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The role of free fatty acid receptor pathways in a selective regulation of TRPA1 and TRPV1 by resolvins in primary sensory neurons

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Abstract

Transient receptor potential ankyrin 1 and vanilloid 1 (TRPA1 and TRPV1, respectively) channels contribute to inflammatory and neuropathic pain, indicating that their pharmacological inhibition could be a novel strategy for treating painful diseases. However, the mechanisms of TRPA1/V1 channel modulation have been mostly characterized to be upregulation and sensitization via variety of exogenous stimuli, endogenous inflammatory mediators, and metabolites of oxidative stress. Here we used calcium imaging of dorsal root ganglion neurons to identify an inhibitor signaling pathway for TRPA1 and TRPV1 regulated by resolvins (RvD1 and RvE1), which are endogenous anti-inflammatory lipid mediators. TRPA1 and TRPV1 channel activations were evoked by the TRPA1 agonist allyl isothiocyanate and the TRPV1 agonist capsaicin. Our results show that RvD1-induced selective inhibition of TRPA1 activity was mediated by free fatty acid receptor 4 (FFAR4)-protein kinase C (PKC) signaling. Experiments assessing RvE1-induced TRPV1 inhibition showed that RvE1 actions required both FFAR1 and FFAR4. Combined stimulation of FFAR1/FFAR4 or FFAR1/PKC mimicked TRPV1 inhibition by RvE1, and these effects were blocked by a protein kinase D (PKD) inhibitor, implying that PKD is an effector of the FFAR/PKC signaling axis in RvE1-induced TRPV1 inhibition. Despite selective inhibition of TRPV1 in the nanomolar range of RvE1, higher concentrations of RvE1 also inhibited TRPA1, possibly through PKC. Collectively, our findings reveal FFAR1 and FFAR4 as key signaling pathways mediating the selective targeting of resolvins to regulate TRPA1 and TRPV1, elucidating endogenous analgesic mechanisms that could be exploited as potential therapeutic targets.

KEYWORDS

dorsal root ganglion neuron, free fatty acid receptor, resolvin, TRPA1, TRPV1

1 | INTRODUCTION

Resolvins are endogenous lipid mediators generated from the omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) during the resolution phase of acute inflammation and have potent anti-inflammatory and pro-resolution actions (Alquier et al., 2009). Recent studies show that resolvins are promising new therapeutics for treating pain associated with inflammation because resolvin D1 (RvD1) and resolvin E1 (RvE1) attenuate inflammatory pain such as arthritic pain (Ji et al., 2011; Lima-Garcia et al., 2011). Current inflammatory pain treatments are limited by their side effects including respiratory depression, sedation, nausea, vomiting, constipation, dependence, tolerance, and addiction after opioid treatment (Stein, 1993), as well as serious cardiovascular effects associated with long-term use of cyclooxygenase-2 inhibitors (Varga et al., 2017). Elucidation of resolvin signaling mechanisms is critical for targeting the resolvin receptors and signaling pathways with small molecule agonists. A number of studies demonstrated that inhibition of transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1) channel function seems to underlie the analgesic effects of resolvins. TRPA1 and TRPV1 are critical TRP channels that are strongly implicated in the genesis of inflammatory pain (Brain, 2011). RvD1 was shown to reduce TRPA1 channel activity, while RvE1 regulates TRPV1 function (Park et al., 2011). Although the RvE1-induced reduction in TRPV1-mediated glutamate release is attributed at least partially to its action on the $G\alpha_i$-protein-coupled receptor ChemR23 (Xu et al., 2010), the detailed molecular mechanisms related to TRP modulation remain unclear. TRPA1 channel inhibition has not raised any significant safety concerns to date, but TRPV1 antagonists exerted adverse effects on body temperature in humans during in vivo testing. For these reasons, regulatory pathways of TRPA1 mediated by resolvins might represent an attractive therapeutic target for pain treatment (Koivisto et al., 2021). However, the identities of the receptors mediating RvD1-induced regulation of TRPA1 remain unknown (Bang et al., 2010; Leuti et al., 2021).

It is well-known that fatty acids affect intra- and intercellular signaling, as well as neuronal membrane fluidity (Horrocks & Farooqui, 2004). In addition to intracellular actions, unbound free fatty acids (FFAs) can also carry out extracellular signaling by stimulating G-protein-coupled receptors (GPCRs). A GPCR deorphanization strategy was recently used to identify multiple FFA receptors (Hirasawa et al., 2008). FFA receptor 1 (FFAR1 receptor or GPR40) and free fatty acid receptor 4 (FFAR4) (previously GPR120) were reported to be activated by middle- to long-chain fatty acids including DHA, EPA, and arachidonic acid (Briscoe et al., 2003; Hirasawa et al., 2005). FFAR1 is expressed in pancreatic β-cells where it mediates insulin secretion (Alquier et al., 2009; Itoh et al., 2003). This receptor is coupled to an intracellular G protein (Gq) that activates phospholipase C and phosphatidylinositol (phosphatidylinositol-4,5-bisphosphate) signaling (Hardy et al., 2005). FFAR4 is important in inflammation and mediates the broad anti-inflammatory effects of omega-3 fatty acids (Talukdar et al., 2010). FFAR4 knockout mice display an accelerated development of osteoarthritis after anterior cruciate ligament transection surgery (Chen et al., 2018), and FFAR4 activation prevents inflammation and apoptosis to protect against focal cerebral ischemic injury (Ren et al., 2019). FFAR4 also improves many aspects of metabolic homeostasis such as insulin sensitivity and adipogenesis (Hilgendorf et al., 2019). As a joint signaling node that integrates the anti-inflammatory effects and improvements of insulin sensitivity and adipogenesis, FFAR4 is a popular drug target for the treatment of obesity-related insulin resistance, type 2 diabetes, and inflammatory diseases (Watterson et al., 2014). FFAR4 activation leads to both β-arrestin recruitment and G-protein dependent signaling through coupling to Gq (Burns et al., 2014; Hirasawa et al., 2005). FFAR1 is widely expressed in the rodent brain (Nakamoto et al., 2012; Zamarbide et al., 2014) and spinal cord (Karki et al., 2015), and that FFAR1 agonists such as DHA and GW-9508 produce antinociceptive effects against chemical-, mechanical-, and thermal-induced pain. Given that FFAR4 mRNA is expressed in the mammalian hypothalamus, hippocampus, and spinal cord, this receptor is also thought to play a role in the nervous system (Kimura et al., 2019). However, the roles of FFAR1 and FFAR4 in resolvin-mediated TRP channel regulation have not been evaluated.

In this study, we investigated the molecular mechanisms underlying specific TRP channel inhibition by resolvins. Using intracellular calcium (Ca^{2+}) imaging in dorsal root ganglion (DRG) neurons, we demonstrated that FFAR4 mediates RvD1-induced suppression of TRPA1 via protein kinase C (PKC) activation. Furthermore, both FFAR1 and FFAR4 signaling pathways contribute to RvE1-induced TRPV1 inhibition. Resolvin-mediated FFAR signaling pathways could represent an underlying mechanism and potential therapeutic molecular target in the analgesic effects of resolvins.

2 | MATERIALS AND METHODS

2.1 | DRG preparation

All experimental procedures were conducted in accordance with the guidelines of the Sungkyunkwan University School of Medicine Institutional Animal Care and Use Committee (approval no. SKKUIACUC2020-11-14-2). We aseptically removed DRGs from 4- to 10-week-old male mice and digested the tissues with collagenase (1 mg/ml;Roche) and dispase II (2.4 units/ml; Roche) for 90 min, followed by 0.25% trypsin for 7 min at 37°C (Xu et al., 2015). We plated cells on glass coverslips coated with poly-D-lysine and laminin and grew them in a Neurobasal defined medium (with 2% B27 supplement) in the presence of 5 μM AraC, at 37°C for 24 h before experiments.

2.2 | Calcium imaging

Ca^{2+} imaging experiments were performed as previously described using fura-2AM (Molecular Probes) as the fluorescent Ca^{2+} indicator. Briefly, DRG neurons were incubated with fura-2 AM (3 μM) for 30 min at room temperature in a normal Tyrode's (NT) solution. For fluorescence excitation, we used a polychromatic light source (xenon

lamp-based; Polychrome-IV; T. I. L. L.-Photonics) coupled to the epi-illumination port of an inverted microscope (IX70; Olympus) via a quartz light guide and UV condenser. Fluorescence intensity was measured via a ×40 objective (Olympus), and images were captured with a charge-coupled device image intensifier camera (Andor Technology). Dual excitation at 340/380 was used with a 400 nm dichroic mirror, and emitted light was collected with a 450 nm long-pass filter. Mean fluorescence intensity ratios (F340/F380) were displayed online every 1 s with Metafluor software (Molecular Devices). All measurements were made at room temperature.

2.3 | Solutions and drugs

The NT solution contained (in mM) NaCl (143), KCl (5.4), $CaCl_2$ (1.8), $MgCl_2$ (0.5), NaH_2PO_4 (0.5), glucose (11.1), and HEPES (5) and was adjusted to pH 7.4 with NaOH. Fura 2-AM was obtained from Thermo Fisher Scientific; RvD1, RvE1, and compound A (Cpd A) were obtained from Cayman Chemical; bisindolylmaleimide I (BIM I) and BIM V were obtained from Merck; DC260126, AH-7614, GW-9508, and kb-NB 142-70 were obtained from Tocris Bioscience; and TAK-875 and AM-1638 were obtained from MedChemExpress. All other drugs were purchased from Sigma-Aldrich. Stock solutions of the drugs were made by dissolving the compounds in deionized water or dimethyl sulfoxide (DMSO) according to the manufacturers' specifications and stored at −20°C. On the day of the experiment, one aliquot was thawed and used. The final concentration of DMSO in the solutions was <0.1%.

2.4 | Statistics

Data were analyzed with Origin (Version 6.1; OriginLab). All results are presented as the mean± SEM with the number of cells (n) used in each experiment. Statistical significance was evaluated using Student's t-tests or Kruskal–Wallis analyses of variance (ANOVA) test for nonnormally distributed data. Comparisons between multifactorial statistical data were made using two-way ANOVAs. Differences were considered significant at $p < 0.05$.

3 | RESULTS

3.1 | FFAR4 mediates the selective inhibition of TRPA1 activity through RvD1

It was reported that RvD1 selectively inhibits TRPA1, while RvE1 inhibits TRPV1 (Park et al., 2011). Thus, we first tried to confirm RvD1's selective modulation of TRPA1 activity in dissociated mouse DRG neurons (Figure 1a,b). Ca^{2+} imaging was performed to measure changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) caused by TRPA1-dependent Ca^{2+} entry with the TRPA1 agonist allyl Isothiocyanate (AITC) (Bautista et al., 2005; Jordt et al., 2004) or TRPV1-dependent Ca^{2+} entry with the TRPV1 agonist capsaicin (Andresen, 2019). Perfusion of DRG neurons with AITC (100 µM) or capsaicin (1 µM) for 20 s elicited marked TRPA1- or TRPV1-dependent Ca^{2+} transients, and repetitive stimulation evoked fairly constant responses. The ratio of the second TRPA1 Ca^{2+} transients versus the first Ca^{2+} transients (P2/P1) was 96.2 ± 3.0% (n = 15), and the ratio of the second TRPV1 Ca^{2+} transients versus the first Ca^{2+} transients (P2/P1) was 96.6 ± 5.0% (n = 16) (Supporting Information: Figure 1). When applied before the second AITC exposure, RvD1 (50 nM) markedly inhibited AITC-evoked TRPA1 activity (Figure 1a), but it did not inhibit TRPV1-dependent Ca^{2+} entry at concentrations up to 500 nM (Supporting Information: -Figure 2). We then investigated the signaling pathways by which RvD1 affected TRPA1 channel activity in DRG neurons. We tested whether FFAR1 or FFAR4 might mediate the RvD1-induced modulation of TRPA1 activity using specific inhibitors (Sparks et al., 2014; Xu & Ji, 2011). As shown in Figure 1b, 10 min pretreatment of DRG neurons with 1 µM of the FFAR1 antagonist DC260126 did not alter RvD1-induced inhibition of TRPA1 activity. However, 1 µM of the specific FFAR4 antagonist AH-7614 blocked RvD1's inhibitory effects, and the extent of RvD1-induced inhibition of TRPA1 was reduced from 70.6 ± 3.1% (n = 20) to −3.0 ± 4.0% (n = 20) by AH-7614 pretreatment ($p < 0.001$; Figure 1c,d). The summary of RvD1-induced TRPA1 inhibition is shown in Figure 1d. AH-7614 or DC260126 alone did not affect TRPA1 activity in DRG neurons (Supporting Information: -Figure 3). These data suggest that FFAR4—but not FFAR1—is involved in RvD1's effects on TRPA1 channels in DRG neurons.

To confirm the role of FFAR4 in TRPA1 regulation, we examined the effects of the FFAR4 selective agonist Cpd A on TRPA1 activity (Oh et al., 2014). We found that Cpd A treatment dose-dependently reduced TRPA1-dependent Ca^{2+} transients in DRG neurons (Figure 1e,f,k). In contrast, when two different FFAR1-selective agonists (TAK-875 and AM-1638) were applied to DRG neurons, neither affected TRPA1 activity (Figure 1g,h,k). We also examined the effects of the dual-acting FFAR1 and FFAR4 agonist GW-9508 on TRPA1 activity. Like Cpd A, GW-9508 induced a dose-dependent inhibition of TRPA1 activity in DRG neurons (Figure 1i–k). Taken together, these data suggest that RvD1 might inhibit TRPA1 activity by stimulating FFAR4.

3.2 | PKC is necessary for RvD1-induced TRPA1 inhibition

Next, we investigated the downstream signaling pathway by which FFAR4 activation mediates RvD1 inhibition of TRPA1 channels. Because FFAR4 is known to be coupled to Gq proteins that can subsequently activate PKC (Hara et al., 2009; Li et al., 2013), we tested whether RvD1 could modulate TRPA1 activity via PKC signaling. As shown in Figure 2a–c, RvD1's inhibitory effects on TRPA1 activity were suppressed by the PKC inhibitor BIM I (1 µM) but not by its inactive analog BIM V (1 µM). These data suggest a critical role of the PKC pathway in RvD1's effects on TRPA1 activity. We then examined whether blocking PKC activity could attenuate

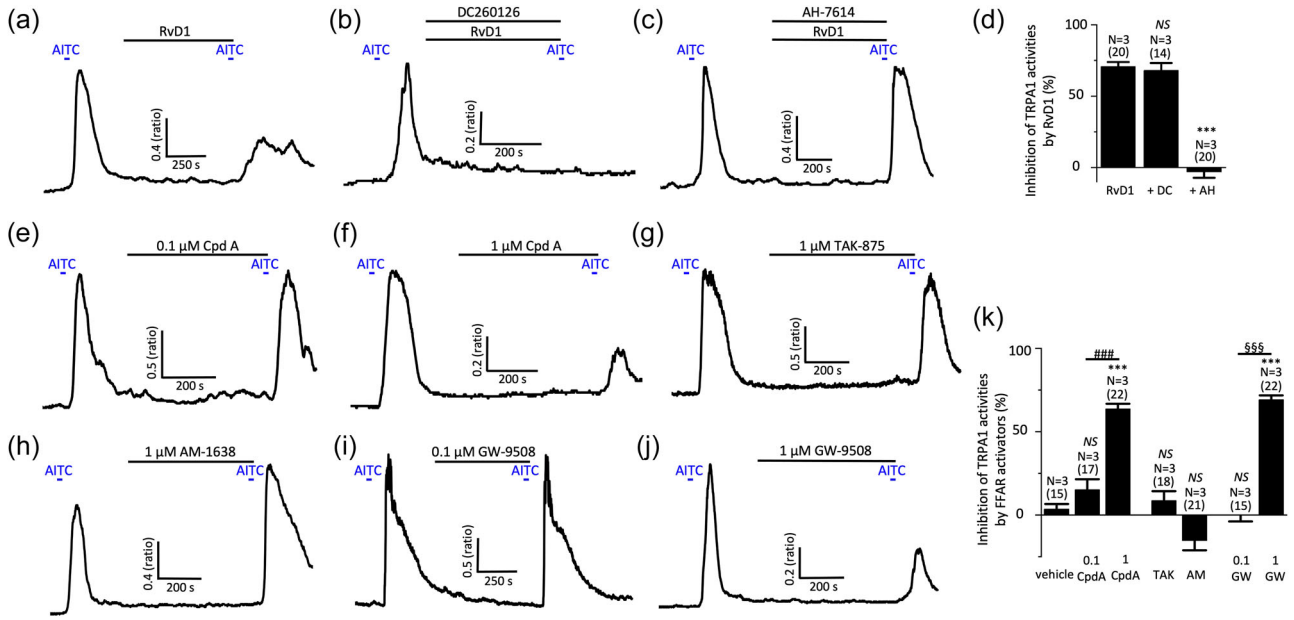

FIGURE 1 RvD1-induced inhibition of TRPA1 activity is mediated by FFAR4 in DRG neurons. (a) Representative traces of $[Ca^{2+}]_i$ responses to repeated application of a saturating concentration of AITC (100 μM, 20 s). RvD1 (50 nM) was pretreated 10 min before the second AITC treatment. Applications of AITC and RvD1 are indicated by the horizontal lines above the trace. (b and c) DC260126 (b) or AH-7614 (c) was given with RvD1 before the second application of AITC. (d) Summary graph of the extent of inhibition of AITC-evoked Ca^{2+} transients by RvD1 in the absence and presence of DC260126 or AH-7614 in DRG neurons. *N*, number of mice; (*n*), cell number. NS, not significantly different; ***$p < 0.001$ versus RvD1-treated group; Kruskal–Wallis. All data are mean ± SEM. (e–j) FFAR4-mediated inhibition of AITC-evoked Ca^{2+} transients. AITC (100 μM) was reapplied after 10 min pretreatment of 0.1 μM Cpd A (e), 1 μM Cpd A (f), 1 μM TAK-875 (g), 1 μM AM-1638 (h), 0.1 μM GW-9508 (i), or 1 μM GW-9508 (j) as indicated by the horizontal lines above the trace. (k) Summary data for the extent of inhibition of AITC-evoked Ca^{2+} transients by various FFAR activators. *N*, number of mice; (*n*), cell number. NS, not significantly different; ***$p < 0.001$ versus vehicle. ###, $p < 0.001$ versus 0.1 μM Cpd A. §§§, ***$p < 0.001$ versus 0.1 μM GW. Kruskal–Wallis test. AH, AH-7614; AM, AM-1638; Cpd A, compound A; DC, DC260126; GW, GW-9508; TAK, TAK-875. AITC, allyl Isothiocyanate; Ca^{2+}, calcium; DRG, dorsal root ganglion; FFAR4, free fatty acid receptor 4; RvD1, resolvin D1; TRPA1, transient receptor potential ankyrin 1.

the inhibitory effects of FFAR4 on TRPA1. As shown in Figure 2d–f, FFAR4 stimulation-induced inhibition of TRPA1 activity was attenuated by pretreatment with BIM I but not BIM V. Alone, neither BIM I nor BIM V affected TRPA1 activity (Supporting Information: Figure 4). Collectively, these results suggest that RvD1 might block TRPA1 activity via FFAR4 and consequent PKC activation in DRG neurons.

3.3 | The selective inhibition of TRPV1 by RvE1 is mediated by FFAR1 and FFAR4

Having delineated the role of FFAR4 in RvD1-induced regulation of TRPA1 activity, we examined whether RvE1-induced modulation of TRPV1 activity is also mediated by FFARs. First, we confirmed that TRPV1 activity was inhibited following 10 min exposure to 5 nM RvE1 (Figure 3a), which is consistent with a previous study (Xu et al., 2010). We next sought to determine whether specific antagonists for FFAR1 and FFAR4 could block RvE1-induced anti-TRPV1 effects (Figure 3b–d). Pretreatment of DRG neurons with the specific FFAR1 antagonist DC260126 (1 μM) for 10 min completely blocked RvE1 inhibition of TRPV1 activity. Notably, RvE1-induced inhibitory effects on TRPV1 were also blocked by the specific FFAR4 antagonist AH-7614. After 10 min pretreatment of DRG neurons with DC260126 (1 μM) or AH-7614 (1 μM), the extent of RvE1-induced inhibition of TRPV1 activity was reduced from 78.6 ± 3.3% ($n = 15$) to −6.2 ± 6.0% ($n = 14$, $p < 0.001$) and −15.6 ± 11.3% ($n = 19$, $p < 0.001$), respectively, indicating that both FFAR1 and FFAR4 are involved in RvE1 inhibition of TRPV1 channels in DRG neurons. AH-7614 or DC260126 alone did not affect TRPV1-dependent Ca^{2+} transients (Supporting Information: Figure 5).

Considering that both FFAR1 and FFAR4 might contribute to RvE1-induced inhibition of TRPV1 channels, we examined the effects of their selective agonists on TRPV1 activity in DRG neurons. We found that selective activation of FFAR1 or FFAR4 had minimal effects on TRPV1 activity ($p > 0.05$ vs. vehicle; Figure 3e,f,i and Supporting Information: Figure 6). However, the dual-acting FFAR1 and FFAR4 agonist GW-9508 markedly inhibited TRPV1 activity in DRG neurons (extent of inhibition: 64.3 ± 6.3%, $n = 15$, $p < 0.001$ vs. vehicle, Figure 3g,i), implying that simultaneous activation of FFAR1 and FFAR4 are necessary to inhibit TRPV1. To further test this possibility, we examined whether combined treatment with FFAR1 and FFAR4 selective agonists (AM-1638 and compound, A, respectively) could suppress TRPV1 channel activity. As shown in Figure 3h,i, simultaneous treatment with the FFAR1 and FFAR4

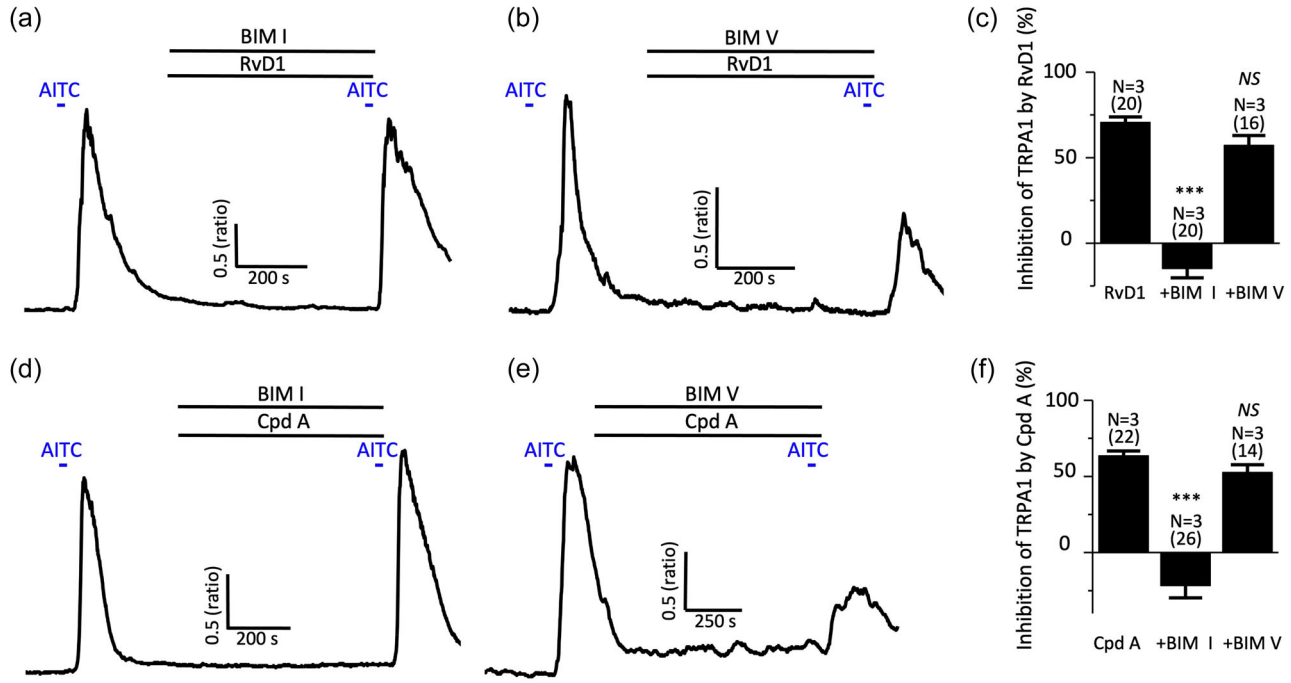

FIGURE 2 The role of PKC in RvD1-induced inhibition of TRPA1 activity in DRG neurons. (a and b) Representative traces showing the effects of the PKC inhibitor BIM I (1 μM, a) or its inactive analog BIM V (1 μM, b) on 50 nM RvD1-induced inhibition of AITC-evoked Ca^{2+} transients in DRG neurons. (c) Summary of the data in (a,b) showing that PKC inhibition with BIM I pretreatment prevented the effects of RvD1 on TRPA1 activity. *N*, number of mice; (*n*), cell number. NS, not significantly different; ***$p < 0.001$ versus RvD1-treated group (redrawn from Figure 1d for comparison); Kruskal–Wallis. All data are mean ± SEM. (d and e) Representative traces showing the effects of BIM I (1 μM, d) or its inactive analog BIM V (1 μM, e) on 1 μM Cpd A-induced inhibition of AITC-evoked Ca^{2+} transients in DRG neurons. (f) Summary of the data in (d,e) showing that PKC inhibition with BIM I pretreatment prevented the effects of Cpd A on TRPA1 activity. *N*, number of mice; (*n*), cell number. NS, not significantly different; ***$p < 0.001$ versus Cpd A-treated group (redrawn from Figure 1k for comparison); Kruskal–Wallis. All data are mean ± SEM. AITC, allyl Isothiocyanate; BIM I, bisindolylmaleimide I; Ca^{2+}, calcium; Cpd A, compound A; DRG, dorsal root ganglion; PKC, protein kinase C; RvD1, resolvin D1; TRPA1, transient receptor potential ankyrin 1.

agonists reduced TRPV1 activity, and the extent of inhibition was 43.3 ± 4.0% ($n = 18$, $p < 0.001$ vs. vehicle). These data suggest that coactivation of FFAR1 and FFAR4 suppresses TRPV1 activity and that anti-TRPV1 effects of RvE1 are mediated by the activation of both receptors. TRPA1 and TRPV1 channels are involved in itch sensation as well as pain, but in distinct subsets of neurons where they mediate chloroquine-indued and histamine-induced itch, respectively (Dong & Dong, 2018). To determine whether RvD1 and RvE1 can regulate TRP channels in itch neurons, we examined their effects on chloroquine-TRPA1 and histamine-induced TRPV1 activities. Our results showed RvD1, RvE1, or FFAR agonists had little effects on chloroquine- and histamine-induced Ca^{2+} transient (Supporting Information: Figures 1 and 7), implying that TRP channels in pain and itch processing systems are differentially regulated by resolvins.

3.4 | PKC-protein kinase D (PKD) signaling pathways contribute to RvE1-induced TRPV1 inhibition

We then explored signaling molecules downstream of FFAR1 and FFAR4 activation that mediate RvE1's inhibitory effects on the TRPV1 channel. Having observed that PKC contributed to FFAR4's effects on TRPA1 activity, we tested whether PKC might also be involved in the inhibitory effects of RvE1. However, PKC activation with 1 μM of phorbol 12,13-dibutyrate (PDBu) enhanced TRPV1 activity (Supporting Information: Figure 8a and c). In fact, this is consistent with a previous study showing that PKC-induced TRPV1 phosphorylation enhances responses to capsaicin (Vellani et al., 2001). Given that RvE1 can activate both FFAR4 and FFAR1 to modulate TRPV1 activity in DRG neurons (Figure 3), we hypothesized that simultaneous activation of FFAR1 signaling with PKC might induce a reduction of TRPV1 activity. To test this possibility, we examined whether combined treatment with a FFAR1 selective agonist and PKC activator could suppress TRPV1 channel activity. Notably, treatment with the FFAR1 agonist AM-1638 and PKC activator PDBu reduced TRPV1 activity, and the extent of inhibition was 72.0 ± 4.5% ($n = 13$, $p < 0.01$ vs. vehicle; Figure 4a,e). These data suggest that when costimulated with FFAR1, PKC might potentiate FFAR1 signaling to inhibit TRPV1 channels. PKD activation has been confirmed as one of the downstream signaling cascades of FFAR1 signaling (Ferdaoussi et al., 2012). To assess PKD's involvement in RvE1-induced inhibition of TRPV1 activity, we used the PKD-specific inhibitor kb-NB-142-70. Treatment with kb-NB-142-70 (1 μM) for

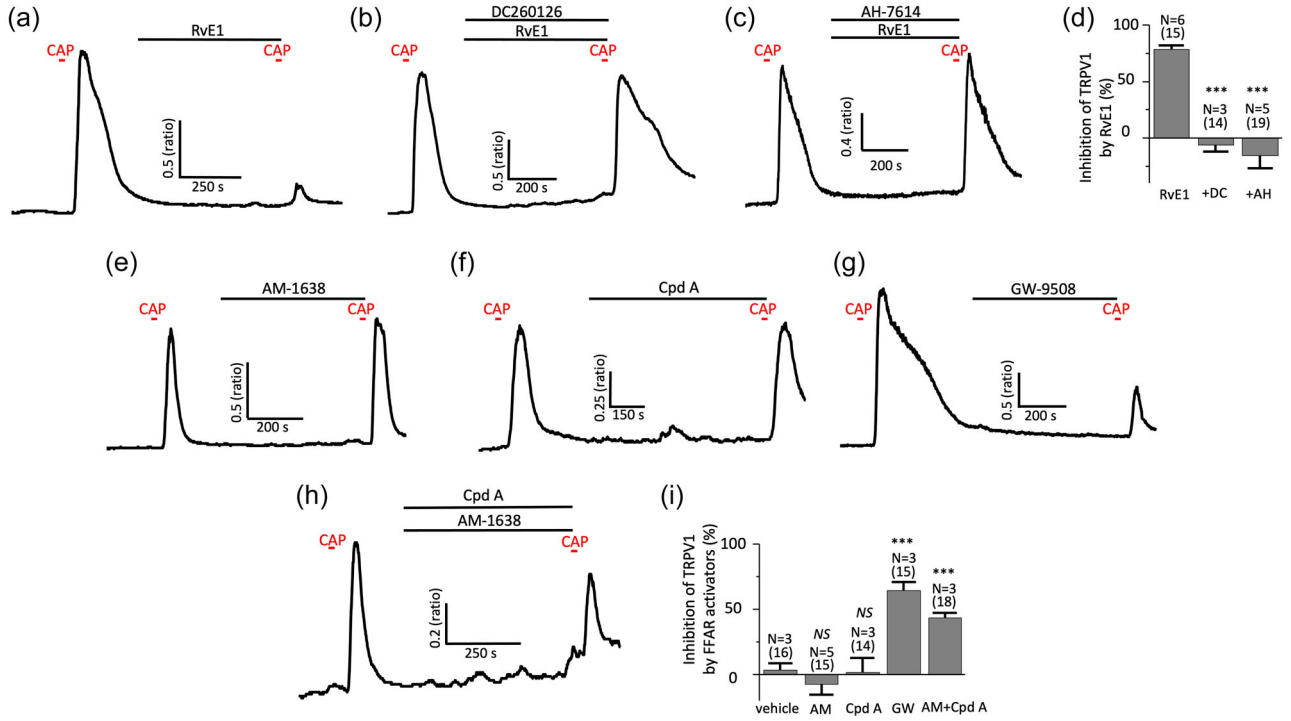

FIGURE 3 RvE1-induced inhibition of TRPV1 activity is mediated by both FFAR1 and FFAR4 in DRG neurons. (a) Representative traces of $[Ca^{2+}]_i$ responses to repeated application of a saturating concentration of capsaicin (1 μM, 20 s). RvE1 (5 nM) was pretreated 10 min before the second application of capsaicin. Capsaicin and RvE1 additions are indicated by the horizontal lines above the trace. (b,c) DC260126 (b) or AH-7614 (c) was pretreated with RvE1 before the second application of capsaicin. (d) Summary graph of the extent of inhibition of capsaicin-evoked Ca^{2+} transients by RvE1 in the absence and presence of DC260126 or AH-7614 in DRG neurons. *N*, number of mice; (*n*), cell number. ***$p < 0.001$ versus RvE1-treated group; Kruskal–Wallis. All data are mean ± SEM. (e–h) Effects of various FFAR activators on capsaicin-evoked Ca^{2+} transients. 1 μM Capsaicin was reapplied after 10 min pretreatment of 1 μM AM-1638 (e), 1 μM Cpd A (f), 1 μM GW-9508 (g), or 1 μM AM-1638 plus 1 μM Cpd A (h) as indicated by the horizontal lines above the trace. (i) Summary data for the extent of inhibition of capsaicin-evoked Ca^{2+} transients by various FFAR activators. *N*, number of mice; (*n*), cell number. NS, not significantly different; ***$p < 0.001$ versus vehicle; Kruskal–Wallis. AH, AH-7614; AM, AM-1638; Cpd A, compound A; DC, DC260126; GW, GW-9508; TAK, TAK-875. Ca^{2+}, calcium; DRG, dorsal root ganglion; FFAR4, free fatty acid receptor 4; RvE1, resolvin E1; TRPV1, transient receptor potential vanilloid 1.

10 min completely blocked RvE1's inhibitory effects on TRPV1 activity in DRG neurons, suggesting a critical role of the PKD pathway in RvE1's effects on TRPV1 activity (Figure 4b,e). We then observed that blocking PKD activity also attenuated the inhibitory effect of GW-9508 on TRPV1 (Figure 4c,e). Furthermore, kb-NB-142-70 blocked TRPV1 inhibition following combined treatment of PDBu and AM1638 (Figure 4d,e). kb-NB-142-70 (1 μM) alone did not affect TRPV1-dependent Ca^{2+} transients (Supporting Information: -Figure 8b and c). Collectively, these data suggest that RvE1 activates both PKD and PKC signaling pathways via FFAR1 and FFAR4, leading to TRPV1 inhibition.

3.5 | High RvE1 concentrations inhibit both TRPV1 and TRPA1 in DRG neurons

The role of PKC in the action of RvE1 raises the question as to whether RvE1-induced activation of PKC might modulate TRPA1 activity. A previous study showed that RvE1 cannot suppress TRPA1 activity at up to 28.5 nM (Park et al., 2011). Consistent with those results, 30 nM RvE1 had little effect on TRPA1 activity in DRG neurons (Figure 5a,e). We then tested the effects of high concentrations of RvE1 on TRPA1 activity. When RvE1 was administered at 100, 300, and 1000 nM, TRPA1 activity was inhibited by 38.5 ± 6.5% ($n = 25$), 71.4 ± 2.5% ($n = 24$), and 74.0 ± 2.0% ($n = 24$), respectively (Figure 5b–e). Effects at concentrations above 300 nM reached saturation, and the effects of 300 and 1000 nM on TRPA1 activity were not statistically different. We further tested whether PKC inhibitors could block TRPA1 inhibition induced by a high concentration of RvE1. The results showed that TRPA1 inhibition by 300 nM RvE1 was blocked by BIM I (Figure 5f,g). This indicates that although PKC activation by RvE1 at lower concentrations (≤30 nM) was not sufficient to inhibit TRPA1 channels, high concentrations of RvE1 (>30 nM) were effective. As expected, 300 nM RvE1 inhibited TRPV1 activity to a similar extent compared to 5 nM (Supporting Information: Figure 9). Taken together, these results suggest that RvD1 activates FFAR4 signaling to selectively inhibit TRPA1, while RvE1 affects FFAR1/FFAR4 signaling to inhibit TRPV1 at low levels and TRPA1 as well as TRPV1 at high levels.

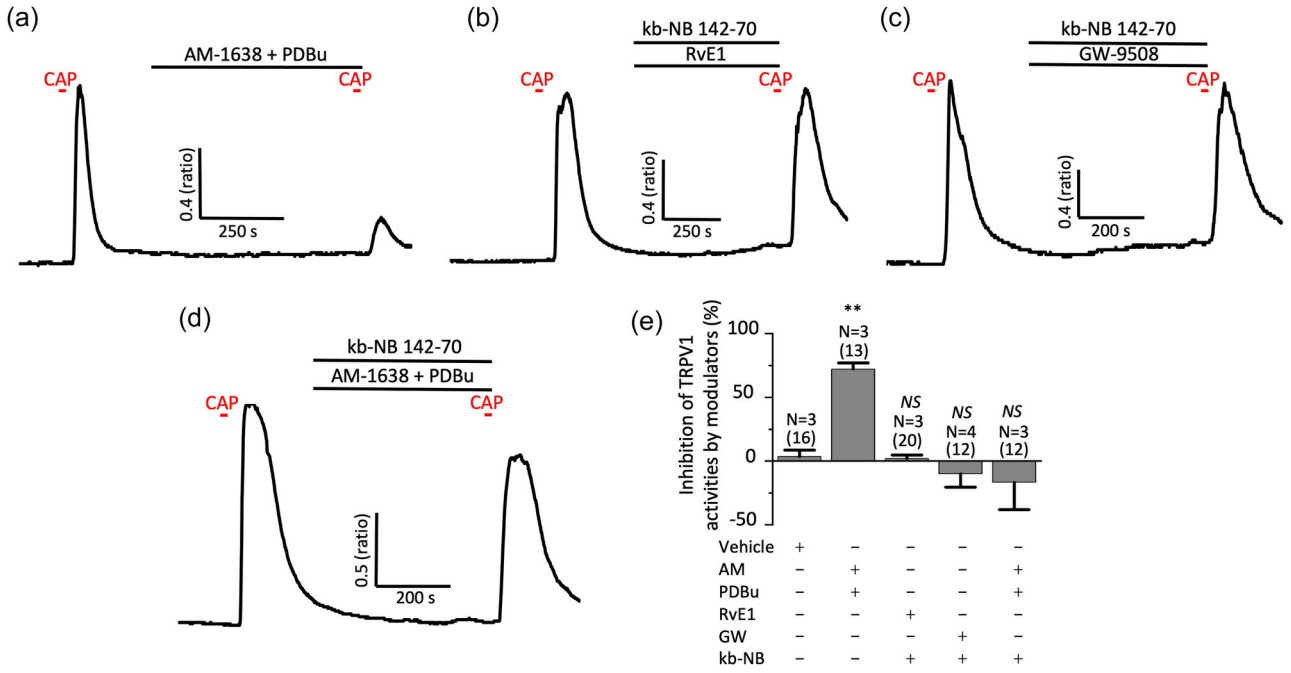

FIGURE 4 Role of the PKC/PKD signaling axis in RvE1-induced inhibition of TRPV1 activity in DRG neurons. (a) Representative traces showing the effects of combined treatment with the FFAR1 agonist AM-1638 (1 μM) and PKC activator PDBu (1 μM) on capsaicin-evoked Ca^{2+} transients in DRG neurons. (b–d) Representative traces showing the effects of the PKD inhibitor kb-NB 142-70 (1 μM) on RvE1-(5 nM, b), GW-9508-(1 μM, c), or combined AM-1638-(1 μM) and PDBu-(1 μM) (d) induced inhibition of capsaicin-evoked Ca^{2+} transients in DRG neurons. (e) Summary of the data in (a–d) showing that PKD inhibition with kb-NB 142-70 pretreatment prevented the effects of RvE1, GW-9508, or simultaneous stimulation of FFAR1 and PKC on TRPV1 activity. *N*, number of mice; (*n*), cell number. NS, not significantly different; $^{**}p < 0.01$ versus vehicle; Kruskal–Wallis. All data are mean ± SEM. AM, AM-1638; GW, GW-9508; kb-NB, kb-NB 142-70. Ca^{2+}, calcium; DRG, dorsal root ganglion; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PKD, protein kinase D; RvE1, resolvin E1; TRPV1, transient receptor potential vanilloid 1.

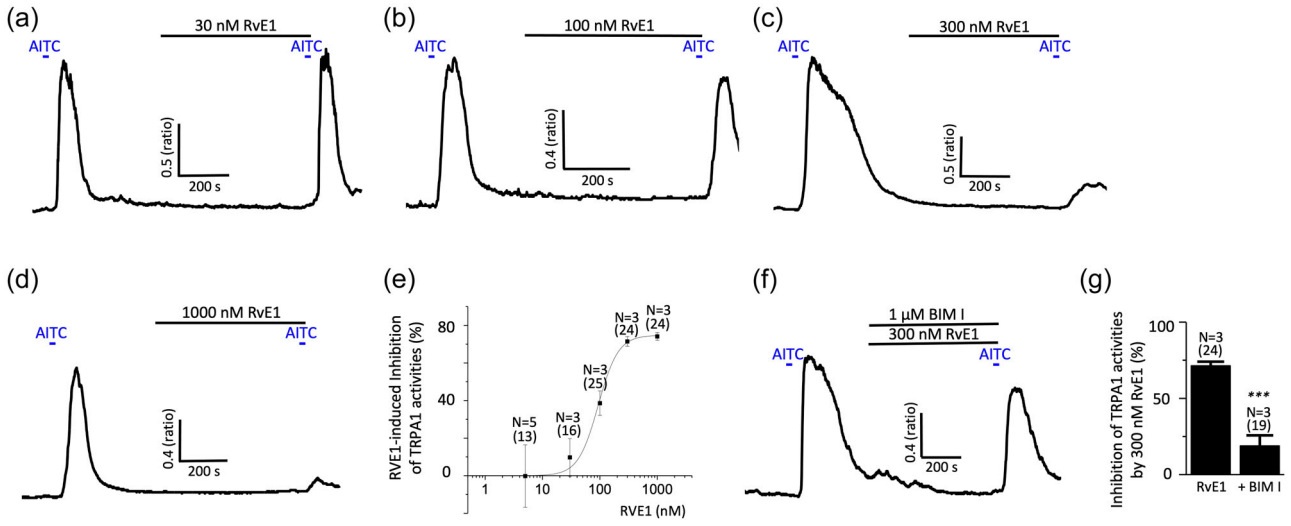

FIGURE 5 High RvE1 concentrations can inhibit TRPA1 activity via effects on PKC signaling. (a–d) Representative traces showing the effects of 30 (a), 100 (b), 300 (c), or 1000 nM (d) RvE1 on AITC-evoked Ca^{2+} transients in DRG neurons. (e) Dose-response curves showing inhibition of TRPA1 activity with high concentrations of RvE1. (f and g) Representative trace (f) and quantification (g) of the effects of BIM I on 300 nM-induced inhibition of TRPA1 activity in DRG neurons. *N*, number of mice; (*n*), cell number. $^{***}p < 0.001$ versus RvE1-treated group; Student's *t*-test. All data are mean ± SEM. AITC, allyl Isothiocyanate; BIM I, bisindolylmaleimide I; Ca^{2+}, calcium; DRG, dorsal root ganglion; PKC, protein kinase C; RvE1, resolvin E1; TRPA1, transient receptor potential ankyrin 1.

4 | DISCUSSION

The main question addressed in the present study was which molecular mechanisms underlie the selective modulation of TRPA1 and TRPV1 by resolvins. We provided evidence that RvD1 might inhibit TRPA1 activity via the FFAR4-PKC signaling pathway. First, RvD1-induced inhibition of TRPA1 activity in dissociated mouse DRG neurons was prevented when cells were pretreated with the specific FFAR4 antagonist AH-7614 but not by the specific FFAR1 antagonist DC260126 (Figure 1a–d). Consistently, TRPA1 activity was inhibited by FFAR4 activation with the selective agonist Cpd A or a dual-acting FFAR1/4 agonist GW-9508, but not when FFAR1 was activated with its selective agonists TAK-875 and AM-1638 (Figure 1e–k). Furthermore the PKC inhibitor BIM I abolished RvD1- or FFAR4 stimulation-induced inhibition of TRPA1 activity (Figure 2), implying that PKC acts as a downstream signal of FFAR4 to control TRPA1. We also demonstrated that TRPV1 responded to different resolvins, and RvE1-induced TRPV1 required both FFAR1 and FFAR4 (Figure 3). RvE1- and GW-9508-induced TRPV1 inhibition was blocked by PKD inhibition (Figure 4). In addition, combined activation of either FFAR1 and FFAR4 or FFAR1 and PKC mimicked the inhibition of TRPV1 activity by RvE1, and these effects were also blocked by PKD inhibition, suggesting that TRPV1 inhibition is mediated through PKC-PKD signaling. We observed selective inhibition of TRPV1 with lower levels of RvE1, while higher concentrations inhibited both TRPV1 and TRPA1. This appears to be at least in part because RvE1 activates both FFAR1 and FFAR4. Consistent with this hypothesis, RvE1-induced TRPA1 inhibition was blocked by the PKC inhibitor BIM I (Figure 5).

Our experiments identified FFAR1 and FFAR4 as key receptors linking the endogenous anti-inflammatory mediator resolvin to nociceptive ion channels. In recent years, particular attention has been given to the effects of numerous endogenous mediators that can fine-tune the sensitivity of TRPA1 and TRPV1 to its activators. The usual activators of TRPA1 are a wide range of chemical compounds, many of which are able to evoke a stinging sensation (Giorgi et al., 2019). An important group of TRPA1 activators are thiol-reactive electrophiles that covalently modify the channels, such AITC and reactive oxygen species. TRPV1 can also be activated by vanilloid ligands such as capsaicin and thermal and proton stimuli (Andresen, 2019). Physiologically, such stimulus levels are only reached under extraordinary circumstances most often associated with overt tissue damage. Several agonists and positive modulators of TRPA1 and TRPV1 activity have been described to date; however, very few naturally occurring inhibitors are known (Morales-Lázaro & Rosenbaum, 2019). Endogenously produced lipid mediators that selectively inhibit TRPA1 and TRPV1 include RvD1 and RvE1 (Bautista et al., 2013). Because resolvins attenuate inflammatory hypersensitivity to thermal and mechanical stimuli without impacting basal pain processing, molecules that target specific resolvin-mediated GPCR signaling pathways may allow selective treatment of TRPA1- or TRPV1-mediated inflammation and pain (Bautista et al., 2013). By revealing the molecular mechanisms underlying TRP channel specificity, our results may provide a basis for novel therapeutic targets. A limitation of our study is the same as any pharmacological study, a potential occurrence of off target effects. In addition, the functions of TRP channels in cultured DRG neurons might be different from those in human nociceptors due to species difference, lack of associated cells including Schwann cells and keratinocytes, and the difference of receptor and ion channel equipment in cell bodies and nerve endings (François et al., 2015; Hou et al., 2011; Klein et al., 2017; Wei et al., 2019). Thus, further studies are required to clarify the relevance of our findings to the clinical diseases.

Our results showed that FFAR4 uses a PKC pathway to inhibit TRPA1 in DRG neurons. This is consistent with a previous study demonstrating that PKC is a downstream effector of FFAR4 (Li et al., 2013). FFAR4 activation by agonists such as synthetic ligands and long-chain fatty acids increases intracellular Ca^{2+} levels without cAMP production in human or mouse FFAR4-expressing cells, suggesting that FFAR4 is coupled with the Gq protein but not the Gi/o or Gs proteins (Hara et al., 2009). Although TRPV1 is known to be activated by PKC phosphorylation, there have been no reports on direct phosphorylation of TRPA1 by PKC (Tiwari, 2011). Furthermore, the major inflammatory mediator bradykinin sensitizes TRPV1 via PKC signaling (Nilius & Owsianik, 2011), while it affects TRPA1 in a PLC- and PKA-dependent manner (Wang et al., 2008). Among several isoforms of PKC expressed in the nociceptive DRG neurons, PKCε and PKCα were shown to be important for TRPV1 activation (Tiwari, 2011). Multiple isoforms of PKC are differentially activated by Ca^{2+} and diacylglycerol and can have opposing effects on a given process (Zoukhri et al., 1998). Thus, different PKC isoforms might play distinct roles in control of TRPA1 and TRPV1 function in DRG neurons. Given that PDBu and BIM we used in this study act on both classical and novel PKC isoforms (Jordt et al., 2004; Sossin, 2007), investigation of the specific PKC isoforms activated by FFAR4 in DRG neurons and inhibition of single isoforms would more accurately demonstrate which PKC isoforms are involved in TRPA1 inhibition. PKD as well as PKC play essential roles in RvE1-induced TRPV1 inhibition. Activation of PKD1 was confirmed as one of the downstream signaling cascades of FFAR1, mediating potentiation of insulin secretion by fatty acids (Ferdaoussi et al., 2012). Notably, we found that a simultaneous activation of FFAR4 and PKC was required for PKD activation in RvE1 signaling. As a downstream signaling molecule of FFAR4, PKC might exert its inhibitory effect on TRPV1 through activation of PKD signaling rather than directly phosphorylating it. In conclusion, FFAR signaling is more complex than expected, and further studies are essential to understand the precise mechanisms and design new treatments for pain.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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