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Involvement of TRPV1 and TDAG8 in pruriception associated with noxious acidosis

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Abbreviation: ASIC, acid-sensing ion channel; CP40/80, compound 40/80; DCA, deoxycholic acid, DRG, dorsal root ganglion; GPCR, G-protein-coupled receptor; H1R, histamine H1 receptor; IB4, isolectin B4; Mrgpr, Mas-related G protein-coupled

receptor; Nav1.8, voltage-gated sodium channel 1.8; TRPV1, transient receptor potential cation channel V1; TDAG8, T-cell death-associated gene 8

Abstract

Itch and pain are closely related but distinct sensations. Intradermal injection of acid generates pain in both rodents and humans; however, few studies have addressed the intriguing question of whether proton can evoke itch like other algogens at the basis of spatial contrast activation of single nociceptors. Here, we report that (1) citric acid (0.2 M) pH-dependently induced a scratching response in mice when applied intradermally to nape or cheek skin; (2) acidified buffer elevated intracellular calcium levels in dorsal root ganglion (DRG) pruriceptors; (3) injection of intradermal citric acid (pH 3.0) into the nape induced a pruritogen-like but not algogen-like c-Fos immunoreactivity pattern in the cervical spinal cord. Using pharmacological and genetic approaches, we identified potential acid-sensing channels/receptors involved in acidic citrate-evoked itch. Results indicate that TRPV1 but neither ASIC3 nor TRPA1 are involved in the acidic citrate-induced scratching response. Furthermore, one of the proton-sensing G-protein-coupled receptors, TDAG8, was highly (~71%) expressed in $Nppb^{+}$ DRG pruriceptors. Acidic citrate but not α-methyl-5-HT, chloroquine, compound 48/80 or bile acid-induced itch was markedly decreased in

TDAG8$^{-/-}$ mice. In a heterologous expression system, TDAG8 potentiated the acid-induced calcium response by regulating TRPV1. Thus, proton could evoke pruriception by acting on TDAG8 to regulate TRPV1 activation with its mechanism of future therapeutic relevance.

Introduction

Itch is defined as an unpleasant sensation that elicits the desire or reflex to scratch. The urge to scratch the affected skin physically is the key episode defining itch and thus protects mammals against possible contact with potential "danger molecules" like parasites or irritants (Ikoma *et al*., 2006). Pain is defined as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (by the International Association for the Study of Pain). Although both somato-sensory sensations activating sensory nerves, itch and pain can be differentiated by psychophysiological and molecular characteristics (LaMotte *et al*., 2014). Dorsal root ganglion (DRG) pruriceptors anatomically represent a subpopulation of DRG nociceptors positive for transient receptor potential cation channel V1 (TRPV1) (Han *et al*., 2013), for example.

Recent progress in itch studies have demonstrated that pruritogens, such as histamine, serotonin, endothelin-1 and chloroquine, induce itch sensations via direct action on their specific receptors, histamine H_1 receptor (H1R), serotonin 5-HT_2 receptor, ET_A receptor and Mas-related G protein–coupled receptors (Mrgpr) A_3

receptor, respectively, in the DRG pruriceptors (Bautista *et al*., 2014). Most, if not all, DRG pruriceptors are TRPV1-expressing, and within this population, only a subset of $Mrgprs^{+}$ pruriceptors express TRPA1. However, itch and pain are somehow distinct because of the recent discoveries of itch-specific effectors, modulators and neurotransmitters (Sun *et al*., 2007; Liu *et al*., 2009; Goswami *et al*., 2014). The label-line theory of itch has gained strong support because genetic knockout or pharmacological ablation of these itch-specific components impede the itch response and leave the pain behavior intact. One unsolved question is why some well-known algogens (e.g., capsaicin) also induces itch (Sikand *et al*., 2009). The opponent theory emphasizes that mechanical/painful scratching inhibits itching, and morphine-like analgesics suppress pain but enhance itching. The opponent action between itch and pain could occur at the spinal cord but not DRG level (Liu *et al*., 2011). Pattern theory, which claims that the ultimate perceived sensation is encoded across the pattern of peripheral nerve activation before being decoded by the central brain, might provide the answer, but supporting evidence is limited (Ikoma *et al*., 2006). Spatial contrast theory reconciles contradictory findings of itch studies, which

states that itch arises from a sharp contrast between individual nociceptors firing among the surrounding silent neighbors; pain would be felt if a more homogenous activation of surrounding nociceptors (Namer & Reeh, 2013).

Increasing evidence indicates that TRPV1 and TRPA1 are downstream effectors in histamine- and Mrgpr–dependent itching, respectively (Shim *et al*., 1997; Wilson *et al*., 2011). Histamine-induced itch is specifically reduced in *TrpV1*$^{-/-}$ but not *TrpA1*$^{-/-}$ mice, but chloroquine-induced itch is specifically impeded in *TrpA1*$^{-/-}$ but not *TrpV1*$^{-/-}$ mice. Histamine, when acting on H1R, may activate phospholipase Cβ (PLCβ) and thus functionally couple to TRPV1 to exert itch. However, chloroquine, activates MrgprA3 to release Gβγ subunits to activate TRPA1.

Acid (proton) is a potent algogen inducing pain in humans and rodents by activating TRPV1, acid-sensing ion channels (ASICs), and proton-sensing G-protein-coupled receptors (GPCRs) such as OGR1, TDAG8, G2A, and GPR4 (Sun and Chen, 2016). Previously, we have shown that acidic citric buffer (pH 3.0) induced itch in histamine-sensitized skin in healthy people and lesional skin in patients with atopic dermatitis (Ikoma *et al*., 2004). However, the molecular mechanism underlying

the citrate-induced itch is unknown. In this study, we provide evidence that acidic citrate can act on TDAG8 coupled with TRPV1 to induce nociception resulting in itch-like behavior in mice.

RESULTS

Intradermal acid solution induced scratching response in mice

To verify whether proton can induce an itch-like sensation in mice, we first screened the scratching behavior in wild-type male mice by intradermal injection of formic acid. When 10 μL formic acidic solution (pH 1.9, 3.0, or 4.7) was injected into the nape skin of mice, only at the highest acidic pH 1.9, mice displayed scratching behavior (Figure 1a).

Since proton without a buffer system is easily diluted in the skin tissue, we next used buffered citric acid to test the scratching response. The amount of 0.2 M citric acid was adjusted with 0.2 M tri-sodium citrate to pH 7.4, 4.7, 3.0 and 1.9. Compared with normal saline, pH7.4 citric acid induced some scratching responses due to a reduction of free calcium, as a similar effect was also observed in mice treated with

EGTA-saline (Figure S1). With [H^+] concentration increased, citric acid pH-dependently increased scratching behavior in the mouse nape-skin model (Figure 1b).

We compared the itch-inducing capacity of citric acid (pH 3.0) with several well-known pruritogens, including deoxycholic acid (DCA), α-methyl-5-HT, chloroquine and compound 48/80 (CP40/80), in different groups of wild-type mice. The order of itch-producing potency in nape-skin model was CP48/80 (100 μg) > chloroquine (200 μg) > α-methyl-5-HT (30 μg) > citric acid (pH 3.0) > DCA (25 μg) (Figure 1c). Because subcutaneous acid treatment has been reported to produce pain in both humans and rodents, we verified whether citric acid (pH 3.0) was a pure pruritogen or combined algogen property in the mouse-cheek assay. Compared with pain-inducing algogen "capsaicin," which induced mainly a wiping response, itch-inducing pruritogen "chloroquine" induced mainly a scratching response. Citric acid induced an equivalent wiping response to capsaicin (which indicates that proton is an algogen) and significantly more scratching response than capsaicin (which indicates that proton is also a pruritogen) (Figure 1d). Chlorpheniramine, an

anti-hi**s**tamine drug, inhibited the citric acid-induced scratching in both nape and cheek skin models (Figure S2). Similar to humans, citric (or even formic) acid (pH3.0)-induced scratching was enhanced in histamine-sensitized skin (Figure 1e,f). Our results support that proton not only induces nociception but also pruriception.

Acid induced calcium transients in DRG pruriceptors and generated itch-specific c-Fos immunoreactivity in the spinal dorsal horn

To harvest DRG pruriceptors for calcium imaging study, we used the double transgenic mice bearing Nav1.8-Cre and the Cre reporter CAG-STOP^{floxed}-Td-tomato to label Nav1.8^{+} DRG neurons, which cover most population of nociceptors and pruriceptors (Shields *et al*., 2012). After loading with Fura-2, Td-tomato^{+} small-to-medium sized Nav1.8-positive DRG neurons were sequentially challenged with histamine (100 μM), chloroquine (1 μM), acidic 1x HBSS (pH 3.0), capsaicin (10 μM) and KCl (75 mM) (Figure 2a-c). In 3 independent trials, 66% (144/215) and 54% (118/215) of the selected neurons responded to capsaicin and acid, respectively. About 20% (43/215), ~16% (36/215) and ~14% (32/215) of the selected neurons responded to histamine,

chloroquine and both, respectively. All acid-responsive neurons were capsaicin-responsive and all histamine- and/or chloroquine-responsive neurons were acid-responsive (Figure 2d). DRG pruriceptors conveyed itch signals to the secondary relay neurons, which are $Npra^{+}$ and Grp^{+}, in the laminae I and II of the spinal dorsal horn, where c-Fos expression is induced after injection of 5-HT (Akiyama *et al*., 2009) or chloroquine (Zhang *et al*., 2014) in the nape-skin model. We verified whether citric acid at pH 3.0 could generate the same itch-specific c-Fos expression. For comparison, we used capsaicin and CP48/80 as a negative and positive control, respectively, because capsaicin did not generate a scratching response and CP48/80 was the most potent pruritogen we found in Figure 1c. After 30 min of scratching with CP48/80 and acid treatment, condensed c-Fos immunoreactivity was observed in the lateral superficial dorsal horn, which was not observed with capsaicin and saline treatment (Figure 2e-h).

Acidic citrate-induced scratching is TRPV1-dependent

In general, itch can be classified into two categories: histamine- and

non-histamine–dependent itch, modulated by downstream activation of TRPV1 and TRPA1 channels, respectively (Bautista *et al*., 2014). Furthermore, TRPV1 and ASIC3 in the peripheral terminals of primary sensory neurons are well known to sense tissue acidosis (Chen *et al*., 2014). We pharmacologically and genetically tested whether TRPV1, TRPA1, and ASIC3 are involved in acidic citrate-induced scratching. In wild-type mice, capsazepine (a selective TRPV1 antagonist), HC-030031 (a selective TRPA1 antagonist) or amiloride (the pan-ASIC blocker) was co-injected intradermally with citric acid (pH 3.0) in the nape skin of wild-type mice. Intradermal injection of capsazepine significantly inhibited the acidic citrate-induced scratching (Figure 3a). Capsazepine also inhibited the enhancement of acidic citrate-induced scratching in histamine-sensitized skin (Figure S3). In *TrpV1*$^{-/-}$ mice, acidic citrate-induced scratching was, however, impeded in part as compared with wild-type controls (Figure 3b). Intradermal HC-030031 had no effect on acidic citrate-induced scratching (Figure 3c); in *TrpA1*$^{-/-}$ mice, acidic citrate-induced scratching was normal (Figure 3d). Intradermal amiloride had no effect on acidic citrate-induced scratching (Figure 3e); in *Asic3*$^{-/-}$ mice, acidic citrate-induced scratching was normal (Figure 3f).

From these *in vivo* studies, we concluded that TRPV1 but not TRPA1 or ASICs played an important role in mediating acidic citrate-induced scratching in mice.

TDAG8 is the most abundant proton-sensing GPCRs in DRG pruriceptors

Because acidic citrate-induced scratching was partially impeded in *TrpV1*$^{-/-}$ mice, activation of TRPV1 in DRG pruriceptors cannot fully explain the mechanism of acid-induced effect. We wondered whether any of proton-sensing GPCRs is expressed in DRG pruriceptors and involved in acidic citrate-induced scratching. We thus harvested DRG pruriceptors and performed single-cell RT-PCR. A recent study has shown that DRG pruriceptors are selectively located within the Nav1.8^{+} /IB4^{-} population and are mainly Nppb^{+} or GRP^{+} (Chiu *et al*., 2014). We verified the result first with green fluorescence–IB4 staining in the cervical DRG culture from Nav1.8-Cre::Td-tomato mice. Td-tomato^{+} DRG neurons were divided into IB4^{+} and IB4^{-} by the appearance of green fluorescence and harvested for single-cell RT-PCR to detect the expression of TrpV1 and Nppb. The pruriceptor marker Nppb was selectively detected in the $Na_V1.8^{+}$/IB4^{-} population (Figure 4a). Among the 128

$Na_V1.8^+$/IB4$^-$ neurons collected, 48 (~38%) showed the expression of both TrpV1 and Nppb. No Nppb$^+$ neurons were found in the $Na_V1.8^+$/IB4$^+$ population. These $Na_V1.8^+$/IB4$^-$ neurons were processed for detection of OGR1, TDAG8, G2A, and GPR4. Among the 48 Nppb$^+$ DRG pruriceptors, 13 were OGR1$^+$ (~27%); 34 were TDAG8$^+$ (~71%); 10 were G2A$^+$ (~21%) and 3 were GPR4$^+$ (~6%). Our result suggested that TDAG8 is the most abundant proton-sensing GPCR in cervical DRG pruriceptors (Figure 4b).

TDAG8 knockout conferred a selective phenotype in acidic citrate-induced itch

To verify whether TDAG8 plays a role in acidic citrate-induced itch, we screened the scratching behavior induced by citric acid (pH 3.0) in TDAG8-knockout (TDAG8$^{-/-}$) mice. Compared with wild-type mice, TDAG8$^{-/-}$ mice showed significantly reduced acidic citrate-induced scratching response (Figure 4c,d).

Interestingly, TDAG8$^{-/-}$ mice showed normal scratching on challenge with chloroquine, α-methyl-5-HT or CP48/80 (Figure S4). Also, we found TDAG8$^{-/-}$ mice showed a normal scratching response to bile acid, another pruritogen that plays a role

in chronic liver disease and acts on TGR5 to generate an itch sensation. Therefore, the pruriception deficit in TDAG8$^{-/-}$ mice was acid-specific.

TDAG8 potentiated acid-induced calcium response by regulating TRPV1

We previously demonstrated that TDAG8 activation by acid (pH 6.4) sensitized TRPV1 response to capsaicin in inflamed DRG neurons (Chen *et al*., 2009). To test whether TDAG8 also sensitizes the TRPV1 response to acid, we first transfected plasmid TDAG8-pIRES-GFP into HEK293T cells. As expected, TDAG8 mediated acid-induced cAMP accumulation at "all" pH values tested, with peak response at pH 6.0~6.8. (Figure 5a). [Ca^{2+}]*i* level was increased by pH value (pH 7.6~5.0) in TDAG8-transfected cells, especially at pH 5.5 and 5.0 (Figure 5b). [Ca^{2+}]*i* level was increased at pH 5.0 in cells with IRES-vector control transfection, so we used pH 5.5 as a standard acid stimulus for further pharmacological study. Increased [Ca^{2+}]*i* level at pH 5.5 was completely inhibited by removal of extracellular calcium with 2 mM EGTA, so [Ca^{2+}]*i* content after acid treatment was from calcium channels (Figure 5c,d). Addition of the TRPV1 antagonist capsazepine inhibited increased [Ca^{2+}]*i*

levels after pH 5.5 treatment. Treatment with the pan-ASIC blocker amiloride at 30 μM had no effect under the same conditions (Figure 5d). Acid may activate TDAG8 and increase $[Ca^{2+}]i$ levels via TRPV1, but not via ASICs. Co-transfection of TDAG8 and TRPV1, TDAG8 dose-dependently potentiated the TRPV1-mediated calcium response elicited by acid treatment (Figure 5e). Furthermore, this synergism was completely impeded in the presence of the TRPV1 antagonist capsazepine at 20 μM. Inhibitors for PLCβ (U73122) and Gβγ (gallein) significantly inhibited the calcium response elicited by acid stimulation (pH 5.5) of TDAG8/TRPV1 co-expressing cells (Figure 5f). Thus, TDAG8 may mediate acidic citrate-induced itch by sensitizing TRPV1 via PLCβ and Gβγ pathways in DRG pruriceptors.

DISCUSSION

In sensitized skin all kinds of nociceptive stimulation including the most unspecific electrical stimulation evokes itch (Ikoma *et al*., 2004; Hosogi *et al*., 2006). Thus the activation of nociceptors is essential for the induction of itch in eczema. In normal skin – without pre-existing sensitization – nociceptive stimulation can induce itch,

when the application is very focal. This is explained by the spatial pattern of discharging nociceptors as the basis of spinal spatial contrast theory (Namer *et al*., 2008; Schmelz, 2010; Namer & Reeh, 2013). Here we provide evidence that acidosis can be one of the causes to evoke itch-like sensation in mouse skin. The acidic citrate-induced itch-like sensation required activation of two proton-sensing receptors, TRPV1 and TDAG8. Especially, TDAG8-mediated pruriception was acid-specific, because TDAG8$^{-/-}$ mice showed normal scratching responses to all other pruritogens. Of note, TDAG8 is more sensitive to acid than TRPV1. Given that acid is gradually diffused in the injected skin and assumed to simultaneously activate a wide range of sensory afferents, it is hard to explain the results based on the labeled line theory. Instead, similar to itch induced by localized nociceptor activation (Sikand et al., 2009), the observed acidic citrate-induced scratching behaviors may be based on the pattern of discharge, including nociceptors, pruriceptors, and low-threshold afferents being activated by reduction of free calcium. Alternatively, the spatial contrast theory could reconcile the dual roles of acid in itch and pain, since acid could evoke sharp contrast activation of nociceptors with TDAG8-TRPV1 coupling among TDAG8-negative

afferents. The interaction of TDAG8 and TRPV1 in itch-like sensation is important under pathological conditions because the number of $TDAG8^+$ (or $TDAG8^+/TRPV1^+$) DRG neurons is increased 24 h after intraplantar CFA or carrageenan injection (Chen *et al*., 2009). Thus, the results of acidic citrate-induced scratching response indicate that a sensitization process that is involved in inflammatory pain could also be operational in itch. Nevertheless, mice have limited types of behavioral responses and that we cannot determine what the mouse is 'feeling' but the responses are manifested the way they are.

Algogen and pruritogen nature of protons

Almost 2 decades ago, the discovery of the acid-sensing capacity in DRG sensory neurons led to studies of acid signaling in pain research because tissue acidosis accompanies many painful inflammatory and ischemic conditions (Krishtal and Pidoplichko, 1981; Bevan and Yeats, 1991; Steen *et al*., 1992). Intradermal infusion of acidic solution-induced pain was reported in humans, with no reduction after repeated capsaicin application; furthermore, co-injection of amiloride (1 mM)

inhibited pain induced by intradermal acid (Ugawa *et al*., 2002; Jones *et al*., 2004). Although these human studies highlighted the importance of ASICs in modulating the proton-induced pain, the selectivity of amiloride is in doubt as it also blocks TRPA1 (Banke, 2011). Here, we demonstrated that pharmacological antagonism and genetic ablation of TRPV1 but not TRPA1 or ASICs impaired acidic citrate-induced scratching in mice. A recent study suggested that acetic acid (pH 3.5~4.0, non-buffered) is not a potent pruritogen and ASIC3 plays a role in acid-mediated potentiation of SL-NH_2-itch (Peng *et al*., 2015). Our result is consistent with this report because formic acid, without a pH buffer capacity such as acetic acid, induced scratching response only at the extreme pH value of 1.9. Under pH-buffered conditions, 0.2 M citric acid at pH 4.7 could significantly generate more scratching responses than at pH 7.4. We conclude that under normal conditions, ASICs do not play a role in an acute, acid-induced scratching response in mice. However, we cannot exclude a possible role of TRPA1 in acidic citrate-induced scratching, because human (but not other species) TRPA1 is exquisitely sensitive to acidosis (de la Roche et al., 2013).

Role of TDAG8 in pruriception

Transcriptome studies of purified neurons indicated that TRPV1, H1R and Nppb are enriched in $Na_V1.8^+/IB4^-$ populations and TRPA1 and MrgprA3 are enriched in $Na_V1.8^+/IB4^+$ populations (Chiu *et al*., 2014). Among 4 proton-sensing GPCRs, TDAG8 was the most abundant in $Nppb^+$ DRG pruriceptors in the $Na_V1.8^+/IB4^-$ populations. $TDAG8^{-/-}$ mice showed deficit to acidic citrate-induced scratching but normal response to other pruritogens including chloroquine, α-methyl-5-HT, CP48/80 and bile acid. In cultured HEK293T cells with proton stimulation, TDAG8 potentiated a TRPV1-mediated calcium response via a Gβγ/PLCβ pathway. TDAG8 may mediate itch by regulating TRPV1 function. Previous studies also showed that TDAG8 mediated acid-induced response via Gs/cAMP/PKA or $G_{12/13}$/RhoA pathway (Ishii et al., 2005; Mogi *et al*., 2009; Ihara *et al*., 2010). Further studies should determine whether the Gs/PKA or $G_{12/13}$/RhoA signaling is involved in the acidic citrate-induced itch-like sensation in DRG pruriceptors.

Conclusion

Acid signaling is polymodal in a somatosensory system and is involved in nociception, pruriception, antinociception, all of which are coupled with distinguishable proton-sensors in sensory neurons on the molecular level. Briefly, ASIC3 and TRPV1 are involved in acid-induced nociception and hyperalgesic priming, TRPV1 and TDAG8 are for pruriception, and a non-ASIC3, non-TRPV1 proton-sensing receptor is required for antinociception in muscle afferent neurons (Sun and Chen, 2016). However, a limitation of the current work is the lack of differentiation between the role of specific sensory transduction pathways (e.g., TRPV1) and the role of spatial contrast of activation patterns of single nociceptors. This limitation is commonly ignored in the discussion of the itch and the pain field and required further clarified in future studies.

MATERIALS AND METHODS

Mice

Wild-type C57BL/6JNarl mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and used as a backcross pool for all lines of genetically modified mutant mice. Mice with knockout of *TrpV1* and *TrpA1* were obtained from the Jackson Laboratory (Bar Harbor, ME); *Asic3*-knockout mice were generated in our laboratory (Chen *et al*., 2002); TDAG8-knockout mice were generated with use of the *Sleeping Beauty* transposon system (Horie *et al*., 2003). Nav1.8-Cre mice were obtained from Dr. John Wood (Stirling *et al*., 2005), and crossed with CAG-Td-tomato Cre-reporter mice. For behavioral studies, all mutant mouse lines were congenic after backcrossing to C57BL/6JNarl for at least 10 generations. All experimental procedures with mice (12~18 weeks old male) were approved by the Institutional Animal Care and Use Committee of Academia Sinica.

Drugs and administration

Chemical compounds including citric acid, sodium citrate, formic acid, capsaicin, chloroquine, amiloride, chlorpheniramine maleate, histamine, and CP48/80 were from Sigma-Aldrich. Capsazepine and HC-030031 were from Tocris. α-Methyl-serotonin was from Abcam. Citric acid (0.2 M) at pH 4.7, 3.0, 1.9 was obtained by mixing pure

0.2-M citric acid and 0.2-M sodium citrate. Formic acid at pH 4.7(~0.000001%, v/v), 3.0 (~0.01%) and 1.9 (~1%) was prepared by sequential dilution of 98% formic acid in sterile saline. To compare the potency of different pruritogens, α-methyl-5-HT (30 μg/10 μL), chloroquine (200 μg/10 μL), DCA (25 μg/10 μL) and CP48/80 (100 μg/10 μL) were freshly prepared, with sterile saline used as a vehicle. To verify the role of TRPV1, TRPA1 and ASIC3 in acid-induced itch, capsazepine (10 μM), HC-030031 (20 μg), or amiloride (1 mM) was co-injected with 0.2 M citric acid (pH 3.0) intradermally (i.d.). Capsaicin (10 μg/10 μL) was dissolved in a vehicle of 7% Tween-80 in saline. Capsazepine and HC-030031 were dissolved in 20% DMSO in saline. Chlorpheniramine (10mg/kg) were intraperitoneally injected 30 min before acid injection.

Itch behavioral study

In the mouse cheek- and nape-skin assay models, mice under anesthesia with isoflurane were shaved at least 24 h before the experiment. On the testing day, mice were placed individually into a glass chamber (25 x 16 x 18 cm) with ~1-cm–thick

fresh bedding sawdust (or without bedding in Figure 1e,f) for 30-min habituation; pruritogen was applied i.d. at a volume of 10 μL with use of a 30-G needle in a Hamilton 25-μL micro-syringe. After drug injection, scratching behavior was recorded for 30 min in the same chamber. For all itch behavioral studies, mouse behavior was videotaped and scored by a well-trained observer blinded to the treatment. In both the nape-skin and cheek models, scratching was defined when the mouse lifted the hindpaw, touched the shaved skin area and returned the paw to the floor. In the cheek assay, wiping/digging was defined when the mouse wiped the shaved skin on cheek unilaterally with the forearm or dig to the bedding (Shimada and LaMotte, 2008). For histamine conditioning, acid was injected to cheek skin at 0.5~0.7cm away from histamine injection site 30 min later (Figure S3).

Immunohistochemistry

Mice were anesthetized with urethane (1.5 g/kg; i.p.) 30 min after the itch-behavioral recording and perfused transcardially with 25 mL of 0.02 M phosphate buffer saline (1x PBS, pH 7.4, at 4°C), then 25 mL cold fixative (4%[w/v] formaldehyde, 0.12 M

PBS (pH 7.4, at 4°C). Cervical spinal cord at the level of C1~C6 was dissected and post-fixed in the same fixative at 4°C for 16 h; PFA-fixed mouse spinal cords were sectioned at 100-μm thick with use of a vibrating tissue slicer (Vibratome 1000 Plus). For free-floating staining, slides were first bleached in PBS containing 0.03% H_2O_2 for 30 min, blocked in PBST (PBS+0.1% Triton X-100) containing 5% bovine serum albumin (BSA) and 5% normal rabbit serum at room temperature for 60 min, then incubated with rabbit-anti-c-Fos antibody (1:1000) in blocking solution overnight at 4°C. Sections were washed 3 times with PBST and incubated for 1 h at room temperature with biotinylated goat-anti-rabbit antibodies (1:500). After 3 PBST washes, sections were incubated in Avidin-Biotin pre-mix solution (1:1000 in 1xPBS, Vector Labs, Burlingame, CA) and immunoreactivity signals were visualized by a Nickel-DAB method. C-Fos immunoreactivity was quantified using ImageJ.

Calcium imaging

DRGs (C1~C6) were cultured as previously described (Chen *et al*., 2014). To measure the calcium response to different pruritogens, DRG neurons were washed

with 1x HBSS and incubated with 5 μM Fura-2-acetoxymethylester (Invitrogen, Carlsbad, CA) containing 0.3% BSA for 45 min at 37°C. Fluorescence measurements involved use of the Zeiss Axioskop FS upright microscope fitted with an ORCA ER CCD camera (Hamamatsu, Japan). Fura-2 was excited at 340 nm and 380 nm (excitation time 200 or 300 ms) with a rapid switching monochromator (TILL Photonics, Germany), and the emitted fluorescence was filtered with use of a 510 nm longpass filter. Background-subtracted mean fluorescence intensity ratios (F340/F380) were displayed by use of Metafluor software (Molecular Devices). The standard bath solution 1x HBSS contained (in mM) 100 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 1 $NaHPO_4$, 4.2 $NaHCO_3$, 12.5 HEPES and 10 glucose (~300 mOsm/kg, pH 7.4).

For heterologous expression system, TDAG8 and TRPV1 cDNAs were cloned into the vector pIRES-hrGFP-2a (pIRES-GFP) and transfected to HEK293T for calcium imaging studies as described (Chen *et al*., 2009). Briefly, at 17 to 19 h after transfection, cells were pre-incubated at 37°C with serum-free DMEM containing 2 μM Fura-2 acetoxymethyl ester (Invitrogen) for 40 min in HEPES/MES buffer (125 mM NaCl, 1 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 8 mM glucose, 10 mM HEPES

and 15 mM MES, pH 7.6). After being washed, cells were supplemented with 300 µl HEPES/MES buffer (pH 7.6), then stimulated with the indicated pH of HEPES/MES buffer (600 µL). For EGTA experiments, 5 mM $CaCl_2$ was removed from HEPES/MES buffer and 2 mM EGTA was added in the buffer.

Single cell RT-PCR

Cultured DRG neurons from Nav1.8-Cre::CAG-Td-tomato double transgenic mice were first processed for green-fluorescent Alexa Fluor 488-IB4 staining and then immersed in the chamber filled with artificial cerebrospinal fluid. Sensory neurons were identified by the presence (or absence) of Td-tomato ($Nav1.8^+$) and green fluorescence ($IB4^+$) under fluorescence microscopy. Single DRG neurons were collected for single-cell RNA isolation and RT-PCR as previously described (Lin *et al*., 2016). Samples with GAPDH signals were selected and processed for two-step nested PCR with intron-spanning primers for TRPV1, Nppb, OGR1, TDAG8, G2A and GPR4 (Table S1).

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis involved use of SigmaState 3.5 (Systat Software, San Jose, CA). Unless otherwise specified, analyses involved Student *t* test or ANOVA, with the Holm-Sidak method for post-hoc analysis; otherwise the non-parametric Mann-Whitney test was used. $P<0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure legends

Figure 1. Proton induced scratching response in wild-type mice. (**a**) Non-buffered formic acid and (**b**) buffered citric acid were injected intradermally to induce itch behavior in the nape skin model (n=10). **P < 0.01 vs. saline or pH 7.4. (**c**) Comparison of itch-inducing potency of well-known pruritogens, including deoxycholic acid (DCA), citric acid (pH 3.0), α-methyl-5-HT, chloroquine and compound 48/80 (n=6~24). (**d**) In cheek assay, algogen "capsaicin" treatment induced wiping response and pruritogen "chloroquine" treatment induced scratching response; citric acid (pH 3.0) injection induced both wiping and scratching response (n=8). *P < 0.05. (**e,f**) Effects of histamine conditioning on citric or formic acid (pH 3.0)-induced scratching in cheek assay (n=6). *P < 0.05. Data are mean ± SEM.

Figure 2. Proton induced calcium response in DRG pruriceptors and generated pruriception-specific c-Fos response in the dorsolateral cervical spinal cord. (**a,b**)

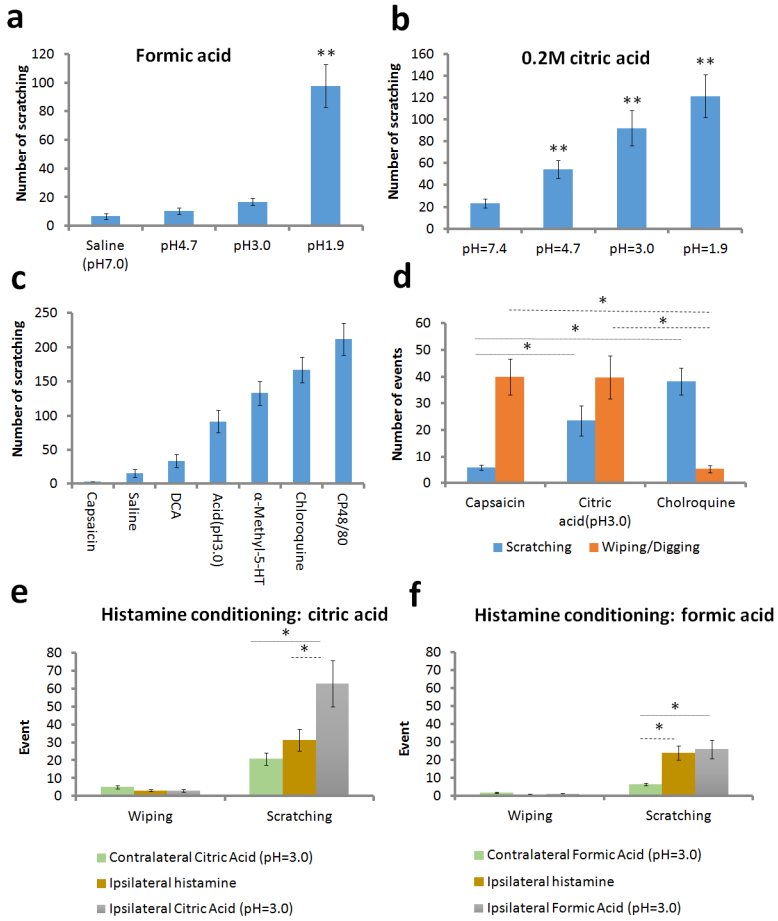
Cervical DRG neurons from Nav1.8-Cre::Td-tomato mice were cultured for calcium imaging study. Scale bar = 100 μm. (**c**) A representative result demonstrates elevated calcium level on treatment with histamine, acidic HBSS (pH=3.0), chloroquine, or capsaicin. (**d**) Proportion of Nav1.8-positive DRG neurons showing response to each treatment (N=3, n=215). (**e-h**) Immunostaining of c-Fos-positive cells responding to capsaicin, citric acid, compound 48/80, or saline treatment 30 min after intradermal injection. Scale bar = 200 μm. (**i**) To quantify the pruritus-related c-Fos signals in the dorsolateral cervical spinal cord, immunoreactivity in the red dashed-line square in (**e-h**) was calculated by using Image J (n=6). Data are mean ± SEM. *P<0.05, **P<0.01.

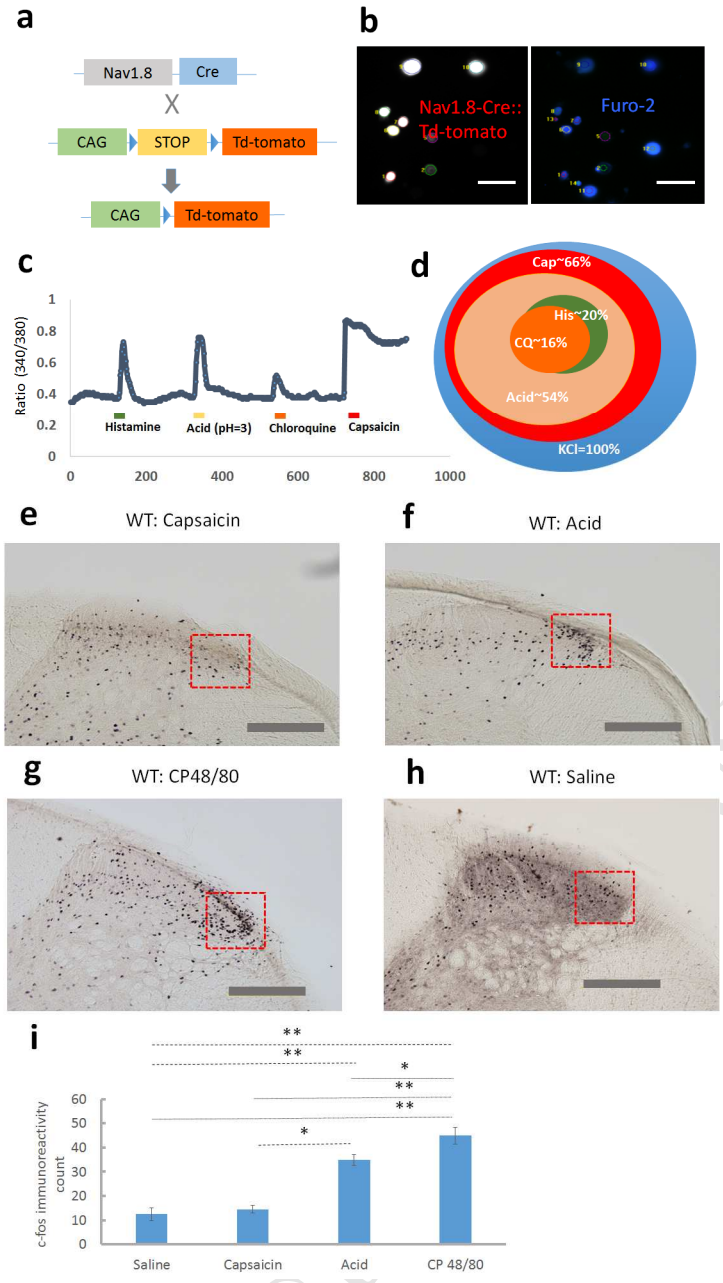
Figure 3. TRPV1 is involved in the acid-induced itch response in mice. (**a**) Citric acid (pH 3.0, 10 μL)-induced scratching response was decreased by intradermal co-injection of capsazepine (10 μM) in nape skin of wild-type (WT) mice. (**b**) Citric acid-induced scratching behavior was partly decreased in *TrpV1*-knockout (KO) mice. (**c**) Co-injection of HC030031 (20 μg/10 μL, i.d.) in nape skin had no effect on citric

acid-induced scratching response in WT mice. (**d**) Citric acid-induced scratching behavior was normal in *TrpA1*-KO mice. (**e**) Co-injection of amiloride (1 mM) in nape skin had no effect on citric acid-induced scratching response in WT mice. (**f**) *Asic3*-KO and WT mice showed no difference in citric acid-induced scratching behavior. N=8~12. Data are mean ± SEM. *P < 0.05 comparison between groups.

Figure 4. A role for TDAG8 in pruriception. (**a**) Single-cell RT-PCR analyses of Nav1.8^{+} DRG neurons. TRPV1^{+} neurons were detectable in both the IB4^{+} and IB4^{-} population, and the DRG pruriceptor marker Nppb was detected only in the IB4^{-} population. (**b**) To screen the expression of the four proton-sensing GPCRs in the pruriceptors, we used 64 Nav1.8^{+}/IB4^{-} DRG neurons for single-cell RT-PCR to detect Nppb, OGR1, TDAG8, G2A and GPR4. TDAG8 was the most abundant proton-sensing GPCR (~71%) in Nppb^{+} DRG pruriceptors. (**c**) Compared with WT mice (n=6), TDAG8-KO mice (n=9) showed decreased scratching behaviors after intradermal injection of citric acid in the nape-skin model. (**d**) Cumulative scratching response over the 30 min after injection. Data are mean ± SEM. *P < 0.05 vs. WT.

Figure 5. TDAG8 potentiates TRPV1-mediated intracellular calcium in HEK293T cells after proton stimulation. (**a**) The pH-dependent cAMP accumulation in TDAG8-expressing cells or vector (IRES)-expressing cells. (**b**) Time course of $[Ca^{2+}]_i$ signal after the addition of the indicated buffer. (**c**) The pH-dependent curves of net calcium increase in TDAG8-expressing cells in the presence or absence of EGTA (ng/ml) or in IRES-expressing cells in the absence of EGTA. At each pH point, peak values of $[Ca^{2+}]_i$ signals (approximately 20 sec after the addition of pH buffer) was presented. (**d**) Peak values of net calcium increase with pH 5.5 stimulation with and without capsazepine (10, 20 μM) or amiloride (30 μM). (**e**) Net calcium increase of TDAG8- and/or TRPV1-transfected cells exposed to pH 5.5 in with and without capsazepine (20 μM). N=20~47. (**f**) TDAG8- and/or TRPV1-transfected cells exposed to pH 5.5 with and without U73122 (5 μM) or gallein (10 μM, 100 μM). N=16~35. Data are mean ± SEM. *P< 0.05, **P<0.01.




a
Nav1.8
Cre
X
CAG
STOP
Td-tomato
CAG
Td-tomato
b
Nav1.8-Cre::
Td-tomato
Furo-2
c
Ratio (340/380)
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
1000
Histamine
Acid (pH=3)
Chloroquine
Capsaicin
d
Cap~66%
His~20%
CQ~16%
Acid~54%
KCl=100%
e
WT: Capsaicin
f
WT: Acid
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