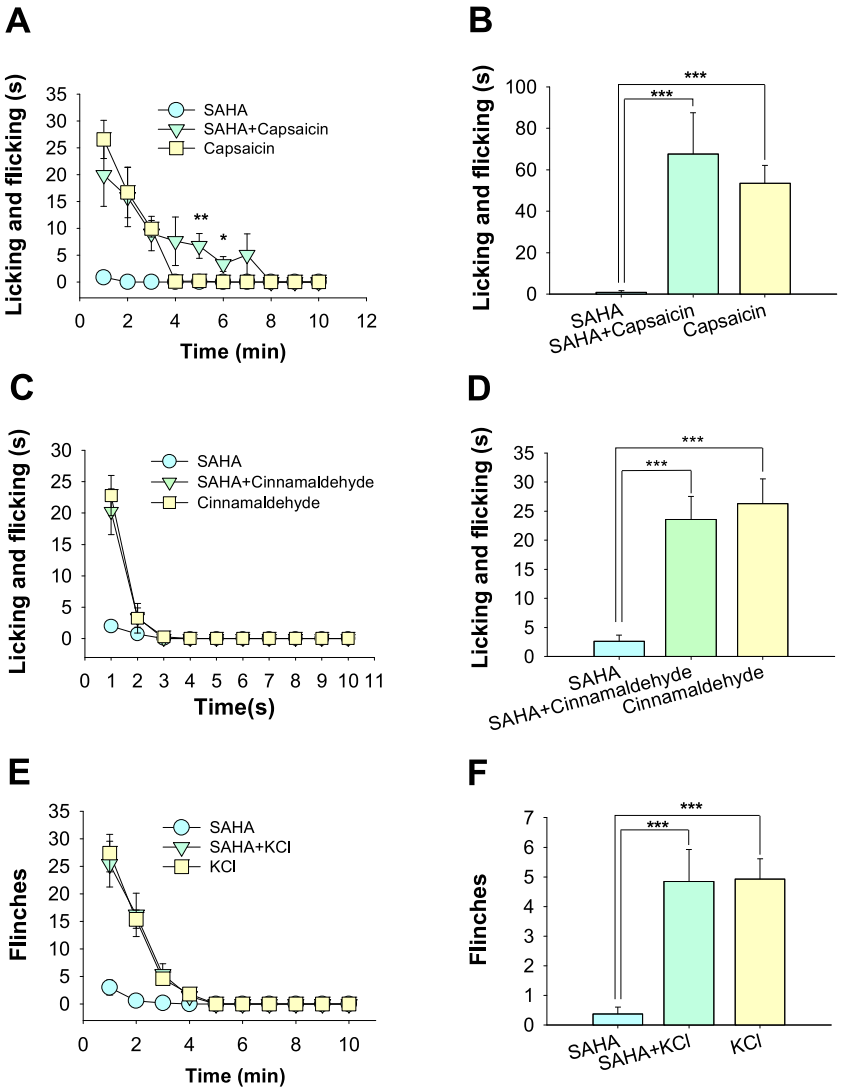

Fig. 3 Effects of SAHA pretreatment on TRPV1-mediated, TRPA1-mediated, and nonspecific depolarization-induced acute behavioral nociception. **a** Summary of the time course of licking/flicking behaviors in mice treated with capsaicin (300 μM in 10 μl) immediately after hind paw intraplantar injection ($n = 5$). Mice were pretreated with SAHA (100 μM in 10 μl) 1 min prior to the capsaicin administration ($n = 5$, gray circle). **b** Summary histograms of the accumulated time engaged in the nociceptive behaviors in **a**. **c** Summary of the time course of licking/flicking behaviors in mice treated with cinnamaldehyde (10 mM in 10 μl) immediately after hind paw intraplantar injection ($n = 5$). Pretreatment with SAHA (100 μM in 10 μl) 1 min before cinnamaldehyde administration failed to prevent such behaviors ($n = 5$). **d** Summary histograms of the accumulated time engaged in the nociceptive behaviors in **c**. **e** Summary of the time course of the licking/flinching behaviors in mice treated with 140 mM KCl solution immediately after hind paw intraplantar injection ($n = 5$ with or without SAHA, respectively). **f** Summary histograms of the cumulative time engaged in the nociceptive behaviors in **e**

and moderately inhibited TRPV4-mediated influx on average (Fig. 4c). Interestingly, the effects of SAHA on TRPV4 appeared to vary on a cell by cell basis. In a fraction of cells ($n = 145$ among 501), SAHA strongly inhibited agonist-activated TRPV4 responses in TRPV4-transfected cells at nanomolar and micromolar ranges (Fig. 4d, e). The inhibitory effect was confirmed in whole-cell voltage clamp experiments (Fig. 4f). Electrical current responses evoked by the TRPV4-specific agonist GSK1016790A in TRPV4-transfected HEK cells were greatly attenuated in the presence of SAHA. Ruthenium red and RN-1734, known TRPV4 blockers, also attenuated the outwardly rectifying currents caused by the agonists, confirming that the agonist-elicited and SAHA-inhibited currents are mediated by TRPV4 activation. Both the Ca^{2+} influx response and the electrophysiological response during SAHA perfusion alone indicate that SAHA does not have partial agonistic activity on TRPV4 or other endogenous components. Despite the structural similarity to capsaicin, no such excitatory case occurred in whole-cell voltage clamp experiments using HEK cells expressing TRPV1, which is the specific target for capsaicin (data not shown, $n = 5$).

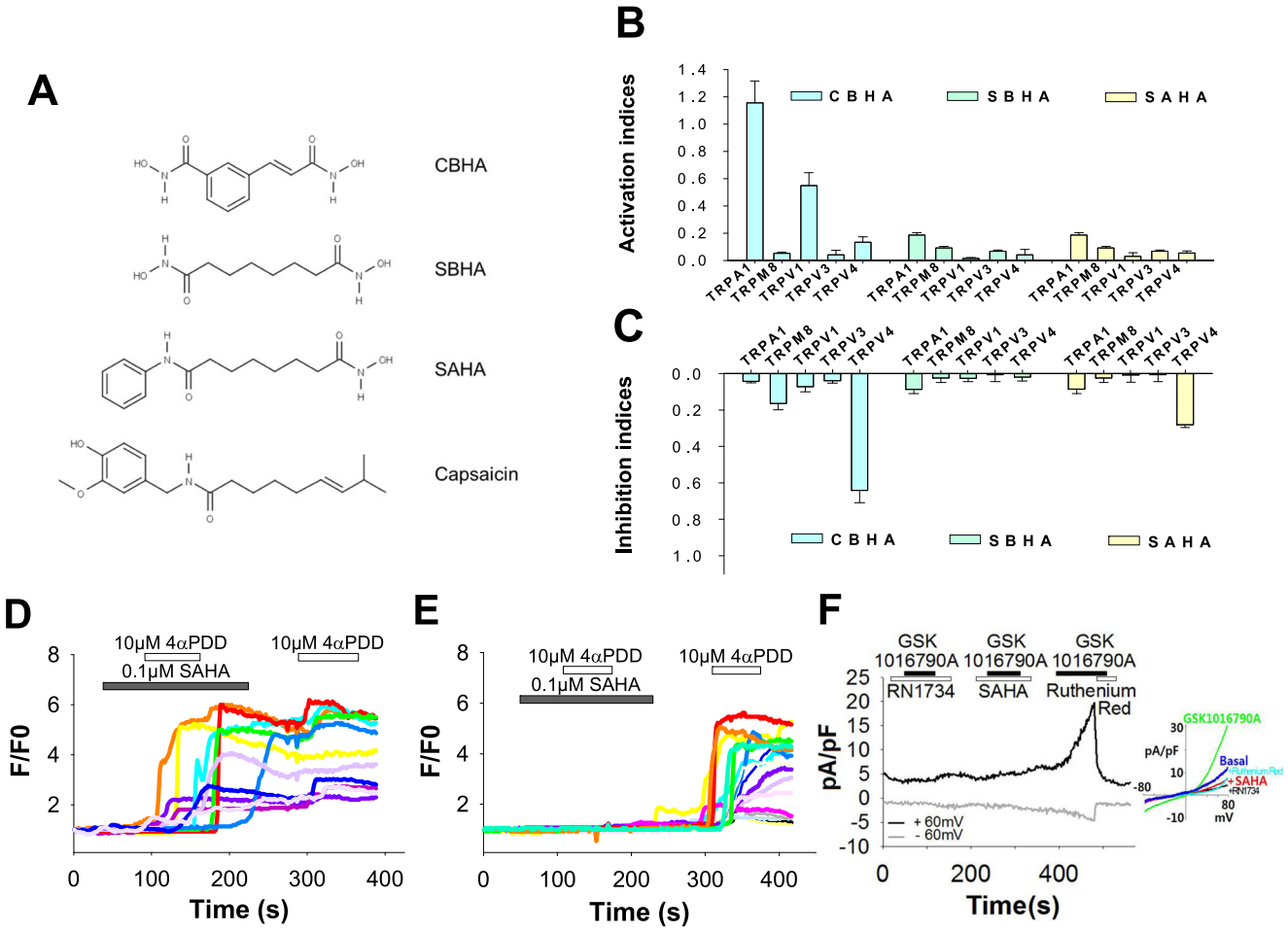

Fig. 4 Effects of SAHA and structurally related compounds on TRP activities. **a** Chemical structures of capsaicin, SAHA, and its structurally related compounds. Abbreviations: CBHA, carboxycinnamic acid bishydroxamide; SBHA, suberoyl bishydroxamic acid. **b**, **c** Pharmacological profiles of CBHA, SBHA, and SAHA for sensory TRP activation and inhibition. Intracellular Ca^{2+} increases were measured in HEK cells transfected with individual TRPs. Intracellular Ca^{2+} increases induced by a compound alone compared to those induced by a standard agonist alone (**b**), and degree of reduction in intracellular Ca^{2+} level by co-application of a compound compared with agonist-induced elevation were shown (**c**). The inhibition data were normalized to the peak responses during agonist application. Concentrations of standard agonists for each specific TRP-expressing cell were as follows: 300 μM cinnamaldehyde for TRPA1, 300 μM menthol for TRPM8, 0.1 μM capsaicin for TRPV1, 4 mM camphor for TRPV3, and 3 μM 4αPDD for TRPV4. Concentrations of compound were 10 μM CBHA, 1 μM SBHA, and 100 nM SAHA (for each data point, $n = 7–70$). 4αPDD did not elicit off-target Ca^{2+} increase in untransfected HEK cells (Supplementary Fig. 3). **d** Representative time-lapse traces where SAHA (100 nM) failed to affect intracellular Ca^{2+} increases in a subset of TRPV4-expressing HEK cells upon treatment with 10 μM 4αPDD ($n = 356$). **e** Representative time-lapse traces where SAHA (100 nM) blocked intracellular Ca^{2+} increases in a different subset of TRPV4-expressing HEK cells upon treatment with 10 μM 4αPDD ($n = 145$). **f** SAHA (100 nM) attenuated current responses to 10 nM GSK1016790A in a similar manner to 10 μM RN-1734 or ruthenium red in whole-cell voltage clamp at ± 60 mV experiments using TRPV4-expressing HEK cells ($n = 5$, respectively). Inset: current-voltage curves from the HEK cell responses were superimposed

Effects of SAHA on Cultured Sensory Neurons

We investigated whether the inhibitory effect of SAHA on TRPV4 and its inertness to TRPV1 were reproducible in cultured sensory neurons. In a subset of cultured mouse DRG neurons, TRPV1-mediated capsaicin responses or TRPV4-mediated GSK1016790A responses were observed during the Fura-2 Ca^{2+} imaging experiments. SAHA consistently failed to alter the capsaicin responses (Fig. 5e, f). On the other hand, as observed in heterologous cell experiments, SAHA acutely blunted GSK1016790A responses in a subset of GSK1016790A-responsive neurons (Fig. 5a–d; $n = 35$ among 73 neurons). Consistent with in vivo results shown in Fig. 3c, d, the Ca^{2+} influx induced by high concentrations of external KCl, which is mediated by native voltage-gated components and reflects the excitable property of these neurons, was not affected by SAHA application, indicating that SAHA does not modify the basic neuronal excitability or the voltage-gated channel function in sensory neurons (Fig. 5g, h). Collectively, these data show that SAHA negatively modulates TRPV4 activity and thus that its treatment can alleviate TRPV4-mediated mechanical pain.

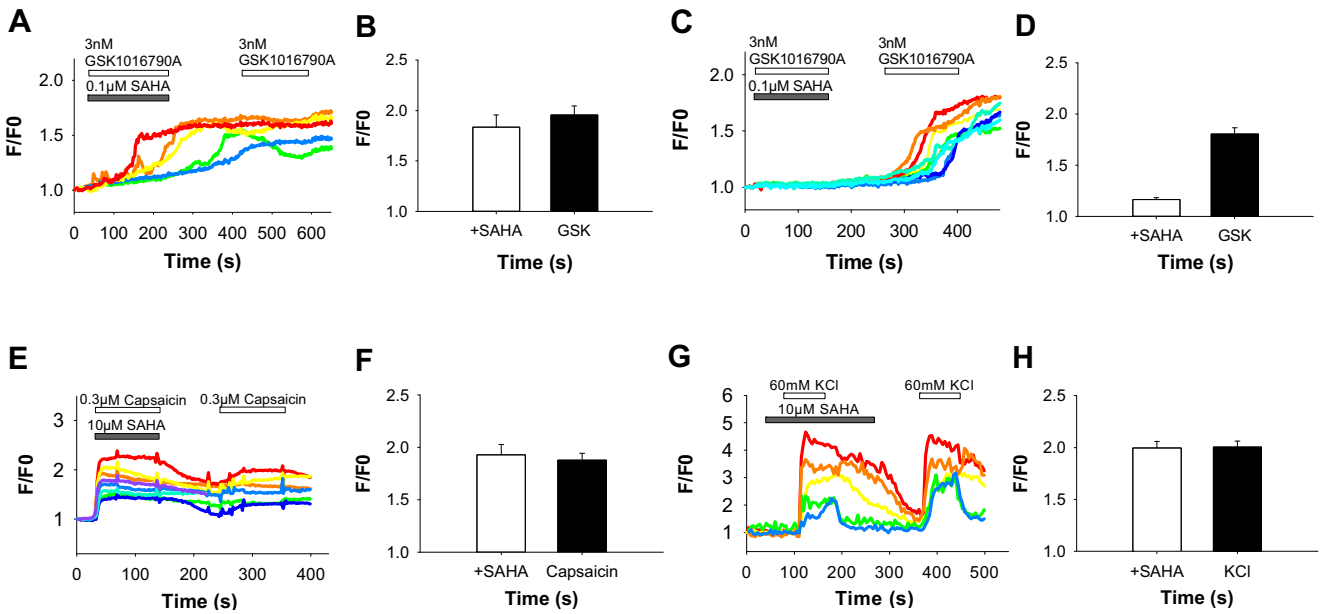

Fig. 5 Effects of SAHA on TRPV4 activation in cultured sensory neurons. **a** Representative time-lapse traces where SAHA (100 nM) failed to affect intracellular Ca^{2+} increases in a subset of cultured mouse DRG neurons upon treatment with 3 nM GSK1016790A. **b** Summary histograms of the averaged peak Ca^{2+} level from experiments including **a** ($n = 40$). **c** Representative time-lapse traces where SAHA (100 nM) blocked intracellular Ca^{2+} increases in a different subset of cultured mouse DRG neurons upon treatment with 3 nM GSK1016790A. **d** Summary histograms of the averaged peak Ca^{2+} level from experiments including **c** ($n = 32$). **e** Representative time-lapse traces where SAHA (10 μM) failed to affect intracellular Ca^{2+} increases in DRG neurons upon treatment with 0.3 μM capsaicin. **f** Summary histograms of the averaged peak Ca^{2+} level from experiments including **e** ($n = 37$). **g** Representative time-lapse traces where SAHA (10 μM) failed to affect intracellular Ca^{2+} increases in DRG neurons upon treatment with 60 mM KCl. **h** Summary histograms of the averaged peak Ca^{2+} level from experiments including **g** ($n = 49$)

Discussion

In the present study, we identified a new modulatory effect and mechanism of the actions of SAHA in pain. The action of SAHA on its conventional target HDACs in malignantly transformed tissues such as cancers results in promotion of the transcription of tumor suppressor genes by interfering with HDAC enzyme activity and is therefore clinically useful for the treatment of cutaneous T cell lymphoma [2, 4, 23]. Some off-target effects that share the same mechanism, namely inhibition of deacetylation, have been reported. For example, accompanying non-histone protein accumulation caused by acetylated histone accumulation or increases in lysine-specific acetylation of non-histone proteins can lead to unpredictable events in normal tissues [5, 24–26]. For pain modulation or sensory neuronal function, previous studies have shown that HDAC inhibitors can relieve pain, focusing on the known epigenetic paradigm [6, 27–30]. Regarding the reversal of sensory neuronal pathology, the similar epigenetic paradigm dominates mechanistic verifications of the therapeutic causes in other previous studies [31–33]. Therefore, throughout the studies, no sensory modality-specific or pain sensor ion channel-associated outcome that does not engage an acetylation-related mechanism has been hypothesized or discussed, particularly with regard to the rapid temporal changes in function that we observed here. Thus, the present study is the first to propose previously unexplored off-target effect for an HDAC modulator, in the context of pain attenuation.

SAHA was synthesized with the aim of finding potent hybrid polar compounds that induce cell growth arrest and terminal differentiation in transformed cells [34]. After the discovery of the action of SAHA on transformed cells, the molecular mechanism of these cellular activities was found to be HDAC inhibition [35]. The hydroxamic residue of the aliphatic side of SAHA coordinates zinc in the bottom of the catalytic site of HDAC, and the other side of the compound occupies the tubular substrate binding pocket of the enzyme, which accomplishes the inhibitory task [23, 36]. It is unlikely that such zinc coordination explains the TRPV4-mediated contribution because the external environment for observing TRPV4 activity in our Ca^{2+} imaging and electrophysiological assays and the intracellular environment in our electrophysiological assays were constantly controlled to be zinc-free. In addition, even SBHA, which contains two hydroxamic residues that coordinate zinc, failed to inhibit TRPV4. However, the possibility that the mechanism involves unknown TRPV4-independent contributors remains since

protein-bound forms or zinc in some microdomains might exist despite the experimental chelation.

CBHA activated TRPA1 and TRPV1 in this study. For TRPA1, the activation is possibly due to its covalent binding potential. It is a central mechanism for TRPA1 activation by its chemical ligands that specific lysine and cysteine residues of the TRPA1 protein can mediate pore gating when they covalently bind to electrophiles containing α,β-carbonyl carbons, which are also present in CBHA [37, 38]. Moreover, chemical size might be another positive factor for its binding because one of the most potent natural TRPA1 activators that use this mechanism is cinnamaldehyde and the CBHA structure appears to mimic this cinnamic backbone [22].

Knowledge on pharmacological antagonism with TRPV4 specificity is limited. A small numbers of antagonists in the synthetic pool (RN-1734, HC-067047, GSK205) with TRPV4 specificity are publicly available. Among these, RN-1734 and HC-067047 have been tested in pain models. RN-1734 was tested in TRPV4-mediated mechanical pain models in our previous study, which also reversely confirmed the contribution of TRPV4 to pain mediation [12]. The analgesic potency of HC-067047 was confirmed in different animal models of pain [12, 39, 40]. SAHA does not share structural similarity with any of these three compounds. Although it is difficult to predict an ideal backbone structure that best fits a ligand binding pocket of TRPV4 as an antagonist with the current knowledge that these three synthetic antagonists are chemically heterogeneous, SAHA might contain a less optimized one based on the instability of its blocking ability.

Other aberrant effects also occurred in our behavioral assays with regard to TRPV1 activation. Although total licking/flicking time was not significantly affected by the injected SAHA, marginal increases in capsaicin-induced licking/flicking behaviors were detected 5–6 min after the injection time point. Those data were somewhat inconsistent with our in vitro observation showing complete inertness in both activation and inhibition indices. SAHA rarely activated TRPV1 at very high concentrations near the upper limit of our buffer solubility (data not shown), which could explain the marginal effect.

We previously reported that intraplantar injection of a TRPV4-specific agonist, DMAPP, elicited mechanical, but not thermal, hypersensitivity in behavioral assays [12]. In contrast, in the present study, injections of a conventional TRPV4 agonist, 4αPDD, elicited behavioral hypersensitivity to both mechanical and thermal stimuli. The 10 times stronger agonistic potency of 4αPDD compared with DMAPP can induce higher Ca^{2+} mobilization in the nociceptive fibers [9]. More amplified signals at the levels of cytosolic Ca^{2+} or its downstream pathway might more readily influence multiple sensory machineries including the thermosensitive one, leading to a broader priming effect. Nonetheless, SAHA effects were limited to mechanical phenotypes, indicating that the acute SAHA-elicited mechanism predominantly involves specific modality-related subcellular components but is dissociated from different parallel components including the thermosensitive machinery and from a more common regulatory mechanism.

Although the in vivo painkilling phenotypes seem to be specific for a TRPV4-dependent modality, only a fraction of TRPV4-expressing cells in vitro showed a reduction in responses in the presence of SAHA. This might be caused by some unknown chemical instability of SAHA when bound to TRPV4 protein. Such variations often occur in agonism and antagonism of TRP channel subtypes: for example, resiniferatoxin on TRPV1 (slow activation and deactivation kinetics), 4αPDD on TRPV4 (irregular onset and strong desensitization), proton on TRPP3 (off-response by proton), and ruthenium red on TRPV3 (voltage-dependent blockage and irreversibility) [41–44]. Alternatively, such in vitro heterogeneity of SAHA responsiveness may reflect the possible involvement of other unknown molecular components. The instability of the presence and absence of SAHA effects occurred not only in HEK cells but also in cultured DRG neurons in our experiments. DRG neurons are composed of subpopulations that differentially express various kinds of molecules that may affect TRPV4 sensitivity to SAHA, and this heterogeneity could accidentally be repeated even in individual HEK cells with different conditions. With given data from the neurons shown in Fig. 5b, d, we analyzed whether different SAHA responsiveness correlate with the soma size which is a standard reflecting the subpopulation of the sensory neurons (Supplementary Figure 4). However, the GSK1016790A responses were only detected in small diameter subpopulations, and no unique differences in the size distribution and average diameters of SAHA-sensitive and SAHA-insensitive neurons were observed.

It could implicate possible acetylation-related mechanisms other than direct interaction. SAHA has recently been shown to target many different deacetylases including not only nuclear deacetylases but also cytoplasmic ones [45]. If the TRPV4 protein itself and/or a critical component for maintaining its activity is sensitive to their acetylation-deacetylation balances and these balances can be ordinarily tuned at a very fast rate, a rapid response change could occur when SAHA alters this equilibrium by nonspecifically inhibiting a participant protein deacetylase. α-Tubulin, acetylation of which is highly modulatable by SAHA, might be structurally important for the subcellular location and function of TRPV4 [46]. Nicotinamide, an endogenous metabolite produced by the action of the deacetylase sirtuin, has recently been proposed as an activator of nematode TRPVs [47]. Sophisticated examination of these possibilities of direct binding or acetylation dynamics is required.

Finding novel off-target effects might open a new avenue for possible applications of drugs of different categories. For

example, different molecular processes appear to account for pancreatic cancer suppression by metformin, an antidiabetic drug with a major mechanism via AMP-kinase activation [48]. Understanding mechanisms for the adverse off-targets common to drugs that have different on-targets can provide an opportunity to actively control target specificity or, alternatively, to utilize the off-targets to reveal novel therapeutic strategies or synthetic leads [49]. In this context, the results in this study may highlight a different aspect for pain modulation than what previous pain studies that have attributed to epigenetic effects did [6–8]. It would also be interesting to carefully assess unpredicted pleiotropic effects in somatosensation, which may be undesirable or beneficial, when SAHA is used for an anti-cancer therapy. In conclusion, the present study suggests that SAHA acutely leads to a peripheral anti-nociceptive outcome that employs a previously unknown mechanism involving TRPV4 inhibition.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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