Coexpression and activation of TRPV1 suppress the activity of the KCNQ2/3 channel

Xu-Feng Zhang, Ping Han, Torben R. Neelands, Steve McGaraughty, Prisca Honore, Carol S. Surowy, and Di Zhang

Neuroscience Research, Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL 60064

Transient receptor potential vanilloid 1 (TRPV1) is a ligand-gated nonselective cation channel expressed predominantly in peripheral nociceptors. By detecting and integrating diverse noxious thermal and chemical stimuli, and as a result of its sensitization by inflammatory mediators, the TRPV1 receptor plays a key role in inflammation-induced pain. Activation of TRPV1 leads to a cascade of pro-nociceptive mechanisms, many of which still remain to be identified. Here, we report a novel effect of TRPV1 on the activity of the potassium channel KCNQ2/3, a negative regulator of neuronal excitability. Using ion influx assays, we revealed that TRPV1 activation can abolish KCNQ2/3 activity, but not vice versa, in human embryonic kidney (HEK)293 cells. Electrophysiological studies showed that coexpression of TRPV1 caused a 7.5-mV depolarizing shift in the voltage dependence of KCNQ2/3 activation compared with control expressing KCNQ2/3 alone. Furthermore, activation of TRPV1 by capsaicin led to a 54% reduction of KCNQ2/3-mediated current amplitude and attenuation of KCNQ2/3 activation. The inhibitory effect of TRPV1 appears to depend on Ca^{2+} influx through the activated channel followed by Ca^{2+}-sensitive depletion of phosphatidylinositol 4,5-bisphosphate and activation of protein phosphatase calcineurin. We also identified physical interactions between TRPV1 and KCNQ2/3 coexpressed in HEK293 cells and in rat dorsal root ganglia neurons. Mutation studies established that this interaction is mediated predominantly by the membrane-spanning regions of the respective proteins and correlates with the shift of KCNQ2/3 activation. Collectively, these data reveal that TRPV1 activation may deprive neurons from inhibitory control mediated by KCNQ2/3. Such neurons may thus have a lower threshold for activation, which may indirectly facilitate TRPV1 in integrating multiple noxious signals and/or in the establishment or maintenance of chronic pain.

INTRODUCTION

The transient receptor potential vanilloid 1 (TRPV1) is a nonselective cation channel of the transient receptor potential superfamily predominantly expressed in peripheral nociceptors of dorsal root ganglia (DRGs) and primary sensory afferents (Caterina et al., 1997). Besides vanilloids, TRPV1 can be activated by noxious heat, acidic pH, and endogenous lipid agonists, and sensitized by proinflammatory agents such as bradykinin and nerve growth factor (Ramsey et al., 2006). It functions as a signal transducer by integration of diverse noxious thermal and chemical stimuli (Tominaga et al., 1998; Venkatachalam and Montell, 2007). TRPV1 plays a key role in pain after inflammation or tissue injury, especially in inflammation-induced thermal hyperalgesia (Caterina et al., 2000).

TRPV1 is permeable to many cations but predominantly to calcium when activated (Marsh et al., 1987; Caterina et al., 1997). The influx of cations through TRPV1 causes membrane depolarization and also initiates a cascade of cellular events, including the activation of

other ion channels that leads to more depolarization. Ultimately, the membrane depolarization reaches a threshold that enables action potential generation and propagation along the sensory afferents of peripheral nociceptors. This leads to the release of a variety of neurotransmitters from the central terminals of the primary nociceptors and the activation of second-order neurons of the dorsal horn. This information will ultimately be perceived in the central nervous system as pain (Szallasi and Blumberg, 1999).

Neuronal excitation caused by capsaicin and other stimuli is likely subject to control by several negative regulatory mechanisms. One such mechanism is mediated by the K_v7 (KCNQ) family of potassium channels, of which the coassembly of KCNQ2 and KCNQ3 forms a major subtype (Wang et al., 1998; Jentsch, 2000). KCNQ2/3 conducts the so-called M current, a low-threshold, slowly activating and noninactivating potassium current found in various central and peripheral neurons, including DRG neurons (Wang et al., 1998; Passmore et al., 2003). By repolarizing the membrane

The Rockefeller University Press $30.00
J. Gen. Physiol. Vol. 138 No. 3   341–352
www.jgp.org/cgi/doi/10.1085/jgp.201110618

341

back toward the resting membrane potential, the M current contributes to the regulation of neuronal subthreshold excitability and responsiveness to synaptic input in several neuronal systems (Gribkoff, 2003). Mutations in KCNQ2 and KCNQ3 have been genetically linked to benign familial neonatal convulsions (Biervert et al., 1998; Charlier et al., 1998; Singh et al., 1998). KCNQ2/3 channel openers retigabine and ICA-27243 exert anticonvulsant and analgesic activities in a broad range of seizure and pain models (Rostock et al., 1996; Blackburn-Munro and Jensen, 2003). These studies revealed the KCNQ2/3 channel as a critical negative regulator of neuronal excitability.

Activation of TRPV1 leads to activation of pronociceptive mechanisms, including neurotransmitter release (Szallasi and Blumberg, 1999). However, whether any TRPV1 activation-mediated event includes crosstalk with negative regulatory mechanisms of neuronal excitability has not yet been reported. In this study, we report on the novel findings of a physical association of TRPV1 with KCNQ2/3 channels and suppression of KCNQ2/3 channel activity by coexpression and activation of TRPV1. These findings imply that repressing the negative regulatory mechanism mediated by KCNQ2/3 channels may be one way in which TRPV1 can facilitate its role in pain and establish a hyperexcitable state.

MATERIALS AND METHODS

Chemicals
Capsaicin, deltamethrin, and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) were purchased from Sigma-Aldrich. XE991 dihydrochloride was obtained from Tocris Bioscience, and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) was purchased from Cayman Chemical. Retigabine, A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], and compound A1 ((±)-*exo*-2-bicyclo[2.2.1]hept-2-yl-*N*-(2-isopropyl-4-oxoquinazolin-3(4H)-yl)acetamide) were synthesized in-house according to published methods (Blackburn-Munro et al., 2005; Gomtsyan et al., 2005; Scanio et al., 2010). BTC-AM dye and cell culture reagents were obtained from Invitrogen. Fluo-3 AM dye was purchased from Molecular Devices.

Cloning
To generate V5-tagged human KCNQ2 (available from GenBank/EMBL/DDBJ under accession no. NM_172107), the coding sequence of a full-length KCNQ2 cDNA clone obtained from OriGene was amplified by PCR and sequence confirmed and cloned into the pcDNA3.1/V5-His TOPO expression vector (Invitrogen) with V5 and His tags in-frame with the C terminus of KCNQ2. To clone flag-tagged human KCNQ3 (GenBank accession no. NM_004519), the coding sequence of KCNQ3 was released from a cDNA clone from OriGene and cloned into the EcoRI and XbaI sites of pcDNA3.1/Zeo(+) expression vector. A flag tag sequence was subsequently amplified by PCR and cloned in-frame with the C terminus of KCNQ3. The cloning of human TRPV1 with an N-terminal HA tag was described previously (Vos et al., 2006).

To generate KCNQ2 truncation mutants, coding sequences for corresponding portions of KCNQ2 (KCNQ2-TM-C, A97-K872; KCNQ2-N-TM, M1-L318; KCNQ2-TM, A97-L318; KCNQ2-C, K319-K872) were amplified by PCR and cloned into the pcDNA3.1/V5-His

TOPO expression vector with V5 tag fused in-frame with the C terminus. To make TRPV1 truncation mutants TRPV1-N-TM and TRPV1-N, a stop codon was introduced after G684 and R433, respectively, by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions.

Antibodies
The following antibodies were obtained from the sources indicated: KCNQ2 antibody (ab22897; Abcam), TRPV1 antibody (sc-12498; Santa Cruz Biotechnology, Inc.), HA tag antibody (Roche), V5 tag antibody (Invitrogen), and flag tag antibody (Sigma-Aldrich).

Transfection and cell culture
Human embryonic kidney (HEK)293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified 5% CO_2, 95% O_2 incubator at 37°C. Cells were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Coimmunoprecipitation and Western blot
HEK293 cells in 10-cm dishes were transiently transfected with equal amounts of the expression constructs (totally 10 µg DNA). When only a single construct was transfected, pcDNA3.1 vector was included to maintain the same final amount of DNA. Cells were lysed 48 h after transfection with IP buffer (50 mM Tris 7.5, 250 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 2 mM EDTA, 0.5 mM DTT, and protease inhibitor cocktail from Sigma-Aldrich). The cell lysates were then centrifuged to remove cell debris, and the antibody used for immunoprecipitation was added to the supernatant. After overnight incubation at 4°C with gentle agitation, immune complexes were precipitated with protein A or protein G agarose beads (Invitrogen), followed by three washes in 1 ml IP buffer. After the final wash, the pellet was resuspended in Laemmli sample buffer, and proteins were resolved by SDS-PAGE (4–12% gel) and transferred to polyvinylidene fluoride membrane for immunoblot analysis.

Whole cell patch clamp
HEK293 cells were transfected with KCNQ2 and KCNQ3 expression constructs and with either TRPV1 or an empty vector (as a negative control). Cells were also cotransfected with a plasmid encoding a GFP reporter (in a 1:10 ratio) to allow identification of positively transfected cells for whole cell patch-clamp measurements. 48 h after transfection, whole cell currents were recorded at room temperature using the standard patch-clamp technique with an amplifier (Axopatch 200B; Axon Instruments), controlled with a personal computer using pCLAMP9 software (Axon Instruments). Borosilicate pipettes with a typical resistance of $\sim$ 2–3 MΩ were filled with a solution containing (in mM): 125 K-aspartate, 20 KCl, 10 EGTA, 1 MgCl$_2$, 5 Mg-ATP, and 5 HEPES, with pH adjusted to 7.2 with KOH. Extracellular solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$ 10 HEPES, and 5 glucose, with pH adjusted to 7.4 with NaOH. KCNQ currents, filtered at 5 KHz using the built-in filter of the amplifier, were recorded from a tail current protocol where cells were held at −100 mV and clamped for 1 s to test potentials between −100 to +60 mV in 10-mV steps, followed by a 200-ms tail pulse to −30 mV. In experiments shown in Fig. 2, as capsaicin causes TRPV1-mediated inward currents, KCNQ2/3 current was not recorded until capsaicin was washed off and the holding current returned to pre-capsaicin level, which usually took $\sim$30–60 s. In experiments shown in Figs. 3 and 4, retigabine- or capsaicin-evoked currents were recorded when cells were held at −40 mV. In experiments shown in Fig. 4 A, Ca^{2+}-free extracellular solution contained (in mM): 140 NaCl, 5 KCl, 5 EGTA, 1 MgCl$_2$ 10 HEPES, and 5 glucose, with pH adjusted to 7.4 with NaOH.

Data analysis

The voltage-dependent activation was determined from peak tail currents and fitted with the Boltzmann equation: $G = (G_{max} - G_{min})/(1 + \exp[(V - V_{1/2})/k]) + G_{min}$, where G_{max} and G_{min} are the maximum and minimum conductance, respectively. Half-activation voltage ($V_{1/2}$) and slope factor (k) were then determined. Average data are presented as mean ± SEM. Statistical significance was determined using Student's t test.

Immunofluorescence

Male Sprague–Dawley rats (200–350 g; Charles River) were deeply anesthetized with CO_2 and perfused through the aorta with buffered saline, followed by 10% formalin. DRGs were dissected and embedded in OCT on dry ice. DRG tissues were cut into 20-µm frozen sections with a cryostat and thaw-mounted onto superfrost glass slides (VWR International). DRG sections were fixed and permeabilized with 4% paraformaldehyde for 10 min. After rinsing twice in PBS, the sections were incubated in blocking buffer (2% FBS and 2% BSA in PBS) for 30 min to reduce nonspecific binding. The sections were then incubated with rabbit anti-KCNQ2 antibody and goat anti-TRPV1 antibody diluted in the blocking buffer for 2 h. After incubating with primary antibodies, the slides were washed once in PBS with 0.25% NP-40 followed by twice in PBS. The slides were then incubated with Alexa Fluor 488–conjugated donkey anti–rabbit IgG (Invitrogen) and Alexa Fluor 555–conjugated donkey anti–goat IgG (Invitrogen) diluted in blocking buffer (1:200 dilution) for 1 h. After washing once in PBS with 0.25% NP-40 followed by twice in PBS, the slides were mounted (Vectashield; Vector Laboratories) for observation using a laser-scanning confocal imaging system (LSM 5 PASCAL; Carl Zeiss).

Ion influx assays

The thallium (Tl^+) influx assay was performed essentially as described previously (Weaver et al., 2004), with some modifications. Cells were seeded in 96-well black-walled, clear-bottomed poly-D-lysine–coated plates (BD) at a density of 10^5 cells per well 24 h before the assay. On the assay day, BTC-AM dye was loaded into the cells by replacing the cell culture medium with 100 µl/well of 4 µM dye in DPBS. Dye loading was allowed to proceed for 2 h at room temperature, and then cells were washed twice in 100 µl/well of assay buffer (in mM: 10 HEPES, pH 7.3, 5 glucose, 140 Na-gluconate,

2.5 K-gluconate, 3.6 Ca-gluconate, 2 $MgSO_4$, and 0.1 Ouabain) to remove unloaded dye. The wash buffer was discarded and the cells were incubated in 100 µl of assay buffer before loading onto a fluorometric imaging plate reader (FLIPR) system (Molecular Devices). Compounds to be assayed were added to the cells in 100 µl of assay buffer along with 6 mM $TlNO_3$ (Sigma-Aldrich) and 10 mM K_2SO_4 to initiate Tl^+ influx FLIPR signals. Fluors were excited using the 488-nm line of an argon laser, and emission was filtered using a 540 ± 30–nm bandpass filter. Fluorescent signals were recorded for 3 min.

The Ca^{2+} influx assay was performed using the fluorescent calcium-chelating dye fluo-3, essentially as described previously (El Kouhen et al., 2005).

RESULTS

KCNQ2/3 activation has no effect on TRPV1 activity measured by calcium influx assay

We first used FLIPR-based ion influx assays to explore any possible functional interaction between TRPV1 and KCNQ2/3 channels. Ca^{2+} influx assay is a well-established approach to study TRPV1 activity (El Kouhen et al., 2005), so we used this assay to assess possible effects of KCNQ2/3 on TRPV1. We transfected HEK293 cells with KCNQ2/3, TRPV1, or KCNQ2/3 and TRPV1 together and evaluated their responses to retigabine, a KCNQ channel opener, and to the TRPV1 agonist capsaicin. Ca^{2+} is not permeable through KCNQ2/3, so cells transfected with KCNQ2/3 alone showed, as expected, no Ca^{2+} influx upon treatment with 10 µM retigabine or 0.1 µM capsaicin (Fig. 1 A). In contrast, HEK293 cells expressing TRPV1 respond to stimulation by 0.1 µM capsaicin with a dramatic increase in Ca^{2+} influx signal but show no response to 10 µM retigabine. This TRPV1-mediated Ca^{2+} influx can be blocked by 1 µM of the TRPV1 antagonist A-425619 (El Kouhen et al., 2005). In cells expressing both TRPV1 and KCNQ2/3, a dramatic increase in
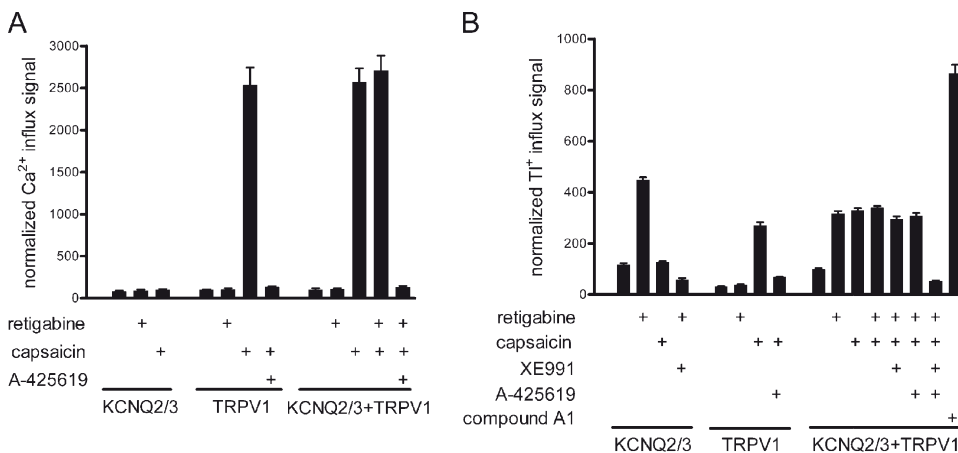
Figure 1. Functional interactions between KCNQ2/3 and TRPV1 channels as revealed by ion influx assays. (A) KCNQ2/3 activation does not affect TRPV1 activity as measured by Ca^{2+} influx assay. HEK293 cells were transfected with KCNQ2/3, TRPV1, or KCNQ2/3 and TRPV1 together, and Ca^{2+} influx into these cells in response to 10 µM retigabine, 0.1 µM capsaicin, and 1 µM A-425619, as indicated, was measured by FLIPR-based Ca^{2+} influx assay. Ca^{2+} influx in cells expressing TRPV1 and KCNQ2/3 with mock treatment was set at 100. Normalized Ca^{2+} influx values expressed as mean ± SEM of six separate assays are shown. (B) Activation of TRPV1 suppresses KCNQ2/3 activity in Tl^+ influx assay. HEK293 cells were transfected with either KCNQ2/3, TRPV1, or KCNQ2/3 and TRPV1 together, and Tl^+ influx into these cells in response to 10 µM retigabine, 0.1 µM capsaicin, 10 µM XE991, 1 µM A-425619, and 10 µM compound A1, in combinations as indicated, was measured by FLIPR-based Tl^+ influx assay. Normalized Tl^+ influx signals (expressed as mean ± SEM of six separate assays) relative to those from cells expressing TRPV1 and KCNQ2/3 with mock treatment are shown.

Ca^{2+} influx was observed when cells were treated with 0.1 µM capsaicin but not with 10 µM retigabine, thus revealing a TRPV1-mediated Ca^{2+} influx signal. When these cells were treated with 10 µM retigabine initially for 3 min and then treated with 0.1 µM capsaicin, the TRPV1-mediated Ca^{2+} influx was not affected by retigabine-mediated KCNQ2/3 activation (Fig. 1 A). In addition, the ability of A-425619 to block the capsaicin-mediated activation of TRPV1 appeared to be unaffected by the presence of KCNQ2/3 and its opener retigabine. Collectively, these data indicate that activation of KCNQ2/3 does not affect the activity of TRPV1.

Activated TRPV1 inhibits KCNQ2/3 channel activation measured by Tl$^+$ influx assay

A Tl$^+$ influx FLIPR assay has been used to measure KCNQ2 activity (Weaver et al., 2004), so we used a similar assay to investigate the effect of TRPV1 activation on KCNQ2/3 activity. HEK293 cells transfected with KCNQ2/3 alone responded to activation by 10 µM retigabine with over a fourfold increase in Tl$^+$ influx signal, which can be blocked by pretreatment with 10 µM XE991, a KCNQ-specific blocker (Fig. 1 B). In contrast, 0.1 µM capsaicin had no effect on KCNQ2/3 activity, as expected. As a nonselective cation channel, TRPV1 can conduct an influx of Tl$^+$. Thus, cells expressing TRPV1 alone showed an increase in Tl$^+$ influx signal upon treatment with 0.1 µM capsaicin, which can be blocked by A-425619. However, 10 µM retigabine showed no effect on the TRPV1 channel, as expected (Fig. 1 B).

We then tested HEK293 cells expressing both TRPV1 and KCNQ2/3. When these cells were treated with either 10 µM retigabine or 0.1 µM capsaicin, we observed KCNQ2/3- or TRPV1–mediated Tl$^+$ influx increase to a similar level (Fig. 1 B). However, when the cells were treated with retigabine and capsaicin together, we observed a combined signal at a level just equivalent to that activated by either retigabine or capsaicin alone. The lack of expected additive effects on the Tl$^+$ influx signal is not a result of saturation of the fluorescence signal because other KCNQ2/3 openers that we have assayed, such as compound A1, can lead to a Tl$^+$ influx signal two- to threefold larger than that caused by 10 µM retigabine (Fig. 1 B). Instead, the results suggested that either the KCNQ2/3- or TRPV1-driven signal may be attenuated. Our data from the Ca^{2+} influx assay suggested that activation of KCNQ2/3 has no effect on TRPV1 activity, so the combined Tl$^+$ influx signal is likely driven only by TRPV1 activity, and the KCNQ2/3-driven Tl$^+$ influx could be inhibited. Consistent with this, when TRPV1 and KCNQ2/3 coexpressing cells were treated with 10 µM XE991 before adding retigabine and capsaicin, there was no significant drop in the combined Tl$^+$ influx (Fig. 1 B), indicating that this signal is primarily driven by TRPV1 activation that is not sensitive to blockade by XE991. Furthermore, when we

pretreated cells with 1 µM A-425619 to inhibit capsaicin-mediated TRPV1 activation, the combined Tl$^+$ influx remained at the same level and was now sensitive to blockade by XE991 (Fig. 1 B). This indicates that blocking TRPV1 activation by A-425619 may relieve the inhibitory effect caused by TRPV1 activation on KCNQ2/3 activity and hence allow Tl$^+$ influx driven by KCNQ2/3 to contribute to the signal observed in cells coexpressing both channels. In summary, data from the Tl$^+$ influx assay suggest that TRPV1 activation may suppress KCNQ2/3 activity.

Coexpression of TRPV1 shifted the voltage-dependent activation of the KCNQ2/3 channel

To confirm and extend our observations in ion influx assays, we turned to electrophysiological studies to evaluate functional interactions between TRPV1 and KCNQ2/3 in more detail. First, a possible effect of coexpression of TRPV1 on the KCNQ2/3 channel properties was studied. Whole cell patch-clamp experiments were performed on HEK293 cells expressing the KCNQ2/3 channel alone or coexpressed with TRPV1 (Fig. 2, A and B). The $V_{1/2}$ of voltage-dependent activation for the KCNQ2/3 channel when expressed alone was -22.0 ± 0.6 mV ($n = 11$). The $V_{1/2}$ value was significantly shifted by +7.5 mV to -14.5 ± 0.6 mV ($n = 11$; P < 0.0001) in cells coexpressing TRPV1 and KCNQ2/3 together (Fig. 2 C). This indicates that coexpression of TRPV1 leads to a depolarizing shift of the voltage dependence of KCNQ2/3 activation.

Activation of TRPV1 by capsaicin suppressed KCNQ2/3 channel activity

We next examined any possible effects of TRPV1 activation on KCNQ2/3 channel activity in cells coexpressing KCNQ2/3 and TRPV1. 3 µM capsaicin was applied to the cells for ~5–10 s. Once TRPV1 was activated, capsaicin was washed off, and the KCNQ2/3 current was then recorded (Fig. 2 B). Capsaicin treatment did not change the $V_{1/2}$ of voltage-dependent activation for KCNQ2/3 any further than observed when the channels were coexpressed without TRPV1 activation (Fig. 2 E). However, KCNQ2/3-mediated current amplitude was reduced by 54% upon treatment with capsaicin (Fig. 2, B and D). To exclude the possibility of a direct action of capsaicin on KCNQ2/3 channels, we examined the effect of capsaicin on cells expressing KCNQ2/3 alone. 3 µM capsaicin had no effect on KCNQ2/3 current amplitude (Fig. 2, A and D) or $V_{1/2}$ of voltage-dependent activation (Fig. 2 F), indicating that the effect of capsaicin on KCNQ2/3 is likely through activation of TRPV1.
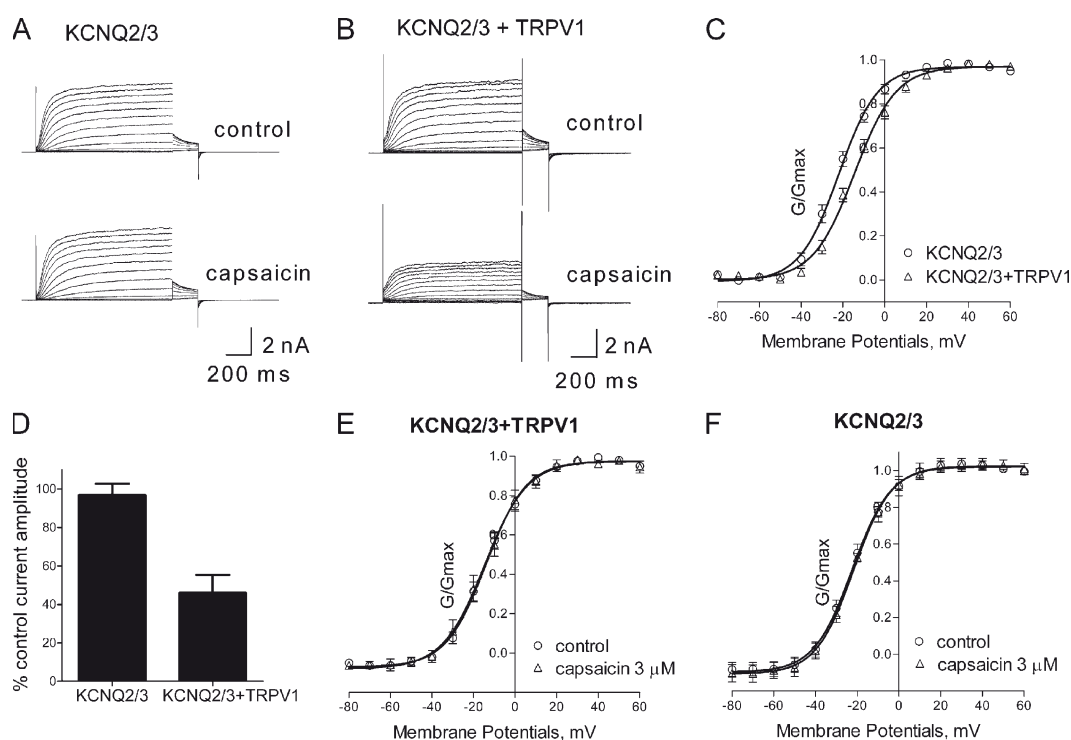
Activation of TRPV1 suppressed the activation of KCNQ2/3 channels by retigabine

Because coexpression and activation of TRPV1 have profound effects on the KCNQ2/3 channel, we next

examined whether activation of TRPV1 affects the ability of the KCNQ opener retigabine to activate KCNQ2/3 channels. At a holding potential of −40 mV, the application of 10 µM retigabine to cells that coexpressed KCNQ2/3 and TRPV1 channels elicited an outward current, which is attributed to the activation of the KCNQ2/3 channel (Fig. 3 A). Subsequent application of 1 µM capsaicin elicited a large inward current because of the activation of the TRPV1 channel. However, after capsaicin was washed off, the reapplication of 10 µM retigabine elicited a much smaller outward current, with current amplitude reduced by 75% compared with that from the first retigabine application (Fig. 3, A and D). This indicates that the enhancement of KCNQ2/3 currents by retigabine was attenuated by activation of TRPV1.

We conducted two additional experiments to confirm that the inhibition of KCNQ2/3 activation was caused by activation of TRPV1. First, when the same compound application scheme was applied to cells that expressed KCNQ2/3 alone, no inward current was observed when capsaicin was applied, owing to the lack of TRPV1 expression. In this instance, the reapplication of retigabine could elicit an outward current with similar amplitude (90%) to that of the first retigabine application (Fig. 3, B and D). Second, when 1 µM of the TRPV1 antagonist A-425619 (El Kouhen et al., 2005) was applied to cells expressing both KCNQ2/3 and TRPV1, no inward current was observed when cells were treated with capsaicin. Under such conditions, activation of the KCNQ2/3 channel was not suppressed and an outward current with similar amplitude (82%) to the initial current was observed after the reapplication of retigabine (Fig. 3, C and D). These data indicate that activation of TRPV1 by capsaicin leads to the suppression of KCNQ2/3 currents evoked by retigabine.

Figure 2. Effects of coexpression and activation of TRPV1 on the KCNQ2/3 channel. HEK293 cells expressing KCNQ2/3 alone or KCNQ2/3 and TRPV1 together were exposed to either buffer (as a control) or 3 µM capsaicin, and then the reagents were washed off. KCNQ2/3 currents were recorded by using 1,000-ms voltage pulses ranging from −100 to +60 mV in 10-mV increments from a holding potential of −100 mV. (A) Representative current traces for cells expressing KCNQ2/3 alone are shown. (B) Representative current traces for cells expressing KCNQ2/3 and TRPV1 together are shown. (C) The voltage-dependent activation curves for KCNQ2/3 coexpressed with or without TRPV1 were plotted. $V_{1/2}$ and slope factor k for KCNQ2/3 alone ($n = 11$) were −22.0 ± 0.6 mV and 9.3 ± 0.6 mV, and for TRPV1/KCNQ2/3 ($n = 11$) were −14.5 ± 0.6 mV and 9.9 ± 0.6 mV. (D) Current amplitudes (measured at +20 mV from a holding potential of −100 mV) retained as a percentage of controls upon capsaicin treatment are shown. In cells expressing KCNQ2/3 alone, 97 ± 6% of current amplitude was retained upon treatment with capsaicin ($n = 6$). In cells coexpressing KCNQ2/3 and TRPV1 together, 46 ± 9% of KCNQ2/3 current amplitude was retained upon treatment with capsaicin ($n = 9$). (E) In cells coexpressing KCNQ2/3 and TRPV1, the voltage-dependent activation curves for KCNQ2/3 with or without exposure to capsaicin were plotted. $V_{1/2}$ and slope factor k in control ($n = 9$) were −14.0 ± 0.7 mV and 9.7 ± 0.7 mV, and upon capsaicin treatment ($n = 9$) were −14.6 ± 1.3 mV and 10.2 ± 1.2 mV. (F) The voltage-dependent activation curves for KCNQ2/3 with or without exposure to capsaicin in cells expressing KCNQ2/3 alone were plotted. $V_{1/2}$ and slope factor k in control ($n = 6$) were −22.4 ± 0.9 mV and 9.2 ± 0.8 mV, and upon capsaicin treatment ($n = 6$) were −21.8 ± 0.8 mV and 9.0 ± 0.7 mV.

Activation of TRPV1 in the absence of extracellular Ca^{2+} had minimal effect on KCNQ2/3 channel activation

The activated TRPV1 channel conducts the influx of several different cations but predominantly of Ca^{2+}. To assess whether the inhibitory effect of TRPV1 on KCNQ2/3 depends on Ca^{2+} influx through the activated channel, we repeated the experiment shown in Fig. 3 A to study the effect of TRPV1 in the absence of extracellular Ca^{2+}. Whole cell currents from HEK293 cells expressing both TRPV1 and KCNQ2/3 were recorded in a Ca^{2+}-free extracellular solution when the cells were held at -40 mV. The application of 10 µM retigabine followed by the application of 1 µM capsaicin elicited a KCNQ2/3-mediated outward current and a subsequent TRPV1-mediated inward current. However, in contrast to the observation in Fig. 3 A, the reapplication of 10 µM retigabine elicited an outward current with 77% of the initial retigabine-evoked current (Fig. 4, A and E). The significant relief of an inhibitory effect on KCNQ2/3 activity in the absence of extracellular Ca^{2+} suggests that Ca^{2+} influx through the activated TRPV1 channel is required to mediate the inhibitory effect.

Mechanisms for suppression of KCNQ2/3 channels by Ca^{2+} influx through activated TRPV1 channels

TRPV1-mediated Ca^{2+} influx may inhibit KCNQ2/3 activity by several possible mechanisms. The excess Ca^{2+} may either bind to calmodulin or activate Ca^{2+}-sensitive protein phosphatase calcineurin, or it may deplete PIP_2 in the cell membrane via Ca^{2+}-induced PLC-mediated hydrolysis; all three of these possibilities have been reported to contribute to KCNQ channel inhibition (Marrion, 1996; Gamper and Shapiro, 2003; Zhang et al., 2003). To assess whether PIP_2 depletion may mediate the inhibitory effect of TRPV1 on KCNQ2/3, we repeated the experiment shown in Fig. 3 A to study the effect of TRPV1 by including 25 µM PIP_2 in the internal pipette solution. In contrast to the observation in Fig. 3 A, the presence of excess PIP_2 relieved the inhibition of KCNQ2/3 channel by TRPV1 activation, and the reapplication of 10 µM retigabine elicited an outward current with similar amplitude (83%) to the first retigabine-evoked current (Fig. 4, B and E). We then studied possible involvement of calmodulin in a similar assay by including 200 µM of the calmodulin inhibitor W-7 in the internal solution (Wu et al., 2005). The addition of W-7 failed to prevent the inhibitory effect of TRPV1 activation, and a repressed retigabine-evoked current (21% of control) was observed after TRPV1 activation (Fig. 4, C and E). To study possible involvement of calcineurin, we added 1 µM of the calcineurin inhibitor deltamethrin in the internal solution (Wu et al., 2005). Similar to the effect of PIP_2 addition, deltamethrin relieved the inhibitory effect of TRPV1 activation, and a robust retigabine-evoked current (90% of control) was observed after TRPV1 activation (Fig. 4, D and E).
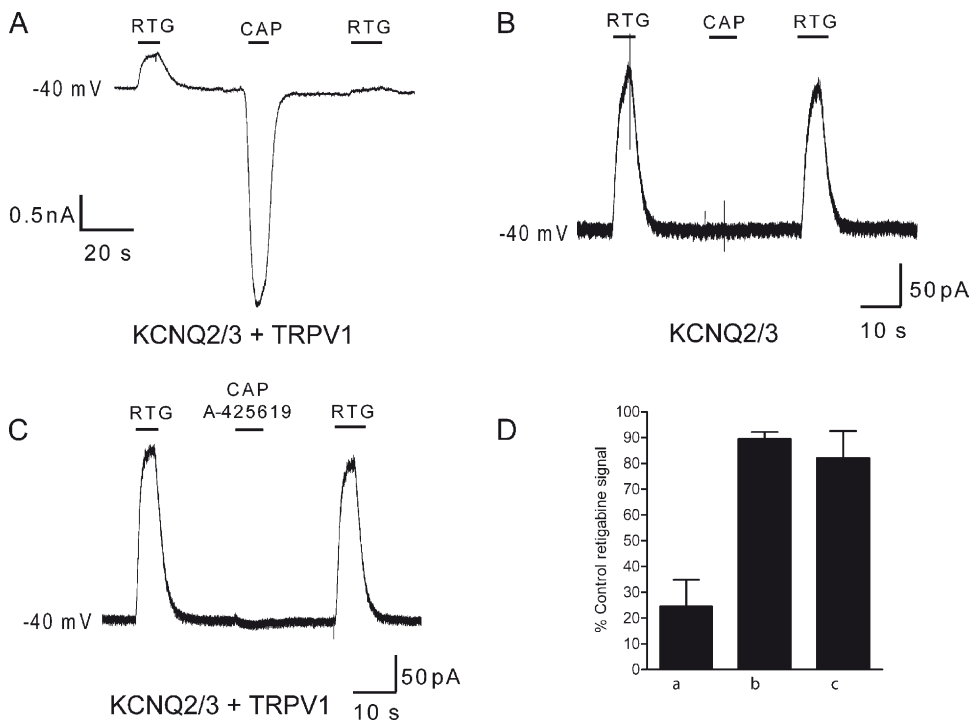
Figure 3. Activation of TRPV1 suppressed the activation of the KCNQ2/3 channel by retigabine. (A) Whole cell currents from HEK293 cells expressing both TRPV1 and KCNQ2/3 were recorded when voltage was held at -40 mV. Compounds were applied to cells sequentially as indicated: 10 µM retigabine, 1 µM capsaicin, and then 10 µM retigabine. A typical current trace recorded is shown. (B) The same experimental scheme as described in A was used, except cells expressing KCNQ2/3 alone were used instead. (C) The same experimental scheme as described in A was used, except 1 µM A-425619 and capsaicin were coapplied to cells expressing both TRPV1 and KCNQ2/3 to block TRPV1 activation. (D) The average current amplitude evoked by the second retigabine application as a percentage of the control, from the first retigabine application, in each of these three cases (shown in a–c) is plotted in a bar graph: case a, $25 \pm 10\%$ ($n = 10$); case b, $90 \pm 3\%$ ($n = 4$); case c, $82 \pm 10\%$ ($n = 7$).

Coimmunoprecipitation of TRPV1 with KCNQ2 and KCNQ2/3 in mammalian cells

The functional interactions between KCNQ2/3 and TRPV1 channels led us to explore whether they may physically associate with each other. We first determined whether TRPV1 has a physical interaction with KCNQ2 by coimmunoprecipitation. HA-tagged TRPV1 was expressed in HEK293 cells either alone (negative control) or together with V5-tagged KCNQ2. The expression of TRPV1 could be detected by Western blot analysis of products immunoprecipitated with TRPV1 antibody as two major bands (Fig. 5 A), which represent different glycosylated forms of TRPV1 (Jahnel et al., 2001; Vos et al., 2006). When KCNQ2 antibody was used in the immunoprecipitation, we observed that the lower band, which represents the less glycosylated form of TRPV1, can be coimmunoprecipitated with KCNQ2 when both were expressed, but not when TRPV1 alone was expressed (Fig. 5 A). In reciprocal experiments, when TRPV1 antibody was used in immunoprecipitation, KCNQ2, detected by V5 antibody, could be coimmunoprecipitated along with TRPV1 only when they were coexpressed in HEK293 cells (Fig. 5 A). These data revealed a physical interaction between KCNQ2 and TRPV1.

We also investigated whether TRPV1 has a physical interaction with the heterotetrameric channel formed by KCNQ2 and KCNQ3 in similar coimmunoprecipitation studies. HA-tagged TRPV1 along with V5-tagged KCNQ2 and flag-tagged KCNQ3 were expressed in HEK293 cells. Immunoprecipitation of either KCNQ2 or KCNQ3 by anti-V5 or anti-flag antibody, respectively, resulted in coimmunoprecipitation of TRPV1 (Fig. 5 B). Reciprocally, both KCNQ2 and KCNQ3 were coimmunoprecipitated with TRPV1, indicating a physical interaction of TRPV1 with both KCNQ subtypes. We also observed under these conditions a physical interaction between KCNQ2 and KCNQ3, by coimmunoprecipitation. This interaction appears to be more robust than that between TRPV1 and KCNQ subtypes, based on band intensity (Fig. 5 B), implying that KCNQ2 and KCNQ3 can still form an intact heterotetrameric channel in addition to their association with TRPV1.

The association of TRPV1 and KCNQ2 is mapped to their respective six-transmembrane domain–containing regions

To investigate which region of the KCNQ2 channel is responsible for the physical association with TRPV1, various KCNQ2 truncations were made and their interactions with TRPV1 were assessed by coimmunoprecipitation. The KCNQ2 channel has a central region with six transmembrane helices flanked by a short N-terminal region and a relatively longer C terminus. We made several KCNQ2 deletion constructs that, when expressed, produced proteins containing only one or two of these three regions (Fig. 6 A). It was observed that KCNQ2 deletion mutants with a truncation in either the N terminus or the C terminus could efficiently coimmunoprecipitate with TRPV1 (Fig. 6 A). Further truncation
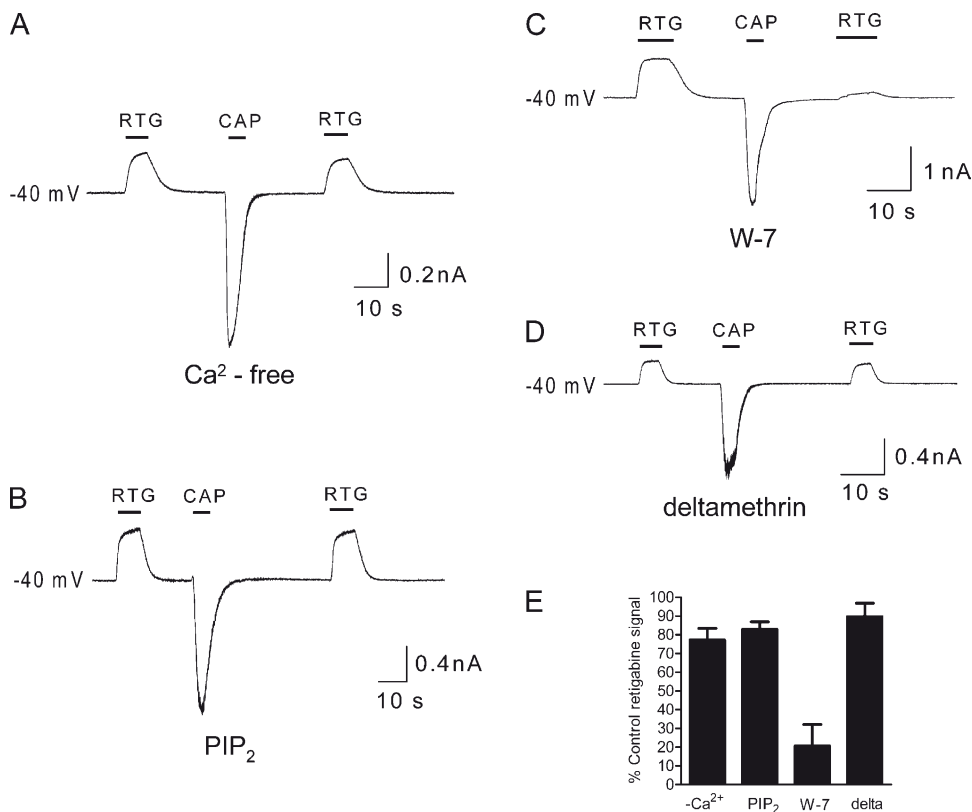
Figure 4. Mechanisms of suppression of retigabine-mediated KCNQ2/3 activation caused by TRPV1 activation. Whole cell currents from HEK293 cells expressing both TRPV1 and KCNQ2/3 were recorded when voltage was held at −40 mV. Compounds were applied to cells sequentially as indicated: 10 µM retigabine, 1 µM capsaicin, and then 10 µM retigabine. A typical current trace (A) with a Ca^{2+}-free extracellular solution used, (B) with 25 µM PIP_2, (C) with 200 µM W-7, and (D) with 1 µM deltamethrin added in the internal solution. (E) The average current amplitudes evoked by the second retigabine application as a percentage of the control from the first retigabine application in these cases were plotted in a bar graph: Ca^{2+}-free (-Ca^{2+}), 77 ± 6% ($n = 6$); PIP_2, 83 ± 4% ($n = 9$); W-7, 21 ± 11% ($n = 7$); deltamethrin (delta), 90 ± 7% ($n = 8$).

revealed that the KCNQ2 six-transmembrane domain–containing region alone still retained full capacity for physical interaction with TRPV1. However, the C-terminal region of KCNQ2, although expressed as robustly as the full-length KCNQ2, loses significant ability to interact with TRPV1. Collectively, these data suggest that the central, six-transmembrane domain–containing region of KCNQ2 mediates its association with TRPV1.

We also mapped which region of TRPV1 mediates its association with KCNQ2 using the same strategy. The N terminus alone and the truncated TRPV1 lacking the C-terminal region expressed successfully (Fig. 6 B). However, the central region of TRPV1 containing only the six-transmembrane domain or the C-terminal region failed to express. Using the truncation constructs that showed successful expression, it was observed that TRPV1 without its C-terminal region coimmunoprecipitates with KCNQ2 as efficiently as the full-length TRPV1, whereas the construct expressing just the N terminus of TRPV1 appears to be relatively poor at interacting with KCNQ2 (Fig. 6 B). These data suggest that the central six-transmembrane domain–containing region of TRPV1 may play a major role in mediating the interaction with KCNQ2.

The shift of the voltage-dependent activation of the KCNQ2/3 channel correlates with its physical association with TRPV1

To explore whether the physical association between TRPV1 and KCNQ2/3 has any effect on KCNQ2/3 channel activity, we studied the truncated TRPV1 lacking the C-terminal region (TRPV1-N-TM), which retains physical interaction with KCNQ2 but loses the ability to function as an ion channel. Whole cell patch-clamp experiments were performed on HEK293 cells coexpressing the KCNQ2/3 channel and TRPV1-N-TM. The $V_{1/2}$ of voltage-dependent

activation for the KCNQ2/3 channel coexpressing with TRPV1-N-TM was -16.6 ± 0.9 mV ($n = 8$), a significant shift of +6.2 mV compared with the value when KCNQ2/3 was expressed alone (-22.8 ± 0.9 mV; $n = 6$; $P < 0.001$; Fig. 6 C). This result establishes a correlation for the physical interaction between KCNQ2/3 and TRPV1, and the functional effect of a depolarized shift in the voltage dependence of KCNQ2/3 activation.

Physical interaction and colocalization of TRPV1 with KCNQ2 in DRG neurons

To investigate whether the interaction between TRPV1 and KCNQ2 channels has a physiological relevance, we turned to DRG neurons in which the expression of both channels has been reported (Passmore et al., 2003; Vos et al., 2006). Rat DRGs were dissected, and the physical interaction between TRPV1 and KCNQ2 was investigated using coimmunoprecipitation. With anti-KCNQ2 antibody, a significant amount of KCNQ2 was precipitated from protein extracts prepared from whole rat DRGs, and TRPV1 was coprecipitated along with KCNQ2, as revealed by Western blot analysis (Fig. 7 A). This indicates a physical interaction between TRPV1 and KCNQ2 in DRGs, in addition to that observed when the two proteins are heterologously coexpressed in HEK293 cells.

To further localize the expression of TRPV1 and KCNQ2, we prepared slides of rat DRG sections and probed for their expression on DRG neurons by immunofluorescent staining. To assess antibody specificity, we performed Western blot analyses on HEK293 cells expressing either KCNQ2 or TRPV1 and detected a protein band specific to each of them with the corresponding antibody (Fig. 7 B). When anti-TRPV1 antibody was used in immunostaining, a robust staining on small- and medium-sized DRG neurons was observed, whereas
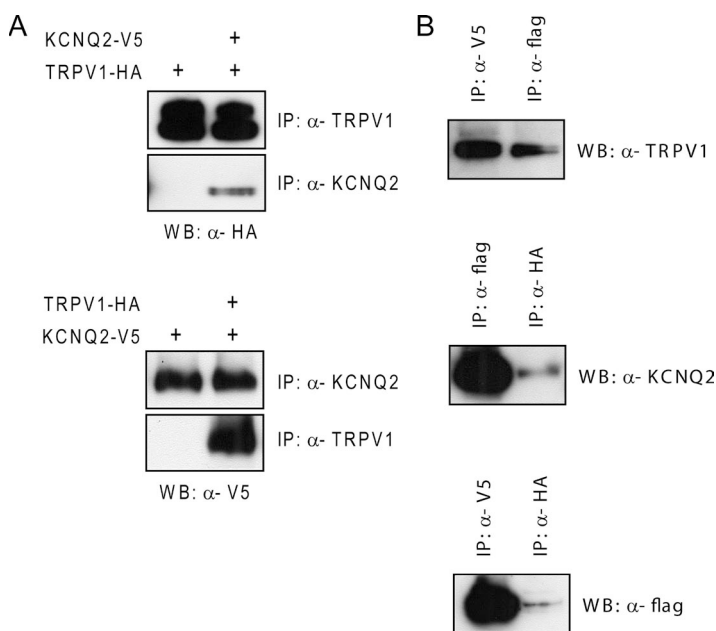
Figure 5. TRPV1 coimmunoprecipitates with KCNQ2 and KCNQ2/3 in HEK293 cells. (A) V5-tagged KCNQ2 (KCNQ2-V5) and HA-tagged TRPV1 (TRPV1-HA) in the indicated combinations were transiently transfected into HEK293 cells. Western blot (WB) of products immunoprecipitated (IP) by the indicated antibodies is shown. When KCNQ2 is specifically immunoprecipitated in cells coexpressing both proteins, TRPV1 was found to coimmunoprecipitate with KCNQ2 (bottom panel of top figure). Likewise, when the TRPV1 protein is specifically immunoprecipitated from cells coexpressing both proteins, KCNQ2 was found to coimmunoprecipitate with TRPV1 (bottom panel of bottom figure). (B) HEK293 cells were transfected with TRPV1-HA, KCNQ2-V5, and flag-tagged KCNQ3. Immunoprecipitation of any one of these ion channel proteins by antibody recognizing its respective tag coimmunoprecipitates both of the other two channel proteins. Western blots shown are representative of experiments repeated at least three times.

almost no specific staining was observed in large DRG neurons (Fig. 7 C). For KCNQ2, a significant signal was observed in small- and medium-sized DRG neurons. In addition, there was relatively weak staining in large DRG neurons (Fig. 7 C). The immunostaining of TRPV1 and KCNQ2 was colocalized in small- and medium-sized DRG neurons, indicating coexpression of these two channels.

DISCUSSION

TRPV1 activation plays a key role in inflammation-induced pain in response to diverse noxious stimuli, and its activation leads to a series of complex events that contribute to this role. Here, we report the novel finding of a physical and functional association between TRPV1 and the potassium channel KCNQ2/3. To our knowledge, although several interactions with other proteins have been identified for either TRPV1 or KCNQ channels, a physical and functional interaction

between these different classes of ion channels is a novel finding.

The most significant observation from our study is the functional interaction between TRPV1 and KCNQ2/3. We did not observe any functional consequence on TRPV1 as a result of association with KCNQ2/3. However, the coexpression and activation of TRPV1 significantly affect the KCNQ2/3 channel in three respects. First, the coexpression of TRPV1 changes the gating of the KCNQ2/3 channel, shifting the $V_{1/2}$ of activation to more depolarized potentials, thus raising the voltage threshold for activation. This change in the activation curve is not likely caused by different expression levels of KCNQ2/3, as indicated by the similar band intensity observed in Western blot analysis of KCNQ channel expression when coexpressed with or without TRPV1 (Fig. 5 A). Second, TRPV1 activation by capsaicin profoundly inhibits KCNQ2/3-mediated current amplitude, although there is no significant further shift of the
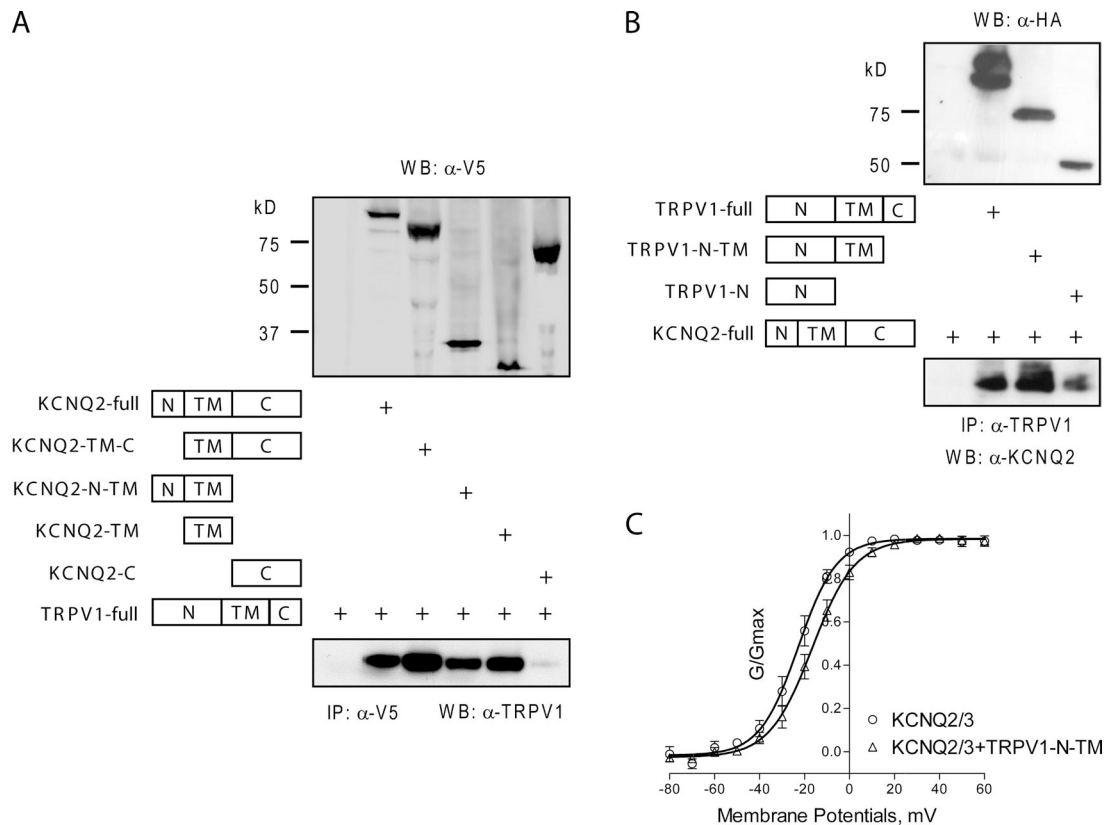
Figure 6. The KCNQ2 and TRPV1 interaction is predominantly dependent on their respective transmembrane domains and correlates with the shift of KCNQ2/3 activation. (A) KCNQ2 full-length (KCNQ2-full) or several truncation mutants lacking one or two of its three regions (the N terminus [N], central region with six transmembrane domains [TM], and the C terminus [C]) were transiently cotransfected into HEK293 cells with full-length TRPV1 (TRPV1-full). KCNQ2 proteins were immunoprecipitated by anti-V5 antibody, and the coprecipitation of full-length TRPV1 was detected by anti-TRPV1 antibody in Western blot analysis. The expression of KCNQ2 mutants is shown in the top panel, and coprecipitation of TRPV1 is shown in the bottom panel. (B) In the reciprocal experiment, full-length or truncated TRPV1 was coexpressed in HEK293 cells with full-length KCNQ2, and coimmunoprecipitation of KCNQ2 was investigated. Western blots shown are representative of experiments repeated at least twice. (C) KCNQ2/3 currents were recorded on HEK293 cells expressing KCNQ2/3 alone or KCNQ2/3 and TRPV1-N-TM together, and the voltage-dependent activation curves for KCNQ2/3 were plotted. $V_{1/2}$ and slope factor k for KCNQ2/3 alone ($n = 6$) were -22.8 ± 0.9 mV and 8.5 ± 0.8 mV, and for KCNQ2/3+TRPV1-N-TM ($n = 8$) were -16.6 ± 0.9 mV and 9.5 ± 0.8 mV.

$V_{1/2}$ of activation. Third, TRPV1 activation results in minimal responsiveness of KCNQ2/3 to the KCNQ channel opener retigabine in both electrophysiological and Tl^+ influx assays. This insensitivity to channel modulation could be caused by the dual effects on KCNQ2/3 channels as a result of TRPV1 coexpression and activation. Although the major effect of retigabine on KCNQ channels is a hyperpolarizing shift of the half-activation voltage, this stimulatory effect is significantly attenuated by the depolarized shift in activation threshold caused by coexpression, and the reduced conductance by KCNQ2/3 caused by activation of TRPV1.

The rapid effect on KCNQ2/3 activity in response to TRPV1 activation suggests that this process does not involve a sudden change in expression level or redistribution of KCNQ2/3 channels on the plasma membrane. The lack of inhibitory effect of TRPV1 on KCNQ2/3 in the absence of extracellular Ca^{2+} observed in our assays suggests that Ca^{2+} influx through the activated TRPV1 channel may cause cellular events that lead to inhibition of KCNQ2/3 channel activity. Inhibition of KCNQ2/3 channels by Ca^{2+}-mediated mechanisms has been reported for heterologously expressed channels and in rat sympathetic neurons (Selyanko and Brown, 1996; Gamper and Shapiro, 2003). For example, bradykinin inhibits the KCNQ current via Ca^{2+} release from IP_3-sensitive Ca^{2+} stores in sympathetic neurons (Cruzblanca et al., 1998). Similarly, Ca^{2+} influx through activated TRPV1 channels could mobilize sufficient Ca^{2+} into cells to inhibit KCNQ2/3.

Ca^{2+} influx through activated TRPV1 may inhibit KCNQ2/3 channel activity by several possible mechanisms. The loss of inhibitory effect on KCNQ2/3 in the presence of excess PIP_2 or the calcineurin inhibitor suggests that depletion of PIP_2 from the cell membrane and activation of calcineurin contribute to functional inhibition of KCNQ2/3 by TRPV1 activation. The C-terminal region of KCNQ channels has binding sites for PIP_2, and the activation of the channels requires a certain level of PIP_2 in the cell membrane (Delmas and Brown, 2005). PIP_2 hydrolysis caused by the stimulation of several G protein–coupled receptors, for example by specific muscarinic acetylcholine receptors, is a common mechanism for these receptors to inhibit KCNQ channels (Zhang et al., 2003). Similarly, Ca^{2+} influx through TRPV1 has been reported to cause depletion of PIP_2 (Liu et al., 2005), which may lead to the inhibition of KCNQ2/3 channel activity. Calcineurin (protein phosphatase 2B) can be activated by a rise in intracellular Ca^{2+} and has been shown to inhibit KCNQ channels in sympathetic neurons (Marrion, 1996; Klee et al., 1998). It has been reported that TRPV1 activation down-regulates voltage-gated calcium channels by dephosphorylation through Ca^{2+}-dependent activation of calcineurin (Wu et al., 2005). It is possible that a similar calcineurin-dependent mechanism may be involved in KCNQ2/3 inhibition by TRPV1. In contrast, assay in the presence of the calmodulin inhibitor revealed no involvement of calmodulin in this process, although Ca^{2+} binding to calmodulin has been reported as a mechanism of KCNQ2/3 channel inhibition (Gamper and Shapiro, 2003). In summary, it seems that inhibition of the KCNQ2/3 channel by Ca^{2+} influx through TRPV1 may involve contributions from two mechanisms. The extent to which each of these mechanisms contributes in vivo remains to be determined and may vary depending on the site of TRPV1 and KCNQ channel coexpression, as well as on specific physiological or pathophysiological stimuli.

The physical association between TRPV1 and KCNQ channels was evident by coimmunoprecipitation. Although only the less glycosylated form of TRPV1 coprecipitated with KCNQ, the cell surface localization of TRPV1 does not depend on its glycosylation state, and both
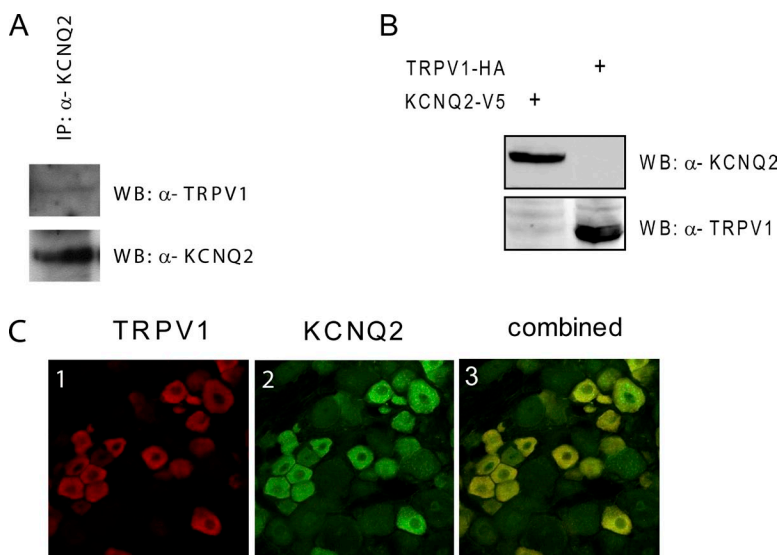
Figure 7. TRPV1 colocalizes and interacts with KCNQ2 in DRGs. (A) Rat DRG protein extracts were prepared and subjected to immunoprecipitation (IP) by anti-KCNQ2 antibody. Western blot analysis of coimmunoprecipitation of TRPV1 is shown. (B) Protein extracts from HEK293 cells expressing either KCNQ2 or TRPV1 were prepared and subjected to Western blot analysis. KCNQ2- and TRPV1-specific bands detected with corresponding antibodies are shown. Western blots shown are representative of experiments repeated twice. (C) Representative immunofluorescence images (experiments repeated at least three times) of DRG neurons from rat DRG sections stained with anti-TRPV1 antibody (1, red) or anti-KCNQ2 antibody (2, green) are shown. The combined image revealed that KCNQ2 and TRPV1 colocalize in small- and medium-sized DRG neurons (3, yellow).

highly glycosylated and less glycosylated forms of TRPV1 have been detected in the plasma membrane (Vos et al., 2006). However, we cannot ascertain through coimmunoprecipitation assays whether their interaction is direct or mediated by other proteins. In addition, because the site responsible for interaction appears to map to the region containing the six transmembrane domains of TRPV1 and KCNQ2/3, an intact membrane structure may also be necessary. Two lines of observation suggest that TRPV1 and KCNQ can still form independent channels, although they associate with each other. First, coimmunoprecipitation revealed an apparently more robust interaction between KCNQ2 and KCNQ3 than between TRPV1 and KCNQ subunits. Second, electrophysiological and pharmacological assays support the existence of independent TRPV1 and KCNQ channels with expected channel properties. We speculate that TRPV1 and KCNQ2/3 may form or be part of an ion channel macrocomplex by association with each other.

Data from our study of a functionally deficient TRPV1 mutant indicates that physical association of TRPV1 with KCNQ2/3 accounts for the depolarized shift of the voltage dependence of KCNQ2/3 activation. The association with TRPV1 may physically cause a conformational change of the KCNQ2/3 channel and hence affect the gating of the channel. Moreover, a physical interaction may also facilitate a fast and localized rise in Ca^{2+} concentration when TRPV1 is activated, which may be required for inhibition of KCNQ2/3 activity. Such an effect contributes to the differential outcome of stimulation of bradykinin versus muscarinic receptors (Delmas et al., 2002). Although activation of both receptors rapidly produces $InsP_3$ as a second messenger, only activation of the bradykinin receptor subsequently results in activation of the IP_3 receptor because of their close coupling. Similarly, the association of TRPV1 with KCNQ2/3 may provide the basis and define the specificity of inhibition of KCNQ activity by TRPV1. Indeed, a recent study revealed that lipid rafts are needed to colocalize both muscarinic receptors and KCNQ2/3 channels to enable receptor-mediated suppression of channel activity (Oldfield et al., 2009). One way to assess the possible role of colocalization is to study mutant KCNQ2/3 or TRPV1 channels that disrupt their physical association.

M currents have been identified in sensory neurons, and the expression of KCNQ2-5 subunits has been reported in small to large DRG neurons (Passmore et al., 2003). A large percentage of small DRG neurons, which would include some expressing KCNQ subunits, has been reported to be sensitive to capsaicin, the agonist for TRPV1 whose expression has been detected in small- and medium-sized DRG neurons (Caterina et al., 1997; Vos et al., 2006). Our observation of colocalization of TRPV1 and KCNQ channels in small- and medium-sized DRG neurons is consistent with these earlier reports. More interestingly, we observed that these two channels physically associate with each other under physiological conditions. TRPV1 in nociceptors plays a key role in pain after inflammation or tissue injury. The findings from our study suggest that the inhibitory effects of TRPV1 on KCNQ2/3 channels may reduce the activation thresholds of the nociceptor to other stimuli. In this regard, it is interesting to note that diverse stimuli not only directly activate TRPV1 but also sensitize and reduce the activation thresholds of the channel to other stimuli (Di Marzo et al., 2002). Besides, intradermal injection of capsaicin or sensitization of TRPV1 signaling by inflammatory mediators induces heat and mechanical hyperalgesia or allodynia (Simone, 1992). Among several possible mechanisms, TRPV1-mediated KCNQ2/3 inhibition may lower the threshold for nociceptor activation, which could indirectly help TRPV1 to integrate diverse noxious stimuli and establish hyperalgesia and allodynia. Besides DRG neurons, the expression of KCNQ and TRPV1 channels has been detected in the central nervous system where TRPV1 can be activated by endogenous ligands such as anandamide (Jentsch, 2000; Szallasi and Di Marzo, 2000; Di Marzo et al., 2008). It remains to be explored whether there are similar physical and functional interactions between these two channels at those sites.

In summary, we report on the novel finding of suppression of KCNQ2/3 channel activity by the coexpression and activation of TRPV1. The functional interaction between these two ion channels could have important pathophysiological consequences in that it could impact the severity of chronic pain.

REFERENCES

Bievert, C., B.C. Schroeder, C. Kubisch, S.F. Berkovic, P. Propping, T.J. Jentsch, and O.K. Steinlein. 1998. A potassium channel mutation in neonatal human epilepsy. *Science*. 279:403–406. doi:10.1126/science.279.5349.403

Blackburn-Munro, G., and B.S. Jensen. 2003. The anticonvulsant retigabine attenuates nociceptive behaviours in rat models of persistent and neuropathic pain. *Eur. J. Pharmacol*. 460:109–116. doi:10.1016/S0014-2999(02)02924-2

Blackburn-Munro, G., W. Dalby-Brown, N.R. Mirza, J.D. Mikkelsen, and R.E. Blackburn-Munro. 2005. Retigabine: chemical synthesis to clinical application. *CNS Drug Rev*. 11:1–20. doi:10.1111/j.1527-3458.2005.tb00033.x

Caterina, M.J., M.A. Schumacher, M. Tominaga, T.A. Rosen, J.D. Levine, and D. Julius. 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*. 389:816–824. doi:10.1038/39807

Caterina, M.J., A. Leffler, A.B. Malmberg, W.J. Martin, J. Trafton, K.R. Petersen-Zeitz, M. Koltzenburg, A.I. Basbaum, and D. Julius. 2000. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science*. 288:306–313. doi:10.1126/science.288.5464.306

Charlier, C., N.A. Singh, S.G. Ryan, T.B. Lewis, B.E. Reus, R.J. Leach, and M. Leppert. 1998. A pore mutation in a novel KQT-like potassium channel gene in an idiopathic epilepsy family. *Nat. Genet.* 18:53–55. doi:10.1038/ng0198-53

Cruzblanca, H., D.S. Koh, and B. Hille. 1998. Bradykinin inhibits M current via phospholipase C and Ca2+ release from IP3-sensitive Ca2+ stores in rat sympathetic neurons. *Proc. Natl. Acad. Sci. USA.* 95:7151–7156. doi:10.1073/pnas.95.12.7151

Delmas, P., and D.A. Brown. 2005. Pathways modulating neural KCNQ/M (Kv7) potassium channels. *Nat. Rev. Neurosci.* 6:850–862. doi:10.1038/nrn1785

Delmas, P., N. Wanaverbecq, F.C. Abogadie, M. Mistry, and D.A. Brown. 2002. Signaling microdomains define the specificity of receptor-mediated InsP(3) pathways in neurons. *Neuron.* 34:209–220. doi:10.1016/S0896-6273(02)00641-4

Di Marzo, V., P.M. Blumberg, and A. Szallasi. 2002. Endovanilloid signaling in pain. *Curr. Opin. Neurobiol.* 12:372–379. doi:10.1016/S0959-4388(02)00340-9

Di Marzo, V., G. Gobbi, and A. Szallasi. 2008. Brain TRPV1: a depressing TR(i)P down memory lane? *Trends Pharmacol. Sci.* 29:594–600. doi:10.1016/j.tips.2008.09.004

El Kouhen, R., C.S. Surowy, B.R. Bianchi, T.R. Neelands, H.A. McDonald, W. Niforatos, A. Gomtsyan, C.H. Lee, P. Honore, J.P. Sullivan, et al. 2005. A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], a novel and selective transient receptor potential type V1 receptor antagonist, blocks channel activation by vanilloids, heat, and acid. *J. Pharmacol. Exp. Ther.* 314:400–409. doi:10.1124/jpet.105.084103

Gamper, N., and M.S. Shapiro. 2003. Calmodulin mediates Ca^{2+}-dependent modulation of M-type K^+ channels. *J. Gen. Physiol.* 122:17–31. doi:10.1085/jgp.200208783

Gomtsyan, A., E.K. Bayburt, R.G. Schmidt, G.Z. Zheng, R.J. Perner, S. Didomenico, J.R. Koenig, S. Turner, T. Jinkerson, I. Drizin, et al. 2005. Novel transient receptor potential vanilloid 1 receptor antagonists for the treatment of pain: structure-activity relationships for ureas with quinoline, isoquinoline, quinazoline, phthalazine, quinoxaline, and cinnoline moieties. *J. Med. Chem.* 48:744–752. doi:10.1021/jm0492958

Gribkoff, V.K. 2003. The therapeutic potential of neuronal KCNQ channel modulators. *Expert Opin. Ther. Targets.* 7:737–748. doi:10.1517/14728222.7.6.737

Jahnel, R., M. Dreger, C. Gillen, O. Bender, J. Kurreck, and F. Hucho. 2001. Biochemical characterization of the vanilloid receptor 1 expressed in a dorsal root ganglia derived cell line. *Eur. J. Biochem.* 268:5489–5496. doi:10.1046/j.1432-1033.2001.02500.x

Jentsch, T.J. 2000. Neuronal KCNQ potassium channels: physiology and role in disease. *Nat. Rev. Neurosci.* 1:21–30. doi:10.1038/35036198

Klee, C.B., H. Ren, and X. Wang. 1998. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J. Biol. Chem.* 273:13367–13370. doi:10.1074/jbc.273.22.13367

Liu, B., C. Zhang, and F. Qin. 2005. Functional recovery from desensitization of vanilloid receptor TRPV1 requires resynthesis of phosphatidylinositol 4,5-bisphosphate. *J. Neurosci.* 25:4835–4843. doi:10.1523/JNEUROSCI.1296-05.2005

Marrion, N.V. 1996. Calcineurin regulates M channel modal gating in sympathetic neurons. *Neuron.* 16:163–173. doi:10.1016/S0896-6273(00)80033-1

Marsh, S.J., C.E. Stansfeld, D.A. Brown, R. Davey, and D. McCarthy. 1987. The mechanism of action of capsaicin on sensory C-type neurons and their axons in vitro. *Neuroscience.* 23:275–289. doi:10.1016/0306-4522(87)90289-2

Oldfield, S., J. Hancock, A. Mason, S.A. Hobson, D. Wynick, E. Kelly, A.D. Randall, and N.V. Marrion. 2009. Receptor-mediated suppression of potassium currents requires colocalization within lipid rafts. *Mol. Pharmacol.* 76:1279–1289. doi:10.1124/mol.109.058008

Passmore, G.M., A.A. Selyanko, M. Mistry, M. Al-Qatari, S.J. Marsh, E.A. Matthews, A.H. Dickenson, T.A. Brown, S.A. Burbidge, M. Main, and D.A. Brown. 2003. KCNQ/M currents in sensory neurons: significance for pain therapy. *J. Neurosci.* 23:7227–7236.

Ramsey, I.S., M. Delling, and D.E. Clapham. 2006. An introduction to TRP channels. *Annu. Rev. Physiol.* 68:619–647. doi:10.1146/annurev.physiol.68.040204.100431

Rostock, A., C. Tober, C. Rundfeldt, R. Bartsch, J. Engel, E.E. Polymeropoulos, B. Kutscher, W. Löscher, D. Hönack, H.S. White, and H.H. Wolf. 1996. D-23129: a new anticonvulsant with a broad spectrum activity in animal models of epileptic seizures. *Epilepsy Res.* 23:211–223. doi:10.1016/0920-1211(95)00101-8

Scanio, M.J., W.H. Bunnelle, W.A. Carroll, S. Peddi, A. Perez-Medrano, and L. Shi. 2010. Potassium channel modulators. Patent WO2010138828 A2.

Selyanko, A.A., and D.A. Brown. 1996. Intracellular calcium directly inhibits potassium M channels in excised membrane patches from rat sympathetic neurons. *Neuron.* 16:151–162. doi:10.1016/S0896-6273(00)80032-X

Simone, D.A. 1992. Neural mechanisms of hyperalgesia. *Curr. Opin. Neurobiol.* 2:479–483. doi:10.1016/0959-4388(92)90183-L

Singh, N.A., C. Charlier, D. Stauffer, B.R. DuPont, R.J. Leach, R. Melis, G.M. Ronen, I. Bjerre, T. Quattlebaum, J.V. Murphy, et al. 1998. A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. *Nat. Genet.* 18:25–29. doi:10.1038/ng0198-25

Szallasi, A., and P.M. Blumberg. 1999. Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.* 51:159–212.

Szallasi, A., and V. Di Marzo. 2000. New perspectives on enigmatic vanilloid receptors. *Trends Neurosci.* 23:491–497. doi:10.1016/S0166-2236(00)01630-1

Tominaga, M., M.J. Caterina, A.B. Malmberg, T.A. Rosen, H. Gilbert, K. Skinner, B.E. Raumann, A.I. Basbaum, and D. Julius. 1998. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron.* 21:531–543. doi:10.1016/S0896-6273(00)80564-4

Venkatachalam, K., and C. Montell. 2007. TRP channels. *Annu. Rev. Biochem.* 76:387–417. doi:10.1146/annurev.biochem.75.103004.142819

Vos, M.H., T.R. Neelands, H.A. McDonald, W. Choi, P.E. Kroeger, P.S. Puttfarcken, C.R. Faltynek, R.B. Moreland, and P. Han. 2006. TRPV1b overexpression negatively regulates TRPV1 responsiveness to capsaicin, heat and low pH in HEK293 cells. *J. Neurochem.* 99:1088–1102. doi:10.1111/j.1471-4159.2006.04145.x

Wang, H.S., Z. Pan, W. Shi, B.S. Brown, R.S. Wymore, I.S. Cohen, J.E. Dixon, and D. McKinnon. 1998. KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science.* 282:1890–1893. doi:10.1126/science.282.5395.1890

Weaver, C.D., D. Harden, S.I. Dworetzky, B. Robertson, and R.J. Knox. 2004. A thallium-sensitive, fluorescence-based assay for detecting and characterizing potassium channel modulators in mammalian cells. *J. Biomol. Screen.* 9:671–677. doi:10.1177/1087057104268749

Wu, Z.Z., S.R. Chen, and H.L. Pan. 2005. Transient receptor potential vanilloid type 1 activation down-regulates voltage-gated calcium channels through calcium-dependent calcineurin in sensory neurons. *J. Biol. Chem.* 280:18142–18151. doi:10.1074/jbc.M501229200

Zhang, H., L.C. Craciun, T. Mirshahi, T. Roháacs, C.M. Lopes, T. Jin, and D.E. Logothetis. 2003. PIP(2) activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron.* 37:963–975. doi:10.1016/S0896-6273(03)00125-9