Retinoids activate the irritant receptor TRPV1 and produce sensory hypersensitivity

Shijin Yin,[1,2] Jialie Luo,[1,3] Aihua Qian,[1,4] Junhui Du,[5] Qing Yang,[1] Shentai Zhou,[5] Weihua Yu,[1,6] Guangwei Du,[1] Richard B. Clark,[1] Edgar T. Walters,[1] Susan M. Carlton,[5] and Hongzhen Hu[1]

[1]Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, Houston, Texas, USA. [2]College of Biomedical Engineering, South-Central University for Nationalities, Wuhan, Hubei, China. [3]Institute of Biomechanics, School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, Guangdong, China. [4]Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University, Shanghai, China. [5]Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, Texas, USA. [6]Department of Anatomy, Chongqing Medical University, Chongqing, China.

Retinoids are structurally related derivatives of vitamin A and are required for normal vision as well as cell proliferation and differentiation. Clinically, retinoids are effective in treating many skin disorders and cancers. Application of retinoids evokes substantial irritating side effects, including pain and inflammation; however, the precise mechanisms accounting for the sensory hypersensitivity are not understood. Here we show that both naturally occurring and synthetic retinoids activate recombinant or native transient receptor potential channel vanilloid subtype 1 (TRPV1), an irritant receptor for capsaicin, the pungent ingredient of chili peppers. In vivo, retinoids produced pain-related behaviors that were either eliminated or significantly reduced by genetic or pharmacological inhibition of TRPV1 function. These findings identify TRPV1 as an ionotropic receptor for retinoids and provide cellular and molecular insights into retinoid-evoked hypersensitivity. These findings also suggest that selective TRPV1 antagonists are potential therapeutic drugs for treating retinoid-induced sensory hypersensitivity.

Introduction

Retinoids are the generic term for over 4,000 known natural and synthetic retinoid molecules structurally and/or functionally related to vitamin A. Retinoids are extremely active biologically and exert a variety of profound effects on vision, cell proliferation, differentiation, apoptosis, inflammation, organogenesis, reproduction, and development (1, 2). There has been considerable public interest and demand for natural and synthetic retinoids because of their proven benefits for a number of therapeutic indications, including but not limited to cancer, skin disorders, and diabetes (2). For instance, the use of all-trans retinoic acid (ATRA, tretinoin) has been very successful in the treatment of acute promyelocytic leukemia (APL) by inducing differentiation and apoptosis of leukemic cells with blood concentrations in the micromolar range (2). Many skin disorders, including acne and psoriasis, are also successfully treated with topical retinoids (3). In fact, tretinoin is the first Food and Drug Administration–approved (FDA-approved) topical retinoid with documented efficacy to treat acne vulgaris, the most common skin condition in the United States (4). Retinol (vitamin A) has been used for cosmetic formulations to reduce wrinkles and improve cellulite and was approved by the FDA for use in anti-aging treatments in 1996 (3).

The pleiotropic effects of retinoids are mediated by 2 known families of nuclear receptors, both belonging to the steroid-thyroid hormone receptor superfamily: the retinoic acid receptors (RARs) (α, β, and γ isotypes) and the retinoid x receptors (RXRs) (α, β, and γ isotypes). RARs and RXRs act as ligand-dependent transcriptional regulators by binding to regulatory regions located in target genes in the form of heterodimers (2, 3). The endogenous ligand ATRA selectively binds to RARs, and 9-cis-retinoic acid (9-cis-RA, alitretinoin) has high affinity for both RARs and RXRs (2).

Despite many beneficial effects, retinoids have substantial irritating side effects. Topical application of retinoids often causes severe local irritation manifested as burning sensation, pruritus, erythema, peeling, or dryness (5), which is commonly termed "retinoid dermatitis." Retinoids also cause severe headache, muscle pain, joint pain, bone pain, and inflammatory back pain when used systemically (6–8). Retinoid-elicited irritation has become a major clinical issue and is the main reason that many patients discontinue retinoid treatment (9–13). Animal studies have shown that oral or intrathecal application of ATRA induced nociceptive behavioral effects, suggesting a sensitization of nociceptive pathways by retinoids (14, 15). However, the molecular mechanisms mediating retinoid-induced sensory hypersensitivity are undetermined, and highly effective treatment options for these side effects are lacking. An understanding of cellular and molecular mechanisms underlying retinoid-elicited sensory hypersensitivity potentially could lead to development of clinically useful treatments.

Skin inflammation is a direct response to noxious chemosensory irritants (16, 17), including retinoids. Epidermal keratinocytes, melanocytes, and fibroblasts release cytokines in response to noxious stimuli, which in addition to other inflammatory effects, can sensitize peripheral nociceptive fibers and produce neurogenic inflammation and pain (18). Alternatively, retinoids can directly increase the excitability of nociceptors and produce neurogenic inflammation (18).

Interestingly, the symptoms of retinoid dermatitis and neurogenic inflammation are very similar (19), raising the possibility that retinoids evoke neurogenic inflammation to induce skin irritation. Primary sensory nerve terminals, especially unmyelinated C-fibers, mediate neurogenic inflammation in the periphery and transmit pain to the CNS (16). Transient receptor potential (TRP) channels expressed by somatosensory neurons are key molecu-

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lar sensors of thermal, chemical, and other sensory stimuli (20). Growing evidence indicates that several temperature-sensitive TRP channels (thermoTRPs) are involved in inflammatory pain and nociception (21). Here, we show that both naturally occurring and synthetic retinoids are specific transient receptor potential channel vanilloid subtype 1 (TRPV1) activators, exciting nociceptive sensory neurons and evoking sensory hypersensitivity, which are inhibited by genetic ablation or pharmacologic inhibition of TRPV1 function. Furthermore, disruption of the "vanilloid-binding pocket" that is required for activation by capsaicin also abolishes activation of TRPV1 by retinoids. Our findings demonstrate that TRPV1 is an ionotropic retinoid receptor that mediates retinoid-induced sensory hypersensitivity in the contexts of tissue damage and some dermatological treatments.

Results

Both naturally occurring and synthetic retinoids activate recombinant TRPV1. Bioactive lipids play important roles in TRP channel signaling (22, 23). To identify novel lipid regulators of thermoTRPs, we screened a bioactive lipid library comprising 195 bioactive lipids (Enzo Bioscience). Screening targets included TRPV1, TRPV3, TRPA1, and TRPM8. An increase of intracellular calcium ($[Ca^{2+}]_i$) was used as a functional readout of activities of TRP channels heterologously expressed in HEK293T cells (fluorometric imaging plate reader [FLIPR]; Molecular Devices) (24). After confirming the excitatory effect of known TRP channel activators including anandamide and lysophosphatidic acid (LPA) (refs. 25, 26, and data not shown), we also observed robust and reproducible signals when 2 retinoid analogs, 4-hydroxyphenylretinamide (4-HPR) or AM580, were applied to TRPV1-expressing HEK293T cells. In contrast, no activity was evoked in cells expressing TRPA1, TRPV3 or TRPM8 (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI66413DS1). The function of TRPA1, TRPV3, and TRPM8 was confirmed by their responsiveness to selective agonists for each respective channel (Supplemental Figure 1).

Of note, both AM580 and 4-HPR (fenretinide) are potent agonists of nuclear retinoid receptors and inhibit proliferation of a variety of cancer cells (3, 27). To ascertain whether other clinically used synthetic retinoids also activate TRPV1, we tested acitretin, tazarotene, and bexarotene, all of which are either second or third generation synthetic retinoids that have been used to treat cancers and skin disorders (3). Since these compounds bear structural similarity to ATRA (Supplemental Figure 2), we were curious to know whether naturally occurring retinoids could also activate TRPV1. We thus examined the effects of β-carotene (provitamin A), retinal (retinaldehyde), retinol, ATRA, 9-cis-RA, 13-cis-RA, and retinol palmitate using whole-cell patch-clamp recordings.

All retinoids tested except β-carotene and retinol palmitate activated membrane currents in TRPV1-expressing HEK293T cells in a concentration-dependent manner (Figure 1, E and F, and data not shown). The current traces in response to a voltage ramp (–100 to +100 mV) for AM580, 9-cis-RA, and ATRA are characterized by outward rectification at low concentrations and being linear at high concentrations, resembling that activated by capsaicin and other TRPV1 agonists (ref. 28, Figure 1, B and C, and data not shown). Interestingly, repeated application of AM580 and other retinoids produced a pronounced desensitization and tachyphylaxis of TRPV1 current in the presence of 2 mM Ca^{2+}, which is a typical property of TRPV1 (ref. 29, Supplemental Figure 3, and data not shown). Furthermore, both AM580 and 9-cis-RA increased single-channel activities in inside-out patches excised from HEK293T cells transfected with TRPV1 but not vector control (Figure 1D and data not shown), suggesting that retinoids activate recombinant TRPV1 without requiring intracellular signaling molecules. However, the potency and efficacy varied among retinoids (Figure 1, E and F, and Supplemental Table 1). The rank of efficacy for retinoids at the maximum soluble doses shows that AM580 and 9-cis-RA are the most potent synthetic and naturally occurring retinoids, respectively (Figure 1, E and F). These results strongly suggest that both naturally occurring and synthetic retinoids are activators and/or modulators of recombinant TRPV1.

Retinoids activate primary nociceptors through TRPV1. We next asked whether AM580, 9-cis-RA, and ATRA could activate native TRPV1 expressed in dissociated mouse dorsal root ganglia (DRG) neurons. The rationale for choosing these 3 retinoids was as follows: (a) they represent both naturally occurring and synthetic retinoids; (b) 9-cis-RA and ATRA are key natural ligands for retinoid nuclear receptors and are clinically used to treat skin disorders and cancers; (c) all 3 retinoids activate recombinant TRPV1 (Figure 1 and Supplemental Table 1). Administration of AM580 (5 μM), 9-cis-RA (30 μM), and ATRA (300 μM) evoked outwardly rectifying currents in dissociated small-diameter wild-type DRG neurons that also responded to capsaicin and allylisothiocyanate (AITC), a selective TRPA1 agonist (Figure 2, A and B, and data not shown). Consistent with TRPV1 mediation of retinoid activation of capsaicin-sensitive nociceptors, coapplication of the selective TRPV1 antagonist AMG9810 (0.1 μM) almost completely abolished AM580-activated current (Figure 2, A–C).

AM580 also evoked membrane depolarizations and increased the number of action potentials in both electrically silent neurons and neurons exhibiting spontaneous activity, which recapitulates the capsaicin-induced response in the same wild-type DRG neurons ($n = 5$ for each group) (Figure 2, D and E). Neither AM580 nor capsaicin evoked excitatory membrane responses in DRG neurons from *Trpv1*$^{-/-}$ mice ($n = 16$). In marked contrast, TRPA1 activator AITC induced comparable responses in DRG neurons isolated from both *Trpv1*$^{-/-}$ and *Trpv1*$^{+/+}$ mice (Figure 2F).

We next used calcium imaging to ask whether retinoids could also elicit TRPV1-dependent Ca^{2+} influx in DRG neurons. Bath application of AM580, 9-cis-RA, or ATRA produced a robust $[Ca^{2+}]_i$ increase in a subpopulation of capsaicin-sensitive wild-type DRG neurons (Figure 3). Consistent with the efficacy rank in TRPV1-expressing HEK293T cells, 5 μM AM580 was the most potent activator inducing a $[Ca^{2+}]_i$ response in about 20% of DRG neurons (Figure 3, A–C),while 30 μM 9-cis-RA and 100 μM ATRA evoked a $[Ca^{2+}]_i$ increase in about 7%–10% of the DRG neurons, respectively (Figure 3, D–I). It was observed that capsaicin application caused a larger number of neurons to respond than retinoids did, suggesting that not all TRPV1-expressing neurons respond to retinoids (Figure 3, C, F, and I). Both retinoid- and capsaicin-evoked $[Ca^{2+}]_i$ responses were totally abolished by genetic ablation of TRPV1 function, while AITC evoked comparable $[Ca^{2+}]_i$ responses in both *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ DRG neurons (Figure 3). These results show that retinoids mimic capsaicin-induced responses from native TRPV1 channels and indicate that TRPV1 is the sole target of retinoid-induced acute excitatory responses in primary sensory neurons.

Retinoids stimulate neuropeptide release and produce paw edema. Neurogenic inflammation is a significant contributor to the pain and swelling that characterize inflamed states (30, 31). TRPV1

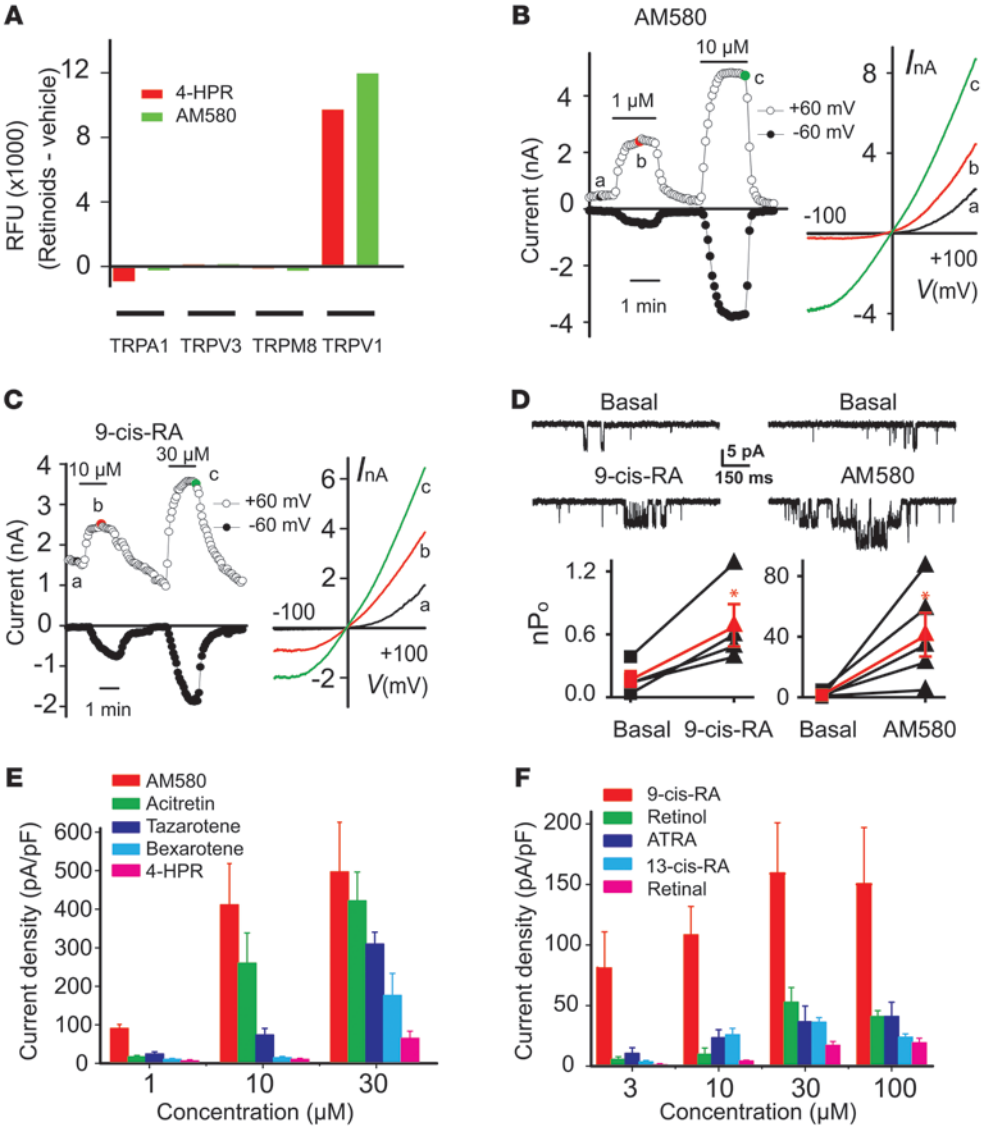

Figure 1

Activation of recombinant TRPV1 by both synthetic and naturally occurring retinoids. (**A**) AM580 (100 μM) and 4-HPR (100 μM) specifically evoked $[Ca^{2+}]_i$ responses in HEK293T cells transfected with recombinant TRPV1. Neither retinoid had an effect on HEK293T cells transfected with TRPA1, TRPV3, or TRPM8. The *y* axis refers to a net increase of relative fluorescence units (RFU) induced by AM580 and 4-HPR after subtraction of the baseline response to vehicle alone. (**B**) Left: representative traces showing that AM580-activated TRPV1-mediated current in a concentration-dependent manner. Right: representative current–voltage (I–V) curves taken at specified time points from the traces on the left illustrate that an outwardly rectifying whole-cell current was evoked by AM580 at 1 μM (*b*), whereas 10 μM (*c*) AM580 activated a current with a linear I-V relationship in a TRPV1-expressing HEK293T cell (*a* refers to the baseline response). (**C**) An endogenous retinoid, 9-cis-RA, also activated TRPV1 current in a concentration-dependent manner similar to that activated by AM580 (*a* refers to the baseline response; *b* and *c* indicate responses activated by 10 and 30 μM 9-cis-RA, respectively). (**D**) Both AM580 (10 μM, $n = 5$) and 9-cis-RA (10 μM, $n = 4$) increased single channel open probability (nPo) in inside-out patches excised from TRPV1-expressing HEK293T cells (*$P < 0.05$). (**E**) Synthetic retinoids activate TRPV1 in a concentration-dependent manner. (**F**) Summary of effects of selected endogenous retinoids on TRPV1-expressing cells. Currents were recorded at a membrane potential of –60 mV. $n = 5$–10 per concentration for all retinoids examined.

activation on nociceptors is a main contributor to neurogenic inflammation (32, 33). Activation of TRPV1-positive nociceptors releases sensory neuropeptides such as calcitonin gene-related peptide (CGRP) from peripheral nerve endings in a number of peripheral tissues including esophagus and colon where TRPV1 is present exclusively in the extrinsic sensory fibers (34–39). Thus, we hypothesized that retinoids should also cause release of CGRP from peripheral nerve terminals by activating TRPV1. To test this possibility, we assessed the ability of retinoids to release CGRP from rat colon segments. AM580, 9-cis-RA, or ATRA (30–1000 μM) evoked a significantly enhanced release of CGRP from rat colon compared with vehicles (Figure 4A). Strikingly, pre- and coapplication of AMG9810 (1 μM) abolished or significantly attenuated CGRP release in response to AM580, 9-cis-RA, or

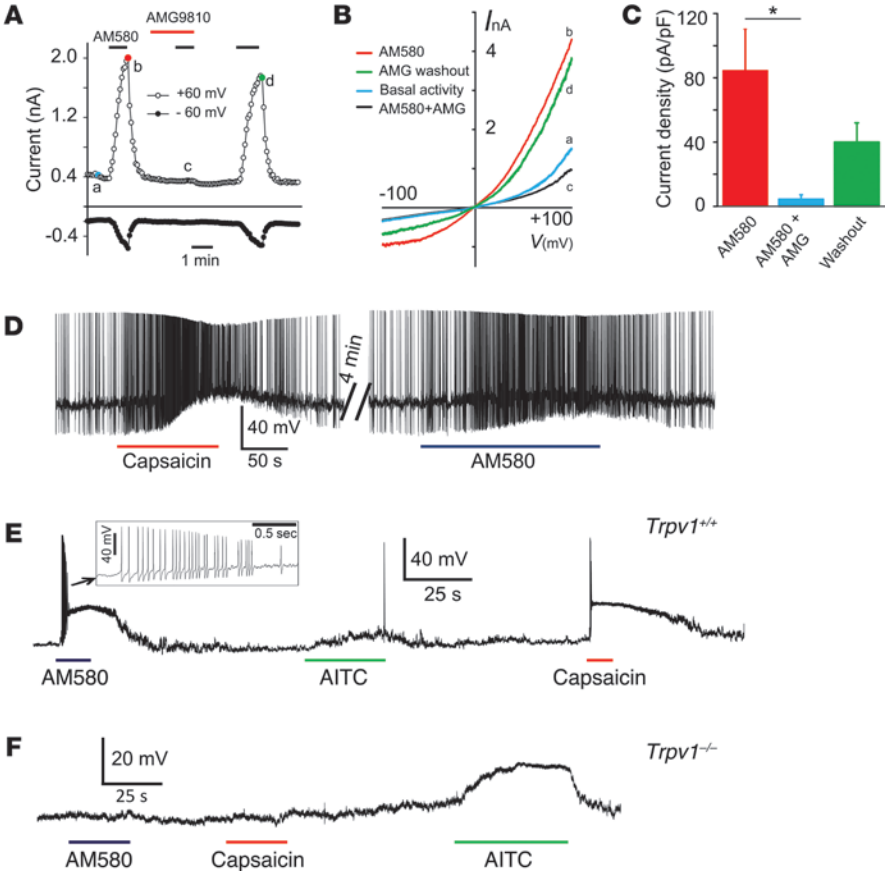

Figure 2

Pharmacological inhibition or genetic ablation of TRPV1 function abolished AM580-evoked membrane current, membrane depolarization, and action potential firing in DRG neurons. (**A**) Representative current traces show that AM580 (30 μM) activated an outward current at +60 mV and an inward current at –60 mV in a wild-type DRG neuron. The whole-cell currents evoked by AM580 were substantially suppressed by the selective TRPV1 antagonist AMG9810 (0.1 μM). The inhibitory effect of AMG9810 was partially reversible after washout. (**B**) Current-voltage relationship of AM580-activated current taken at time points specified in **A**. (**C**) Quantification of current responses to AM580 in DRG neurons voltage-clamped at –60 mV. AMG9810 (0.1 μM) significantly inhibited the AM580 response (*$P < 0.05$, $n = 6$). (**D**) Representative voltage traces illustrate that AM580 evoked a membrane depolarization and increased firing rate of a capsaicin-sensitive, tonically firing wild-type DRG neuron. (**E**) AM580 produced membrane depolarization and action potential firing in an electrically silent DRG neuron from a wild-type mouse that was also sensitive to both AITC and capsaicin. (**F**) AITC but not AM580 or capsaicin induced a membrane depolarization in a DRG neuron from a *Trpv1*$^{-/-}$ mouse. Drug concentrations: AM580, 30 μM; capsaicin, 1 μM; and AITC, 100 μM. MP, membrane potential.

ATRA (Figure 4A). Thus, each of these retinoids was capable of evoking release of CGRP from the peripheral sensory nerve endings of the TRPV1-positive neurons.

It is well known that sensory neuropeptides such as SP and CGRP, when released from the sensory nerve endings, promote plasma extravasation and inflammatory edema (19, 40). Consistent with this mechanism, we found that intraplantar administration of AM580, 9-cis-RA, or ATRA (100–600 nmol/20 μl) produced a significant increase in paw volume in the hind paws of wild-type mice (Figure 4B). Pretreatment with AMG9810 (30 mg/kg, i.p. injection) abolished or markedly attenuated this response (Figure 4B). Moreover, retinoid-induced increases in paw volume were abolished in *Trpv1*$^{-/-}$ mice. These results suggest that retinoids produce paw edema in a TRPV1-dependent manner.

Retinoids evoke nocifensive pain behavior. We next asked whether retinoids can elicit nocifensive behavior in vivo. Injection of AM580, 9-cis-RA, or ATRA into hind paws of mice (40–600 nmol/20 μl) immediately induced nocifensive behavior, including flinching and licking of the injected paw compared with vehicle-injected mice. The nocifensive responses were dose dependent (Supplemental Figure 4). Strikingly, the nocifensive responses to all 3 retinoids were either abolished or significantly inhibited by AMG9810 (50 mg/kg, i.p. injection) applied 30 minutes before intraplantar injections of individual retinoids (Figure 5). Furthermore, none of the retinoids evoked significant nocifensive behavior in *Trpv1*$^{-/-}$ mice (Figure 5). Therefore, retinoids evoke TRPV1-dependent nocifensive behavior in mice.

Retinoids produce TRPV1-dependent inflammatory hyperalgesia. TRP channels function as polymodal detectors of noxious chemical and physical stimuli and integrate information from various endogenous and environmental cues (20). TRPV1 plays a critical role in peripheral sensitization of nociceptors and is essential to thermal hypersensitivity in both acute and chronic inflammatory pain models (21, 41, 42). We thus asked whether injections of retinoids into a hind paw could produce heat hyperalgesia, which was assessed with the Hargreaves method (43). Paw injection of AM580, 9-cis-RA, or ATRA induced a robust and sustained thermal hypersensitivity lasting for at least 120 minutes in a dose-dependent manner (Figure 6, A–C, and Supplemental Figure 5, A–C). Consistent with TRPV1 being the mediator of retinoid-evoked thermal pain behaviors, pretreatment with AMG9810 (10 mg/kg, i.p. injection) for 30 minutes markedly inhibited the AM580-, 9-cis-RA– or ATRA-induced decrease of paw withdrawal latency upon heat stimulation (Figure 6, A–C). Strikingly, thermal hyperalgesia evoked by injections of AM580, 9-cis-RA, or ATRA was completely absent in *Trpv1*$^{-/-}$ mice (Figure 6, A–C).

Growing evidence indicates that TRPV1 also mediates mechanical hypersensitivity in a number of pain models including but not limited to inflamed bladder or colon, bone cancer pain, sickle cell disease, and pain after nerve injury or cutaneous inflammation (44–47). We therefore investigated whether AM580, 9-cis-RA, or ATRA evoked mechanical allodynia through stimulation of TRPV1. Indeed, all 3 retinoids produced a robust and sustained mechanical hypersensitivity lasting for at least 90 minutes following paw injections in a dose-dependent manner (Figure 6, D–F, and Supplemental Figure 5, D–F). Remarkably, genetic ablation of TRPV1 or pretreatment with the selective TRPV1 antagonist AMG9810 (10

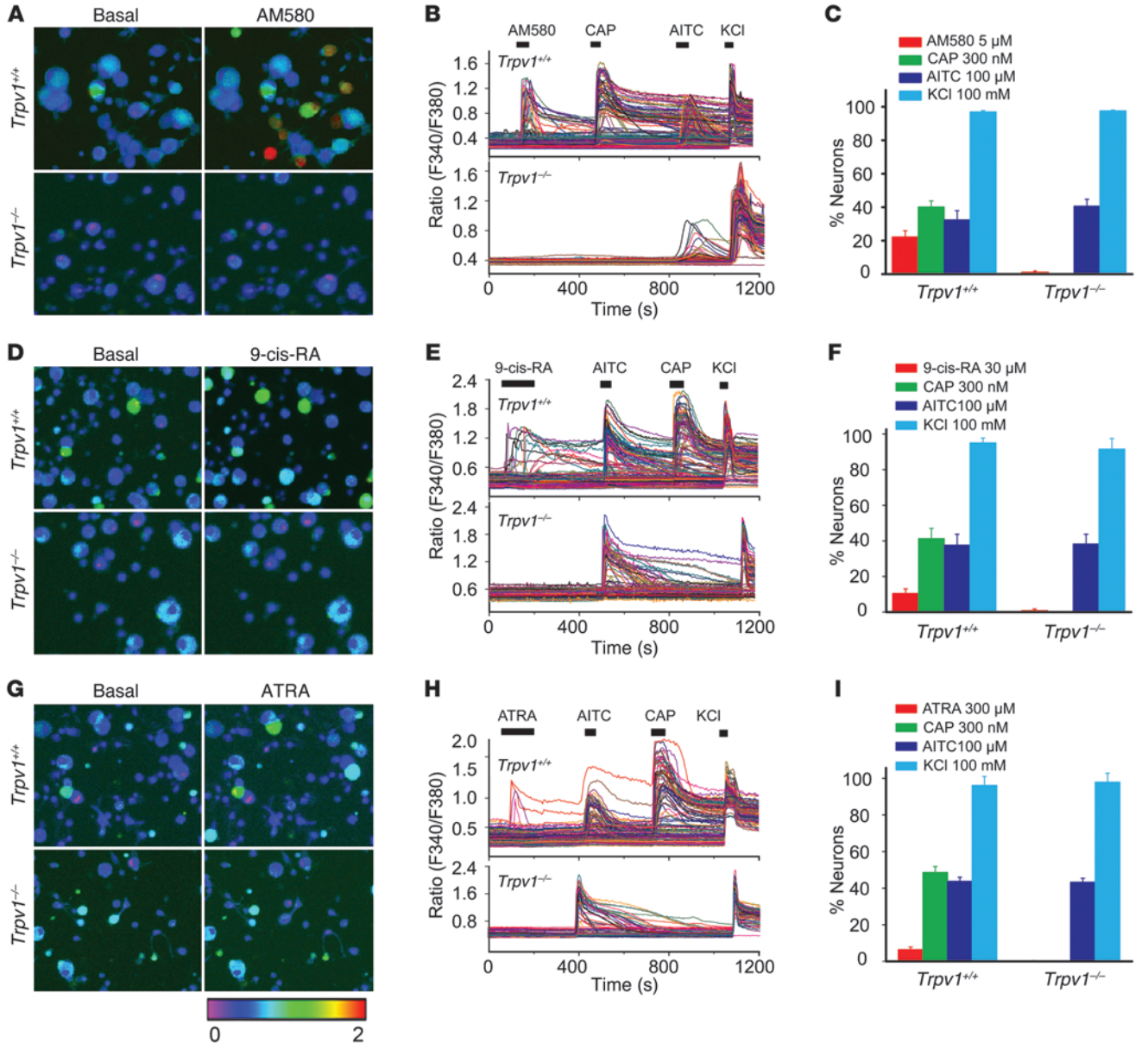

Figure 3

TRPV1 is the sole target of retinoids in sensory nociceptors. AM580 (**A**–**C**), 9-cis-RA (**D**–**F**), ATRA (**G**–**I**), and capsaicin evoked $[Ca^{2+}]_i$ responses in a subset of DRG neurons from *Trpv1*$^{+/+}$ but not *Trpv1*$^{-/-}$mice. (**A**, **D**, and **G**) Representative Fura-2 ratiometric images of cultured DRG neurons show that AM580 (**A**), 9-cis-RA (**D**), and ATRA (**G**) evoked $[Ca^{2+}]_i$ responses in a subset of DRG neurons from *Trpv1*$^{+/+}$ but not *Trpv1*$^{-/-}$ mice. The color of the neurons switching from blue to green or red indicates the increase of $[Ca^{2+}]_i$. (**B**, **E**, and **H**) Representative traces illustrate that AM580 (**B**), 9-cis-RA (**E**), ATRA (**H**), or capsaicin elicited $[Ca^{2+}]_i$ responses in *Trpv1*$^{+/+}$ but not *Trpv1*$^{-/-}$ DRG neurons. AITC evoked similar $[Ca^{2+}]_i$ responses in both *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ DRG neurons. Each trace corresponds to the change of fluorescence ratio in a single neuron. Neurons were exposed to each retinoid (5 μM AM580, 30 μM 9-cis-RA, or 300 μM ATRA), 0.3 μM capsaicin, 100 μM AITC, and 100 mM KCl for the indicated times. (**C**, **F**, and **I**) Percentage of DRG neurons responding to AM580 (**C**), 9-cis-RA (**F**), ATRA (**I**), capsaicin, AITC, and KCl in neurons isolated from *Trpv1*$^{+/+}$ or *Trpv1*$^{-/-}$ mice ($n \geq 330$ per genotype for AM580; n 350 per genotype for 9-cis-RA and ATRA).

mg/kg, i.p. injection) for 30 minutes abolished or substantially reduced AM580-, 9-cis-RA–, or ATRA-evoked mechanical hypersensitivity (Figure 6, D–F). Furthermore, injection of either vehicle or AMG9810 alone had no effect on baseline mechanical and thermal responses (Figure 6 and data not shown). These results demonstrate that TRPV1 senses retinoids in vivo and mediates both thermal and mechanical hypersensitivity produced by retinoids.

RAR antagonists are potent TRPV1 agonists and induce inflammatory hyperalgesia. Previous studies have shown that the pan RAR antagonist LE540 and the selective RARβγ antagonist AGN193109 could suppress retinoid-induced irritating responses (14, 48). We thus asked whether RARs also contribute to direct activation of nociceptors by retinoids. We tested the effect of LE540 and AGN193109 on AM580-activated membrane currents in DRG neurons. Unex-

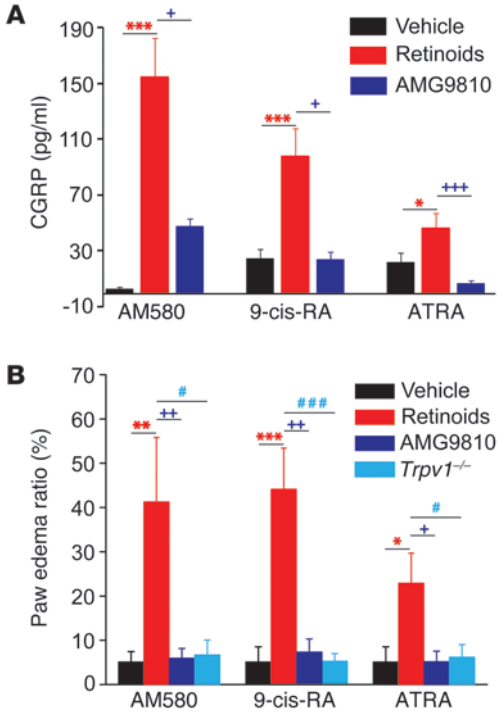

Figure 4
TRPV1 mediates retinoid-evoked CGRP release and paw edema. (**A**) AM580 (30 μM), 9-cis-RA (300 μM), and ATRA (1000 μM) increased CGRP levels in the perfusates from rat colon segments. AMG9810 (1 μM) significantly reduced the effect of all retinoids tested (n = 6). $^{*}P < 0.05$ and $^{***}P < 0.001$ versus vehicle; $^{+}P < 0.05$ and $^{+++}P < 0.001$ versus AMG9810. (**B**) Intraplantar injections of 20 μl of each AM580 (100 nmol), 9-cis-RA (100 nmol), or ATRA (600 nmol) significantly increased paw volume compared with that injected with vehicle controls. The paw edema ratio is the percentage increase of paw volume induced by retinoids. The effects of retinoids were markedly attenuated by AMG9810 (30 mg/kg, i.p. injection) or abolished in the *Trpv1*$^{-/-}$ mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle; $^{+}P < 0.05$, $^{++}P < 0.01$ versus AMG9810; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus *Trpv1*$^{-/-}$. n = 6–10 animals per condition.

pectedly, both LE540 and AGN193109 activated large membrane currents when applied to DRG neurons alone (Supplemental Figure 6, A–C). Both responses were abolished by pretreatment with AMG9810, suggesting that LE540 and AGN193109, like other structurally related retinoids, are indeed potent TRPV1 activators (Supplemental Figure 6, A–C). Consistent with these findings, both LE540 and AGN193109 activated recombinant TRPV1 in a concentration-dependent manner (Supplemental Figure 6, D–F). We further used calcium imaging to determine whether LE540 and AGN193109 could evoke Ca^{2+} influx in DRG neurons. LE540 produced a $[Ca^{2+}]_i$ response in about 5% of DRG neurons that were also capsaicin sensitive. Genetic ablation of TRPV1 function completely abolished LE540-induced $[Ca^{2+}]_i$ responses (Supplemental Figure 6, G–I, and data not shown). Responses to AGN193109 could not be measured because of large artifacts when administered to Fura-2–loaded DRG neurons, presumably caused by a direct interaction between AGN193109 and Fura-2. We next assessed the impact of paw injection of LE540 or AGN193109 on thermal pain behavior. Both LE540 and AGN193109 caused sustained thermal hyperalgesia lasting for 60 to 90 minutes, which was completely abolished by either pharmacological or genetic ablation of TRPV1 function (Supplemental Figure 7, A and B). These results provide what we believe is the first evidence that LE540 and AGN193109 directly activate nociceptors through TRPV1. Taken together, our results support a model in which diverse retinoids including RAR antagonists activate TRPV1 to produce pain and inflammation that is independent of retinoid-sensitive nuclear receptor activities.

Retinoids activate TRPV1 via the "vanilloid-binding pocket". Although TRPV1 integrates many pain-producing chemical and physical stimuli, distinct modular domains are involved in activation of TRPV1 by different modalities (49). Capsaicin and resiniferatoxin (RTX) bind to the "vanilloid-binding pocket" in the cytosolic side to initiate TRPV1 gating while extracellular protons and Mg^{2+} interact with acidic residues on the TRPV1 extracellular pore loop (50, 51), where several specific amino acid residues are also required to confer heat sensitivity to TRPV1 (Figure 7A and ref. 52). In contrast to mouse and human TRPV1, chicken TRPV1 expressed in HEK293T cells was insensitive to AM580 but retained sensitivity to acid (Figure 7B and Supplemental Figure 8).

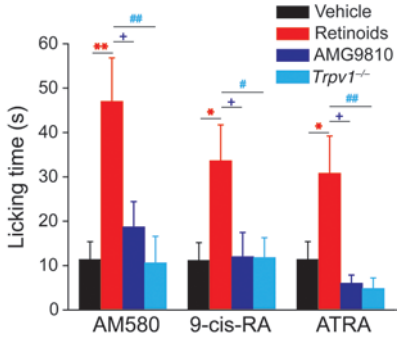

Figure 5
Ablation of retinoid-induced nocifensive responses by genetic deletion or pharmacological blockade of TRPV1. Intraplantar injection of 20 μl of each AM580 (100 nmol), 9-cis-RA (100 nmol), or ATRA (600 nmol) produced flinching and licking behaviors that were significantly reduced by i.p. injection of AMG9810 (50 mg/kg) 30 minutes before paw injection of retinoids. Genetic ablation of TRPV1 function totally abolished the nocifensive responses evoked by retinoids. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle; $^{+}P < 0.05$ versus AMG9810; and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus *Trpv1*$^{-/-}$. n = 6–10 animals per condition.

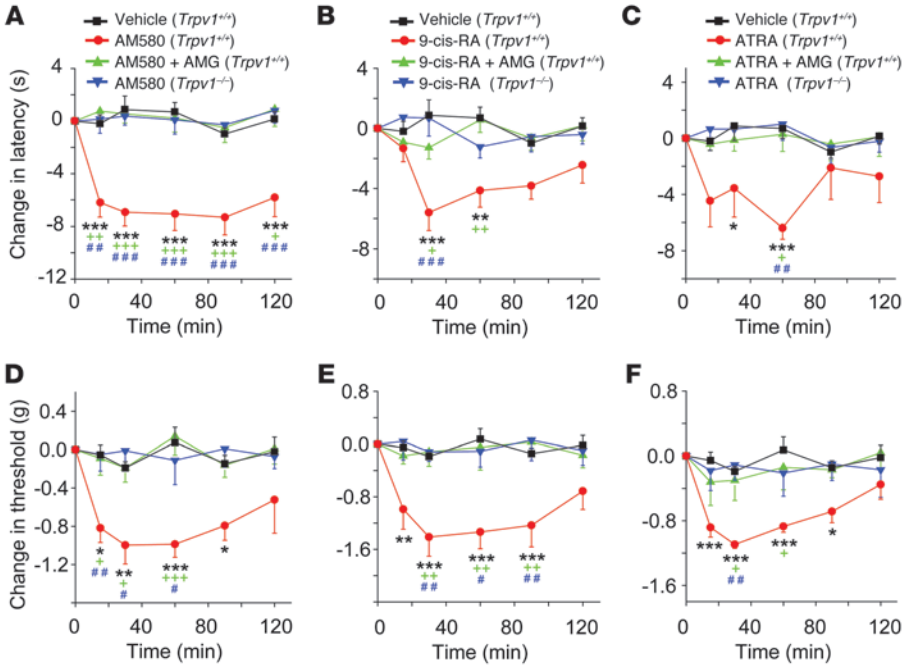

Figure 6

Pharmacological or genetic ablation of TRPV1 function abolishes retinoid-induced sensory hypersensitivity. (**A**–**C**) Time course of thermal hypersensitivity in animals treated with AM580 (**A**), 9-cis-RA (**B**). or ATRA (**C**). Intraplantar injection of 10 μl of each retinoid (AM580, 2 nmol; 9-cis-RA, 3 nmol; and ATRA, 30 nmol; red traces) induced thermal hyperalgesia in *Trpv1*$^{+/+}$ mice. AMG9810 (10 mg/kg; i.p. injection;green traces) abolished the effect of selected retinoids. Retinoid-elicited thermal hypersensitivity was also abolished in *Trpv1*$^{-/-}$ mice (blue traces). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle; $^{++}P < 0.01$, $^{+++}P < 0.001$ versus AMG9810; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus *Trpv1*$^{-/-}$. (**D**–**F**) Time course of mechanical allodynia in animals treated with AM580 (**D**), 9-cis-RA (**E**), and ATRA (**F**). Intraplantar injection of 10 μl of each retinoid (AM580, 2 nmol; 9-cis-RA, 3 nmol; and ATRA, 30 nmol; red traces) produced mechanical hypersensitivity in *Trpv1*$^{+/+}$ mice, which was abolished by i.p. injection of AMG9810 (10 mg/kg; green traces). Retinoid-elicited mechanical hypersensitivity was also abolished in the *Trpv1*$^{-/-}$ mice (blue traces). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ versus AMG9810; and $^{\#}P < 0.05$, $^{\#}P < 0.01$ versus *Trpv1*$^{-/-}$. Please note that no effect was observed upon injection of 10 μl vehicle alone (0.9% saline; black traces). n = 5–10 animals per condition. Baseline values for mechanical and thermal testing are listed in Supplemental Table 2.

This is reminiscent of the insensitivity of the chicken TRPV1 to capsaicin and structurally related analogs (53). The vanilloid-binding pocket is composed of residues facing the cytoplasm (R115, E762) and residing within the inner leaflet of the lipid bilayer (Y512, S513, M548, and T551) (Figure 7A and ref. 54). Mutants carrying Y512A or S513Y residues display substantially reduced sensitivity to capsaicin without altered proton activation (53). We thus examined whether these mutants also show altered sensitivity to retinoids. AM580-activated currents were dramatically decreased in the Y512A, S513Y, T550I, Y512A/S513Y, and Y512A/S513A mutants (Figure 7C). EC_{50} values for AM580 were increased by at least 10-fold in these mutants (Figure 7C and Supplemental Figure 9, A–C). Similarly, 9-cis-RA–activated current was also strongly reduced in Y512A and S513Y mutants but not the S503A mutant in which protein phosphorylation is severely compromised (54) (Figure 7D). EC_{50} values for AM580 in mutants S513A, M548L, and T550I were also increased, although not as much as in Y512 and S513 mutants (Figure 7C). On the other hand, in TRPV1 mutants with disrupted sites for protein phosphorylation, proton and heat activation did not affect AM580-induced responses (Figure 7A, Supplemental Figure 9, and Supplemental Table 3). These results demonstrate that structural elements required for vanilloid-binding to TRPV1 are also essential to retinoid interaction with TRPV1.

Discussion

Retinoids that are extensively used in the treatment of skin disease and cancer can cause toxicity and produce sensory hypersensitivity associated with burning, pruritus, and inflammation in humans when applied topically or systemically (6–8, 13). Oral or intrathecal application of vitamin A derivatives also was shown to increase nocifensive responses in rodents with or without tissue inflammation (14, 15). However, the cellular and molecular basis of retinoid signaling in the pain pathway had not been explored. In this study, we investigated the excitatory action of retinoids on pain-initiating TRPV1 channels and discovered that TRPV1 is a primary molecular target for both naturally occurring and synthetic retinoids, and TRPV1 is both necessary and sufficient for retinoid-induced activation of primary nociceptors and retinoid-evoked sensory hypersensitivity, most convincingly by results of genetic or pharmacological ablation of TRPV1 function. Our work suggests that this signaling pathway is capable of generating the irritating side effects of retinoids independently of gene regulation.

Pain results from complex processing of neural signals at multiple levels (21). TRPV1 at both peripheral and central terminals of nociceptors is one of the key players that initiate both pruritus and pain sensation (55). Activation and sensitization of nociceptors by retinoids should induce pain and neurogenic inflammation. Indeed, our findings show that retinoids at the

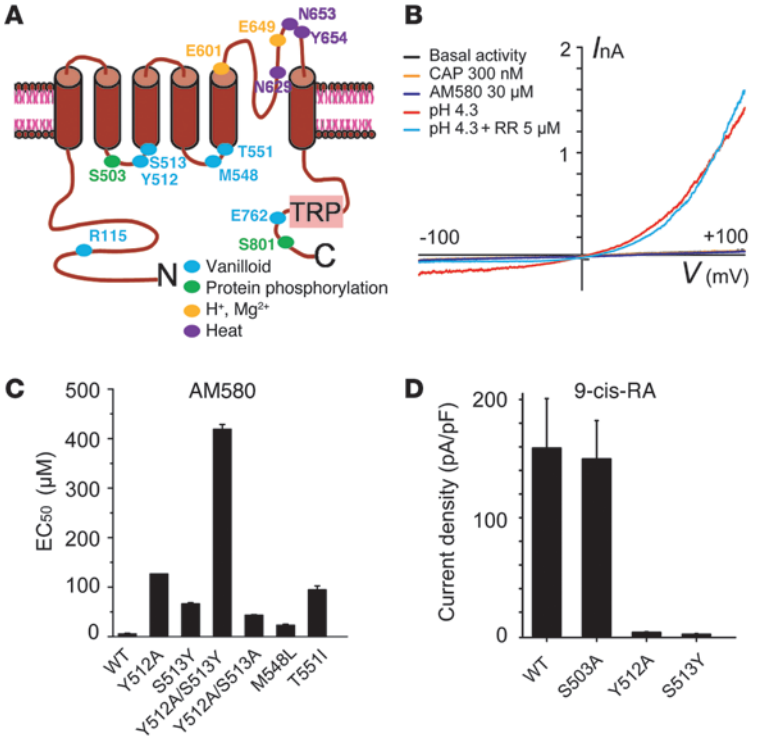

Figure 7
The vanilloid-binding pocket of the TRPV1 confers retinoid sensitivity. (**A**) Schematic diagram illustrates structural elements required for activation/modulation of TRPV1 by capsaicin (blue circle), protein phosphorylation (green circle), protons (yellow circle), and heat (purple circle). (**B**) Representative I-V curves illustrate that the chicken TRPV1 was activated by pH 4.3 but not AM580 or capsaicin (n = 5). The nonselective TRP channel blocker ruthenium red (RR) abolished the inward but not the outward proton-activated current. (**C**) Quantification of EC_{50} values for AM580-activated currents at –60 mV in wild-type or TRPV1 mutants with disrupted vanilloid-binding pocket. (**D**) 9-cis-RA–activated (30 μM) membrane currents (at –60 mV) were nearly abolished in TRPV1 Y512A or S513Y mutant but not S503A mutant (n = 4–7 per condition).

doses well within the range that occurs clinically produce powerful sensitization of both thermal and mechanical responses. TRPV1-mediated responses to exogenous retinoids are likely to produce irritation or mild pain that could be a warning response to overexposure of retinoids, especially under a preexisting inflammatory state where TRPV1 function is upregulated, as in acne or sensitive and aged skin (56–58).

While we have not as yet pursued a possible role of endogenous retinoid in regulation of TRPV1, our finding that TRPV1 acts as an ionotropic receptor for retinoids raises the question of whether TRPV1 can also sense endogenous retinoids. Vitamin A is stored as retinyl esters (REs) in liver and delivered into the bloodstream as retinol bound to retinol-binding proteins (RBPs) (59). In fact, retinoids are highly enriched in the spinal cord and brain, and the CNS seems to synthesize retinoids more efficiently than other tissues through an unknown mechanism (60). Retinoids are critically involved in spinal neuronal development and promote recovery from spinal cord injury–induced motor dysfunction (61). Retinoids also play important roles in axonal outgrowth, elongation, regeneration, myelin formation, neural plasticity, and peripheral neuropathy in diabetes etc. (62, 63). Our results show that 9-cis-RA and ATRA are potent endogenous retinoids activating TRPV1 in vitro and in vivo and produce neurogenic inflammation and sensory hypersensitivity following intraplantar injections. Changes in endogenous retinoid metabolism during inflammation and nerve injuries could alter TRPV1 function and thus participate in nociception, allodynia, and hyperalgesia (64).

Our results show that the vanilloid-binding pocket is required for retinoid activation of TRPV1, disruption of which leads to a decreased potency of retinoids to activate TRPV1. Thus, we do not expect that retinoids directly activate epidermal keratinocytes through TRPV1 since keratinocytes are vanilloid resistant (65). On the other hand, retinoid signaling can also regulate TRPV1 function indirectly. For instance, ATRA treatment enhances TRPV1 protein expression and function in TRPV1-expressing SHSY5Y neuroblastoma cells (66). Retinoid signaling can also influence levels of other proinflammatory mediators, such as NGF and prostaglandins, which could change pain sensation under disease conditions such as diabetes mellitus (62, 67).

In our characterization of the panel of retinoid agonists and antagonists, we found that AGN193109 and LE540, 2 RAR antagonists, are potent TRPV1 activators and excite primary nociceptors to elicit thermal hypersensitivity in a TRPV1-dependent manner. Both AGN193109 and LE540 are retinoid analogs that bind to but do not activate RARs, thus displaying antagonist activities to RARs (68, 69). This result is not surprising given the differences in the ligand-binding pockets for the RARs and TRPV1. The effectiveness of AGN193109 and LE540 to activate TRPV1 might result from their structural similarities to the TRPV1-activating retinoids (Supplemental Figure 2). Similarly, a recent report shows that an antagonist of LPA receptors with a structure similar to that of LPA also activates TRPV1 (25). From these findings, application of these drugs would be expected to both inhibit RAR activity and simultaneously activate TRPV1. However, paradoxically, it has been reported these RAR antagonists inhibit retinoid-induced irritation (14, 48). A possible explanation for this paradox is that the inhibition results from desensitization of TRPV1 instead of inhibition of retinoid nuclear receptors. It has been established that TRPV1 activators such as capsaicin cause TRPV1 desensitization (70), and we have found that the retinoid agonists used in this study also cause desensitization (Supplemental Figure 3 and data not shown). It is possible that preapplication and/or coapplication of RAR receptor antagonists might desensitize and attenuate the irritating action evoked by subsequent application of retinoids, since they both activate TRPV1.

In summary, this study demonstrates that TRPV1 is an ionotropic retinoid receptor that mediates retinoid-evoked activation of nociceptors and provides a plausible mechanism to explain the phenomenon of retinoid-induced toxicity. Our results provide further insight into the diversity of noxious signals that are sensed by TRPV1 to initiate neurogenic inflammation and sensory hypersensitivity. These studies point to what we believe is a novel therapeutic target for retinoid-evoked irritating side effects that can benefit from the availability of selective TRPV1 antagonists. Although systemic application of TRPV1 antagonists has a pain-inhibiting effect, it causes hyperthermia in many species, including humans (71). Recent studies revealed that inhibition of proton binding to TRPV1 might be the mechanism underlying hyperthermia induced by TRPV1 blockers (72). New blockers that spare the proton activation of TRPV1 might be effective and safe drugs that can be used to treat pain and inflammation (73). In addition, topical instead of systemic application of TRPV1 antagonists with retinoids might be an attractive approach to suppress retinoid-induced irritation locally without causing hyperthermia. Furthermore, peripheral manipulation of TRPV1 function should not alter nuclear receptor activation, which increases the promise of TRPV1 as a therapeutic target to reduce inflammation and pain resulting from the clinical use of retinoids.

Methods

Animals. *Trpv1*$^{+/+}$and congenic *Trpv1*$^{-/-}$ mice on the C57BL/6J background were obtained from Jackson Laboratories and were bred at the University of Texas Health Science Center at Houston. Mice were housed in a temperature- and humidity-controlled environment on a 12-hour light/12-hour dark cycle with free access to food and water.

Chemicals. β-carotene, and all trans-retinal, retinol, acitretin, capsaicin, and AMG9810 were purchased from Sigma-Aldrich; 9-cis-retinoic acid and 13-cis-retinoic acid were from MP Biomedicals LLC; AM580 was from Tocris; all trans-retinoic acid and 4-HPR were from Enzo Bioscience; bexarotene was from CT-BEX ChemieTek; tazarotene was from Selleckchem; AITC was from ACROS; AGN 193109 was from Santa Cruz Biotechnology Inc.; papain and collagenase (type 2) were from Worthington. LE540 was a generous from Hiroyuki Kagechika (Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan).

Molecular biology, HEK293T cell culture and transfection. HEK293T cells were grown as a monolayer using passage numbers less than 30 and maintained in DMEM (Life Technologies), supplemented with 10% FBS (Life Technologies), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO_2. The cells were transiently transfected with cDNA for mouse TRPV1 (mTRPV1), mTRPV1 mutants, human TRPV1 (hTRPV1) (a gift from Huai-hu Chuang, The Institute of Molecular Biology, Academia Sinica, Taiepi, Taiwan), or chicken TRPV1 (cTRPV1) using Lipofectamine 2000 (Invitrogen) with a ratio of 0.8:2. Following transfection, the cells were maintained in DMEM at 37°C for 24 hours before use. All TRPV1 mutants were made using the QuikChange II XL Mutagenesis Kit (Agilent Technologies Inc.) according to the manufacturer's directions. All mutations were confirmed by DNA sequencing.

Isolation and short-term culture of mouse DRG neurons. The spinal column was removed and placed in ice-cold HBSS; laminectomies were performed and bilateral DRG were dissected out. Neurons were acutely dissociated and maintained as described (24). In brief, after removal of connective tissues, DRG were transferred to a 1-ml Ca^{2+}/Mg^{2+}-free HBSS containing 2 μl saturated $NaHCO_3$, 0.35 mg L-cysteine, and 20 U papain (Worthington) and incubated at 37°C for 10 minutes. DRG were spun down, the supernatant was removed, and 1-ml Ca^{2+}/Mg^{2+}-free HBSS containing 4 mg collagenase type II and 1.25 mg dispase type II (all from Sigma-Aldrich) was added and incubated at 37°C for 10 minutes. After digestion, neurons were pelleted, suspended in Neurobasal medium containing 2% B-27 supplement, 1% L-glutamine, 100 U/ml penicillin plus 100 μg/ml streptomycin, and 50 ng/ml nerve growth factor (NGF), plated on a 12-mm coverslip coated with poly-l-lysine (10 μg/ml), and cultured under a humidified atmosphere of 5% CO_2/95% air at 37°C for 18–24 hours before use.

Ratiometric measurement of intracellular free Ca^{2+}. Cultured DRG neurons and TRPV1-expressing HEK293T cells were loaded with 4 μM Fura-2 AM (Life Technologies) in culture medium at 37°C for 60 minutes. Cells were then washed 3 times and incubated in HBSS at room temperature for 30 minutes before use. Fluorescence at 340 nm and 380 nm excitation wavelengths was recorded on an inverted Nikon Ti-E microscope equipped with 340-, 360-, and 380-nm excitation filter wheels using NIS-Elements imaging software (Nikon Instruments Inc.). Fura-2 ratios (F340/F380) reflect changes in $[Ca^{2+}]_i$ upon stimulation. Values were obtained from 100–250 cells in time-lapse images from each coverslip. Threshold of activation was defined as 3 SD above the average (~20% above the baseline).

Patch-clamp recordings. Whole-cell and single-channel patch-clamp recordings were performed using an EPC 10 amplifier (HEKA Elektronik) at room temperature (22–24°C) on the stage of an inverted phase-contrast microscope equipped with a filter set for GFP visualization. Pipettes pulled from borosilicate glass (BF 150-86-10; Sutter Instrument) with a Sutter P-97 pipette puller had resistances of 2–4 and 8–10 megaohms for whole-cell and single-channel recordings, respectively, when filled with pipette solution containing 140 mM CsCl, 2 mM EGTA, and 10 mM HEPES with pH 7.3 and 315 mOsm/lin osmolarity. Symmetrical solutions with the same components as that in the pipette solution were used for single-channel recordings. Except for TRPV1 desensitization experiments, a Ca^{2+}-free extracellular solution was used for whole-cell recording to avoid Ca^{2+}-dependent desensitization of TRPV1, containing the following: 140 mM NaCl, 5 mM KCl, 0.5 mM EGTA, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to ≈340 mOsm/l with sucrose). The whole-cell membrane currents were recorded using voltage ramp from –100 to +100 mV during 500 ms at holding potential of 0 mV. Data were acquired using Patchmaster software (HEKA Elektronik). Currents were filtered at 2 kHz and digitized at 10 kHz. Data were analyzed and plotted using Clampfit 10 (Molecular Devices). Single-channel events were identified on the basis of the half-amplitude threshold-crossing criteria. Open probability was determined from idealized traces as the ratio of the sum of all open durations to the total trace duration.

Retinoid-induced release of CGRP. CGRP release was determined as previously described with modifications (74). In brief, adult male Sprague-Dawley rats (7 to 8 weeks) were anesthetized with isoflurane. The colon was excised at the junction of the ascending colon and cecum and at the junction of the rectum and anus. Colon segments (1.5-cm each) were transferred to the Krebs solution and bubbled with 95% O_2 and 5% CO_2 at 37°C for 30 minutes for equilibration. Each segment was transferred to an individual tube with 400 μl Krebs solution in the shaking bath and incubated for 20 minutes before elutions (100 μl) were collected for baseline measurement. Each segment was then transferred to another tube with retinoids as indicated and incubated for another 20 minutes. 30 μM AM580 containing 0.1% DMSO, 300 μM 9-Cis-RA, and 1000 μM ATRA containing 3% DMSO and 0.2% Tween 80 were applied. The TRPV1 antagonist AMG9810 was pretreated for 30 minutes before addition of retinoids. The CGRP-LI was measured with a CGRP EIA kit according to the manufacturer's instructions (Cayman Chemicals) and read at 414 nm by the Flexstation 3 (Molecular Devices) (75). The concentration of CGRP-LI released into the medium is determined by comparing the light absorption values of the samples to that of their standard curves.

Paw edema test. Edema was induced by intraplantar injection of 20 μl of AM580, 9-cis-RA, or ATRA freshly prepared in vehicle (5% DMSO + 0.25% Tween 80) into the right-hind paws of *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$mice. Paw volumes were measured just before and then 2 hours after injection of retinoids using a plethysmometer (IITC) according to the manufacturer's instructions. To pharmacologically suppress TRPV1, AMG9810 (50 mg/kg, i.p. injection) was applied 30 minutes prior to injection of retinoids. The increase in percentage of paw volume was calculated based on the volume difference between the normal and abnormal paws (with or without injection of retinoids). The following equation was used: paw edema ratio (%) = (paw volume after injection of retinoids – paw volume before injection)/paw volume before injection × 100%.

Nocifensive response. Intraplantar injection of retinoids was used to induce nociceptive responses as described (24). Immediately after injection, mice were placed inside a Plexiglas chamber. Total time spent licking and lifting the injected hind paw was measured from video recordings (5 minutes). AMG9810 was administered i.p. 30 minutes before intraplantar injection of retinoids. Control mice received equivalent volumes of the relevant vehicle (5% DMSO + 0.25% Tween 80).

Thermal and mechanical behavioral tests. Hargreaves apparatus (Plantar Analgesia meter) and von Frey apparatus (Dynamic Plantar Aesthesiometer) were from IITC Life Science Inc. Mechanical or thermal hyperalgesia assays were performed as described (41, 76). Briefly, mice were acclimated for 60 minutes to the testing environment prior to all experiments. Paw withdrawal latencies in response to radiant heat were measured using the Hargreaves apparatus. Briefly, each mouse was placed individually in clear Plexiglas chambers (8 × 8 × 12 cm) and acclimated for at least 1 hour before testing. Left hind paws of mice were injected intraplantarly with 10 μl vehicle (saline + 1% DMSO + 0.1% Tween 80) with or without chemicals. For assessment of thermal nociception, left hind paw withdrawal latencies were measured before (0 minutes) and 15, 30, 60, 90, and 120 minutes after injections. The infrared intensity was adjusted to obtain basal paw withdrawal latencies of 10 to 15 seconds. An automatic 20-second cut-off was used to prevent tissue damage. For assessment of mechanical allodynia, starting with the 0.4 g filament, von Frey filaments ranging from 0.04 to 4 g bending force were applied to the plantar skin of the left hind paw, using the up-down method to determine threshold sensitivity. von Frey threshold was measured at 15, 30, 60, 90, and 120 minutes after injection. AMG9810 (10 mg/kg, i.p. injection) was given 30 minutes before paw injections of retinoids. To reduce the effects of baseline variability among animals, withdrawal responses were expressed as differences from baseline across groups (77). All experiments were performed blind with respect to genotype and treatment.

Statistics. All data are presented as mean ± SEM for *n* independent observations. Student's *t* test was used to analyze statistical significance between control and experimental groups. ANOVA and repeated measures tests were used to test hypotheses about effects in multiple groups occurring over time. $P < 0.05$ was considered significantly different.

Study approval. All experiments involving mice and rats were approved by The University of Texas Health Science Center at Houston Animal Welfare Committee.

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Address correspondence to: Hongzhen Hu, Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, 6431 Fannin Street, Houston, Texas 77030, USA. Phone: 713.500.7525; Fax: 713.500.7524; E-mail: Hongzhen.Hu@uth.tmc.edu.

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