Diacylglycerol kinases regulate TRPV1 channel activity

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Luyu Liu, Yevgen Yudin, and Tibor Rohacs*

From the Department of Pharmacology, Physiology, and Neuroscience, Rutgers New Jersey Medical School, Newark, New Jersey, USA

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The transient receptor potential vanilloid 1 (TRPV1) channel is activated by heat and by capsaicin, the pungent compound in chili peppers. Calcium influx through TRPV1 has been shown to activate a calcium-sensitive phospholipase C (PLC) enzyme and to lead to a robust decrease in phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$] levels, which is a major contributor to channel desensitization. Diacyglycerol (DAG), the product of the PLC-catalyzed PI(4,5)P$_2$ hydrolysis, activates protein kinase C (PKC). PKC is known to potentiate TRPV1 activity during activation of G protein–coupled receptors, but it is not known whether DAG modulates TRPV1 during desensitization. We found here that inhibition of diacylglycerol kinase (DAGK) enzymes reduces desensitization of native TRPV1 in dorsal root ganglion neurons as well as of recombinant TRPV1 expressed in HEK293 cells. The effect of DAGK inhibition was eliminated by mutating two PKC-targeted phosphorylation sites, Ser-502 and Ser-800, indicating involvement of PKC. TRPV1 activation induced only a small and transient increase in DAG levels, unlike the robust and more sustained increase induced by muscarinic receptor activation. DAGK inhibition substantially increased the DAG signal evoked by TRPV1 activation but not that evoked by M1 muscarinic receptor activation. Our results show that Ca$^{2+}$ influx through TRPV1 activates PLC and DAGK enzymes and that the latter limits formation of DAG and negatively regulates TRPV1 channel activity. Our findings uncover a role of DAGK in ion channel regulation.

The TRPV1 ion channel is a major noxious heat sensor; it is expressed in primary sensory neurons of the dorsal root ganglia (DRG) and trigeminal ganglia. TRPV1 is an outwardly rectifying nonselective cation channel with high permeability to Ca$^{2+}$; its activation depolarizes the neuron and induces a robust Ca$^{2+}$ signal (1). In accordance with its role as a heat sensor, activation of TRPV1 by capsaicin, the pungent compound in chili peppers, evokes a burning pain sensation.

Proinflammatory mediators such as bradykinin, prostaglandins, and extracellular ATP sensitize TRPV1 to activation by heat, capsaicin, and extracellular protons, which manifest as a left shift of the dose–response curves of these activators (2, 3). Most of these proinflammatory mediators exert their effects through activation of G protein–coupled receptors that act via G$_{q/11}$ and stimulate PLC$_{b}$ enzymes. Sensitization of TRPV1 activity has been shown to be mediated mainly by phosphorylation of the channel by PKC at residues Ser-502 and Ser-800 (4). The in vivo relevance of phosphorylation of the Ser-800 residue (Ser-801 in mice) has also been demonstrated recently (5).

Upon sustained maximal stimulation by capsaicin, TRPV1 activity decreases despite the presence of the agonist, a phenomenon termed desensitization (6). The decrease in TRPV1 activity during desensitization has been shown to be due to a right shift in the capsaicin dose response (7). We and others have shown earlier that activation of TRPV1 leads to activation of a Ca$^{2+}$-sensitive PLC, most likely a PLC$_{b}$ isoform (8), which leads to a robust decrease in the levels of PI(4,5)P$_2$ (7, 9, 10). Because PI(4,5)P$_2$ is required for TRPV1 activity, depletion of this lipid is a major factor in desensitization (11). The products of PI(4,5)P$_2$ hydrolysis by PLC are inositol 1,4,5-trisphosphate and DAG. DAG activates PKC; although the role of this enzyme is very well established in sensitization (4, 12, 13), the role of endogenous DAG in desensitization is essentially unexplored.

Here we find that inhibition of DAGK enzymes that phosphorylate DAG leads to diminished desensitization of capsaicin-induced currents of native TRPV1 in DRG neurons and recombinant TRPV1 expressed in HEK293 cells. The effect of DAGK inhibition was eliminated when the two key PKC phosphorylation residues, Ser-502 and Ser-800 (4), were mutated. We also found that activation of TRPV1 induced only a small and transient increase in DAG levels, in contrast to the robust and sustained increase evoked by activation of the G$_{q/11}$-coupled M1 muscarinic receptors. Inhibition of DAGK activity substantially increased DAG accumulation induced by TRPV1 activation but not by muscarinic receptor activation. These data indicate that DAG production upon TRPV1 activation is limited by DAGK activation. Our findings uncover a novel role for DAGK enzymes in regulation of TRPV1 activity.

Results

DAGK inhibition reduces capsaicin-induced desensitization of TRPV1 currents

To examine the role of DAGK enzymes in TRPV1 channel desensitization, we measured inward currents evoked by capsaicin in mouse DRG neurons at ~60 mV holding potential, using the whole-cell patch clamp technique. Consistent with earlier results, capsaicin-induced currents decayed substantially after an initial peak during 75-s application of 1 μM capsaicin. When capsaicin was reapplied 3 min later, it evoked smaller currents (Fig. 1A) whose amplitudes, on average, were less than 20% of the first peak current (Fig. 1D). The decrease in the amplitude of the second application of capsaicin is sometimes referred to as tachyphylaxis to differentiate it from the
current decay during the first capsaicin application, which is termed acute desensitization (6). When the neurons were treated with DAGK inhibitor 1 (DK1), also called R59022 (14), the peak amplitude during the first and second capsaicin application became significantly larger (Fig. 1, B and C). When the current amplitudes were normalized to the first peak in both groups, the relative currents evoked by the second capsaicin application were significantly larger in the DK1-treated group (Fig. 1D), indicating reduced tachyphylaxis. DK1, on average, did not alter the kinetics and extent of acute desensitization (data not shown).

Next we tested the involvement of PKC phosphorylation of TRPV1. We transfected HEK293 cells with WT TRPV1 or the S502A/S800A mutant of TRPV1 that lacks the critical phosphorylation sites for PKC-induced sensitization (4). Fig. 2, A, B, E, and F, show that, in TRPV1-expressing cells treated with DK1, the peak amplitude of the capsaicin-induced current was higher than in cells not treated with DK1 in the first capsaicin application (Fig. 1D), indicating reduced tachyphylaxis. DK1, on average, did not alter the kinetics and extent of acute desensitization (data not shown).

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DAGK inhibition potentiates DAG accumulation in response to TRPV1 activation

The effect of the DAGK inhibitor DK1 suggests that DAG accumulation is limited by DAGK activity in response to capsaicin. To test this, next we measured cellular DAG signals using fluorescent sensors in response to TRPV1 stimulation. First, we transfected HEK293 cells with a green fluorescent DAG sensor that responds with an increase in fluorescence intensity to DAG formation, a red fluorescent Ca^{2+} sensor (Red-GECO) (15), as well as TRPV1 and muscarinic M1 receptors to measure cytoplasmic Ca^{2+} levels simultaneously with DAG levels. Fig. 3A shows that activation of the G_{q}–coupled M1 receptors by carbachol induced a large increase in DAG and cytoplasmic Ca^{2+} levels in these cells. When capsaicin was
applied after carbachol, it evoked an increase in Ca\(^{2+}\) but a decrease in fluorescence of the DAG sensor. When capsaicin was applied first, it induced a clear increase in cytoplasmic Ca\(^{2+}\) and a decrease in fluorescence of the DAG sensor, preceded by a small transient increase (Fig. 3B). Carbachol applied after capsaicin did not induce any Ca\(^{2+}\) signal or DAG signal (Fig. 3B). We also found that capsaicin induced a large decrease in PI(4,5)P\(_2\) levels, indicating PLC activation (Fig. 3, E–G), which is consistent with previous publications (9, 10). This decrease in PI(4,5)P\(_2\) levels eliminates the substrate for PLC and thus explains why carbachol did not induce any DAG or Ca\(^{2+}\) signal after capsaicin.

Next we tested whether DAGK inhibition can rescue the DAG signal in response to TRPV1 activation. Fig. 4, A and B,
shows that, in cells treated with DK1, the DAG signal in response to capsaicin was larger than without the inhibitor. The effect of capsaicin on the peak of the DAG signal in the presence of DK1 was not statistically significantly different from that induced by M1 activation by carbachol. DK1 also potentiated the Ca\(^{2+}\) responses to capsaicin (Fig. 4, A and C), which is
consistent with its effect on TRPV1 desensitization (Figs. 1 and 2). Pretreatment with DAG kinase inhibitor 2 (DK2; R59949) (14) or the DAG lipase (DAGL) inhibitor RHC80267 (16), on the other hand, had no effect on DAG or Ca\textsuperscript{2+}/H\textsubscript{11001} signals induced by capsaicin (Fig. 4, A–C).

Our data show that, without DAG Kinase inhibition, capsaicin induced a negligible DAG signal (Figs. 3 B and 4 A), despite clear PI(4,5)P\textsubscript{2} hydrolysis (Fig. 3, E–G). Next we tested whether this apparent lack of DAG accumulation was due to the low affinity of the DAG sensor we used. To this end, we transfected HEK293 cells with the high-affinity DAG sensor containing the tandem C1 domains (C1ab) of protein kinase D1 fused to the C terminus of GFP (GFP-PKD C1ab) as well as TRPV1 and M1 muscarinic receptors (17). The GFP-PKD C1ab sensor translocates from the cytoplasm to the plasma membrane in response to an increase in DAG concentration, which we monitored using confocal microscopy. We found that, in response to capsaicin, this sensor displayed clear translocation to the plasma membrane (Fig. 5, A and C), but the effect was smaller and more transient than the translocation induced by carbachol (Fig. 5, B and D). TRPV1 activation by capsaicin also prevented carbachol from inducing a DAG signal (Fig. 5 A). In cells pretreated with DK1, capsaicin induced a more sustained DAG signal than in the absence of the inhibitor (Fig. 5, A–C), but DK1 had no effect on the translocation induced by carbachol (Fig. 5, B and D). The DAG signal induced by capsaicin in the presence of DK1 was comparable with that induced by carbachol (Fig. 5, C and D).

Our data indicate that DAGK, but not DAGL activity, decreases DAG accumulation in response to TRPV1 activation.

To confirm this conclusion, we used additional pharmacological tools: the DAGK inhibitor ritanserin (18) and the DAGL inhibitor DO34 (19). Fig. 6, A–C, shows that the transient DAG signal induced by TRPV1 activation became sustained in cells treated with ritanserin but not in cells treated with DO34.

PKC inhibition accelerates capsaicin-induced acute desensitization, but not tachyphylaxis, in DRG neurons

Our data with the two different DAG sensors show that TRPV1 activation induces a DAG signal even though it is transient and smaller than that induced by muscarinic receptor activation. Next, we tested whether this small transient increase in DAG has any effect on desensitization. We measured capsaicin-induced inward currents in DRG neurons in the presence

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**Figure 4. DK1 transiently rescues DAG levels.** A, HEK293 cells were transfected with Green-up (DAG)/Red-GECO(Ca\textsuperscript{2+}) sensors and either TRPV1 or M1 muscarinic receptors. TRPV1 expressing cells were stimulated with capsaicin (caps), M1 expressing cells with carbachol (carb). Shown are representative time courses of normalized green (DAG) and red (Ca\textsuperscript{2+}) fluorescence signals upon application of 100 \textmu M carbachol or 1 \textmu M capsaicin with or without 1-min preapplication of the DAGL inhibitor RHC80267 (RHC, 50 \textmu M) or the DAGK inhibitors DK1 (50 \textmu M) or DK2 (50 \textmu M), respectively. Con, control. B and C, statistical analysis of baseline, peak, and end of application of capsaicin or carbachol. B, normalized green fluorescence for DAG level changes. C, normalized red fluorescence for Ca\textsuperscript{2+} level changes. Data are shown as mean ± S.E. and scatterplots. Statistical significance was calculated with two-way analysis of variance and Fisher’s post hoc test; F(17,210) = 27.0, p = 1.3 \times 10^{-13} (B); F(17,210) = 12.3, p = 2.3 \times 10^{-11} (C).
**Figure 5. Application of DK1 increases DAG levels upon capsaicin-induced TRPV1 activation.** A and B, representative confocal fluorescence images of HEK293 cells transfected with the high-affinity PKDc1ab-GFP (DAG) sensor, TRPV1, and M1 with or without 1-min preapplication of DK1 (top and bottom rows, respectively). Time courses of fluorescence changes (normalized to baseline) in the plasma membrane are shown on the right next to the representative images for each cell. A, cells were stimulated by 1 μM capsaicin (Caps), followed by 100 μM carbachol (Carb). Con, control. B, cells were first stimulated by 100 μM carbachol, followed by 1 μM capsaicin.

C, statistical analysis of cells first stimulated by capsaicin in the control group (black, n = 7) and in the DK1 group (red, n = 13). D, statistical analysis of cells first stimulated by carbachol in the control group (black, n = 3) and DK1 group (red, n = 6). Data are shown as mean ± S.E. and scatterplots. Statistical significance was calculated with two-way analysis of variance with Fisher’s post hoc test; F(11, 108) = 6.05, p = 1.23 × 10⁻⁷ (C); F(11, 41) = 4.59, p = 0.00016 (D).
and absence of two different PKC inhibitors (Fig. 7, A–F). We found that neither bisindolylmaleimide IV (BIM-IV) nor Gö6976 had a significant effect on the peak current amplitudes evoked by two consecutive 1-min applications of 1 μM capsaicin (Fig. 7, A–D). BIM-IV pretreatment accelerated the current decay during the initial phase of the first capsaicin application (acute desensitization) (Fig. 7, A, C, E, and F). Gö6976, on the other hand, induced only a marginal effect on acute desensitization (Fig. 7, A, B, E, and F), which did not reach statistical significance. This is consistent with Gö6976 not inhibiting PKCe (20), the isoform that plays a major role in TRPV1 phosphorylation (12). Neither BIM-IV nor Gö6976 had an effect on the peak amplitude (Fig. 7D) or the decay kinetics (data not shown) of the second capsaicin application.
The small effect of PKC limited to the initial phase of acute desensitization is consistent with the transient DAG signal in response to capsaicin (Fig. 5). In agreement with the patch clamp experiments, in DRG neurons loaded with the low-affinity Ca\textsuperscript{2+}/H11001 indicator FuraFF, the Ca\textsuperscript{2+} response to 1 M capsaicin became more transient in the presence of BIM-IV but not Gö6976 (Fig. 7, G and H).

Overall, our data show that DAGK activation reduces DAG accumulation in response to TRPV1 activation, which limits the role of PKC to modulating the initial phase of acute desensitization. Fig. 8 shows our overall model of the involvement of DAGK and PKC in TRPV1 regulation during desensitization.

**Discussion**

The sensitivity of TRPV1 to capsaicin and other stimuli is not static. Upon activation of Gq-coupled receptors, the channel becomes more sensitive to capsaicin, which manifests as a left shift in the capsaicin concentration response relationship but...
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Figure 8. Cartoon explaining the role of DAGK and PKC in the regulation of TRPV1.

no change in the maximal response (3). This increased sensitivity is largely mediated by direct phosphorylation of the channel by PKC at residues Ser-502 and Ser-800 (4). When the channel is activated by high concentrations of capsaicin, channel activity desensitizes (6), which manifests as a right shift in the capsaicin concentration–response curve (7). It has been shown that Ca\(^{2+}\) influx via TRPV1 activates a Ca\(^{2+}\) sensitive PLC, which leads to depletion of PI(4,5)P\(_2\) (7, 8, 10), and that loss of this lipid is an important factor in desensitization (11). Although it has been shown that pharmacological activation of PKC with phorbol 12-myristate 13-acetate slows desensitization (21, 22), it is not known whether endogenous DAG and consequential PKC activation modulate desensitization. Here we found that, despite robust PLC activation, TRPV1 stimulation leads to only a small and transient increase in DAG levels as opposed to the robust DAG signal induced by activation of G\(_q\)-coupled M1 muscarinic receptors. The DAG signal induced by TRPV1 activation was potentiated by DAGK inhibition, indicating that DAGK activity limited DAG accumulation. DAGK inhibition, on the other hand, had no effect on the DAG signal induced by activation of G\(_q\)-coupled M1 receptors. Consistent with positive regulation of TRPV1 by PKC, DAGK inhibition also reduced capsaicin-induced desensitization of TRPV1 in a PKC-dependent manner.

We used two different sensors of DAG in this study. The first sensor is based on a circularly permutated GFP placed between the pseudosubstrate domain and the DAG binding C1 domain of PKC8 (23) and responds with an increase in fluorescence in response to G\(_q\)-coupled receptor stimulation and phorbol esters (15). In our experiments, this sensor showed only a very small and transient increase in DAG levels in response to TRPV1 activation, whereas it showed a robust but still transient increase to M1 muscarinic receptor activation (Figs. 3 and 4). The high-affinity GFP-PKD C1ab DAG probe has been shown to translocate to the plasma membrane in response to G\(_q\)-coupled receptor stimulation, application of diC\(_8\) DAG, and phorbol esters (17). The GFP-PKD C1ab probe detected a clear increase in DAG levels in response to TRPV1 activation, which was smaller and more transient than that induced by muscarinic receptor activation (Fig. 5). In both cases, treating the cells with the DAGK inhibitor DK1 increased the DAG signal evoked by TRPV1 activation. This indicates that DAG is generated when TRPV1 is activated but that its levels are diminished by DAGK activation, making the signal small and transient.

We studied TRPV1 desensitization using two consecutive capsaicin applications. During the first application of capsaicin, current levels decreased substantially despite the continuous presence of capsaicin; this decrease is often referred to as acute desensitization. The peak amplitude of the second capsaicin application is a lot smaller than that of the first one; this decrease in the response to the second application is sometimes referred to as tachyphylaxis. Although depletion of PI(4,5)P\(_2\) plays a crucial role in these phenomena (8, 10), in this study, we found different effects on acute desensitization and tachyphylaxis when we pharmacologically modified the PKC pathway at two different targets. The DAGK inhibitor DK1 had a more robust effect on tachyphylaxis (Figs. 1 and 2); the PKC inhibitor BIM-IV, on the other hand, only affected acute desensitization (Fig. 7). These data are consistent with our measurements showing that DAG formation in response to capsaicin is transient, which explains why the PKC inhibitor BIM-IV only affected the early phase of acute desensitization. The presence of a DAGK inhibitor, on the other hand, allows increased DAG levels at both capsaicin applications, explaining why tachyphylaxis was affected. Overall, we conclude that rapid phosphorylation of DAG by DAGK limits the role of PKC to a small effect during the initial phase of acute desensitization of TRPV1.

Interestingly, pretreatment with DK1 also increased the amplitude of the peak current evoked by the first capsaicin application in DRG neurons (Fig. 1) and in HEK cells (Fig. 2), and this effect was more pronounced in DRG neurons. A potential explanation for this is the following. With the relatively slow whole-chamber perfusion system in our patch clamp experiments, even during the upstroke of the current, some desensitization may occur, limiting the maximum macroscopic current. The increased DAG formation in DK1-treated cells could counteract this process, leading to an increase in peak amplitude. It has been shown that components of desensitization and sensitization, including PKC, are anchored to TRPV1 by the scaffolding protein AKAP79/150, making such fast signaling possible (13).

DAG formed upon PLC activation can be metabolized by DAGK enzymes that phosphorylate DAG and generate phosphatidic acid and DAGL enzymes that remove one acyl chain, leading to formation of 2-arachidonylglycerol (2-AG) (24). We
found that inhibition of DAGK by DK1 and ritanserin potentiated the DAG signal evoked by TRPV1 activation, indicating that DAGK limited DAG accumulation. Inhibition of DAGL enzymes using RHC80267 or DO34, on the other hand, did not influence the DAG signal, indicating that DAGL enzymes do not play a role in metabolizing DAG generated in response to TRPV1 activation. DAG can also potentially be converted to triacylglycerol by DAG acyltransferase enzymes (25); exploring their role in TRPV1 regulation will require further studies.

There are 10 isoforms of DAGK in mammals, divided into five different groups based on the presence of conserved domains (26). Type 1 enzymes (α, β, and γ) are activated by Ca\(^{2+}\); therefore, they are potential candidates for the isoenzymes activated by TRPV1 activation. These isoforms, however, are inhibited similarly by DK1 and DK2 (14, 27), and our finding that DK1, but not DK2, potentiated DAG formation by TRPV1 activation argues against involvement of these isoforms. An additional argument against involvement of Ca\(^{2+}\)-sensitive DAGK isoforms is the finding that the DAG signal induced by M1 muscarinic receptor activation was not potentiated by DK1 (Fig. 5B) despite carbachol inducing a Ca\(^{2+}\) signal similar to that induced by capsaicin (Fig. 4C).

Ritanserin was originally described as a serotonin receptor antagonist; it is structurally similar to DK1, and it has been shown to inhibit DAGKα (18), but there is no information on the effect of this compound on other DAGK isoforms. Similar to DK1, ritanserin also potentiated the DAG signal evoked by TRPV1 activation.

It has been reported that DAGKε and DAGKθ are inhibited by DK1 but not by DK2 (14), raising the possibility that these isoforms are responsible for the effect of DK1 in our study. The literature regarding the selectivity of these inhibitors, however, is conflicting: inhibition of DAGKθ by DK1 was confirmed by Tu-Sekine et al. (28) but contested by Boroda et al. (18). Another report showed that DK2 also inhibits DAGKθ (29). Thus, it is hard to draw firm conclusion regarding the selectivity of DK1 and DK2 on DAGKθ.

Given the reported differential of effect of DK1 and DK2 on DAGKε (14) and the differential of effect of DK1 and DK2 on DAG production induced by TRPV1 activation (Fig. 4), DAGKε is a potential candidate for involvement in TRPV1 regulation. This isoform is highly specific for arachidonoyl-stearyl DAG (30, 31), which is the dominant lipid formed by PLC activation. P(4,5)P\(_2\) has been reported to inhibit DAGKε with very high affinity (32, 33). P(4)P, the precursor of P(4,5)P\(_2\), also inhibits DAGKε, but it is less potent than P(4,5)P\(_2\) (32). We found earlier that TRPV1 activation induces a more robust decrease in P(4,5)P\(_2\) and P(4)P levels than that evoked by G\(_{q}\) coupled receptor activation (8, 9). Relief from inhibition by P(4,5)P\(_2\) and P(4)P thus gives a potential mechanism for activation of DAGKε. DAGKε is also potentiated by Ca\(^{2+}\) (33), giving an additional mechanism for its stimulation by TRPV1 activation. Overall, however, the large number of DAGK isoforms and their conflicting pharmacological characterization makes it beyond the scope of this study to identify the DAGK isoform involved in TRPV1 regulation.

Relatively little is known about the role of DAGK enzymes in ion channel regulation. For the P(4,5)P\(_2\)-dependent KCNQ2/3, channel it has been reported that DAGK or DAGL inhibition had no effect on channel inhibition by muscarinic receptor activation (34). In Drosophila photoreceptors, mutation of the rdgA gene that encodes a DAGK induces retinal degeneration and constitutive activity of the DAG-sensitive TRP channels (35). Our work uncovers a novel role of DAGK in the regulation of the heat- and capsaicin-activated TRPV1 channels.

**Experimental procedures**

**DRG neuron isolation and preparation**

All animal procedures were approved by the Institutional Animal Care and Use Committee at Rutgers New Jersey Medical School. DRG neurons were isolated from 3- to 6-month-old C57BL6 mice. DRG neurons were isolated from mice of either sex and anesthetized with i.p. injection of ketamine (100 mg/kg) and xylazine (12 mg/kg). The detailed methods for DRG neuron isolation and digestion have been described previously (36). After digestion with 3 mg/ml type I collagenase (Worthington) and 5 mg/ml dispase, neurons were seeded onto glass coverslips coated with a mixture of poly-d-lysine (Invitrogen) and laminin (Sigma). Neurons were then cultured for 16–48 h before measurements in DMEM–F12 supplemented with 10% FBS (Thermo Scientific), 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidity-controlled tissue culture incubator maintaining 5% CO\(_2\) at 37°C.

**HEK293 cell culture and preparation**

HEK293 cells were obtained from the ATCC (catalog no. CRL-1573) and cultured in minimal essential medium (Invitrogen) containing supplements of 10% (v/v) Hyclone-characterized FBS (Thermo Scientific), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Transient transfection was performed at ~70% cell confluence using Effectene reagent (Qiagen) according to the manufacturer’s protocol. After transfection, cells were incubated overnight (16–20 h) with transfection reagents containing the lipid–DNA complexes. Then the cells were trypsinized and replated on to poly-d-lysine–coated glass coverslips and cultured at least for an additional 2 h (in the absence of the transfection reagent) before measurements in a humidity-controlled tissue culture incubator maintaining 5% CO\(_2\) at 37°C. The complementary DNA used for transfecting HEK293 cells was prepared using the Endo-Free Plasmid Maxi Kit from Qiagen. The WT rat TRPV1 clone was provided by Dr. David Julius (University of California San Francisco). The S502A/S800A TRPV1 mutant was from Dr. Makoto Tominaga (National Institute for Physiological Sciences). The Green-up (DAG)/Red-ECO (Ca\(^{2+}\)) sensor was from Dr. Thomas Hughes (Montana State University). The PKD1αb-GFP DAG sensor and PLCβ-H-RFP P(4,5)P\(_2\) sensor were from Dr. Tamas Balla (National Institutes of Health).

**Electrophysiology**

Whole-cell patch clamp recordings were performed at room temperature. Patch pipettes were pulled from borosilicate glass capillaries (1.75 mm outer diameter, Sutter Instruments) on a P-97 pipette puller (Sutter Instrument) to a resistance of 4–6 megaohm. After formation of the gigaohm seal, the whole-cell configuration was established, and currents were measured at a holding potential of −60 mV for DRG neurons and HEK293.
cells using an Axopatch 200B amplifier (Molecular Devices). Currents were filtered at 2 kHz using the low-pass Bessel filter of the amplifier and digitized using a Digidata 1440 unit (Molecular Devices). Recordings were conducted using the Clampex software, and data analysis was performed using Clampfit software (Molecular Devices).

Measurements were conducted in solutions based on Ca\(^{2+}\) containing extracellular (EC) medium consisting of 137 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose, and 2 mM CaCl\(_2\) (pH adjusted to 7.4 with NaOH) (8). Intracellular solutions for DRG measurements consisted of 130 mM K-gluconate, 10 mM KCl, 1 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose, and 2 mM CaCl\(_2\) (pH adjusted to 7.4 with NaOH) (8).

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Ca\(^{2+}\) imaging

Ca\(^{2+}\) imaging measurements were performed on an Olympus IX-51 inverted microscope equipped with a DeltaRAM excitation light source (Photon Technology International). DRG neurons were loaded with 1 \(\mu\)M low-affinity Fura-2FF/AM (Invitrogen) for 50 min in Ca\(^{2+}\) containing EC solution supplemented with 0.1 mg/ml bovine serum albumin before the measurement at room temperature. Images at 340-nm and 380-nm excitation wavelengths were recorded with a Roper Cool-Snap digital CCD (charge coupled device) camera using a \(\times20\) objective; the emission wavelength was 510 nm. Measurements were conducted in Ca\(^{2+}\)-containing (EC) solution (8). Cells were analyzed for increases or decreases in fluorescence intensity of the whole cell. Image analysis was performed using Image Master software (PTI). Drugs were applied using a gravity-driven whole-chamber perfusion system.

Confocal fluorescence imaging

Confocal measurements were conducted with an Olympus FluoView-1000 confocal microscope in the frame scan mode using a \(\times 60\) water immersion objective at room temperature (\(\sim25\) °C). Green fluorescence was measured using an excitation wavelength of 473 nm; emission was detected through a 515/50-nm band-pass filter. Red fluorescence was measured using an excitation wavelength of 559 nm; emission was detected through a 585/50-nm band-pass filter. Image analysis was performed using Olympus FluoView-1000 and ImageJ. Measurements were performed in the same extracellular solution as used for electrophysiology. Drugs were directly applied to the experimental chamber, followed by gentle mixing.

Wide-field fluorescence imaging

Wide-field fluorescence measurements were performed on an Olympus IX-81 inverted microscope equipped with an ORCA-FLASH 4.0 camera (Hamamatsu) using a \(\times40\) oil immersion objective. Cells were imaged live at room temperature. To detect the green fluorescence from the Green-up DAG sensor, we used 480 ± 15 nm excitation and 535 ± 20 nm emission filters. Red-GECO Ca\(^{2+}\) sensor fluorescence was detected with 575 ± 25 nm and 640 ± 25 nm excitation and emission filters. For simultaneous imaging, the motorized filter turret was utilized to switch filter cubes between measuring the green and red fluorescence. Cells were analyzed for increases or decreases in fluorescence intensity of the whole cell. Data were analyzed using the Metamorph and Image J softwares. Measurements were performed in the same extracellular solution as used for electrophysiology. Drugs were directly applied to the experimental chamber, followed by gentle mixing.

Data analysis and statistics

Data analysis was performed in Excel and Microcal Origin. Data collection was randomized. Data were plotted as mean ± S.E. and scatter plots for most experiments. Data were analyzed with analysis of variance with Fisher’s post hoc test. The results of the analyses of variance are reported in the figure legends.

Data availability

All data are contained within the manuscript.

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Abbreviations—The abbreviations used are: DRG, dorsal root ganglia; PLC, phospholipase C; PI(4,5)P\(_2\), phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; DAGK, diacylglycerol kinase; DAGL, diacylglycerol lipase; BIM-IV, bisindolylmaleimide IV; PI(4)P, phosphatidylinositol 4-phosphate; PKC, protein kinase C; EC, extracellular.

References

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