THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 14, pp. 9600–9610, April 4, 2014

Furanocoumarins Are a Novel Class of Modulators for the Transient Receptor Potential Vanilloid Type 1 (TRPV1) Channel*

Received for publication, November 20, 2013, and in revised form, February 21, 2014 Published, JBC Papers in Press, February 25, 2014, DOI 10.1074/jbc.M113.536862

Xingjuan Chen[‡1], Weiyang Sun[‡§1], Nicholas G. Gianaris[‡1], Ashley M. Riley[‡], Theodore R. Cummins[¶‖], Jill C. Fehrenbacher[¶‖], and Alexander G. Obukhov[‡¶2]

From the Departments of [‡]Cellular and Integrative Physiology and [‖]Pharmacology and Toxicology and [¶]Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, Indiana 46202 and the [§]School of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan 250355, Shandong, China

Background: Furanocoumarin imperatorin is a major active component of *Angelica dahurica* root extracts exhibiting analgesic properties.
Results: Imperatorin inhibited formalin- and capsaicin-induced nocifensive responses, facilitated TRPV1 desensitization, and sensitized TRPV1 to acid activation.
Conclusion: Furanocoumarins represent a novel class of TRPV1 partial agonists exhibiting analgesic potential.
Significance: Imperatorin is a lead compound for drug discovery aimed at developing new analgesics.

Furanocoumarin imperatorin is the major active component of *Angelica dahurica* root extracts, widely used in traditional medicine to treat headache, toothache, and orbital eye pain. In this study, we investigated the mechanisms that may underlie the pain-relieving effects of the compound. We found that imperatorin significantly inhibited formalin- and capsaicin-induced nocifensive responses but did not alter baseline thermal withdrawal thresholds in the rat. We established that imperatorin is a weak agonist of TRPV1, a channel implicated in detecting several noxious stimuli, exhibiting a 50% effective concentration (EC_{50}) of 12.6 ± 3.2 μM. A specific TRPV1 antagonist, JNJ-17203212 (0.5 μM), potently inhibited imperatorin-induced TRPV1 activation. Site-directed mutagenesis studies revealed that imperatorin most likely acted via a site adjacent to or overlapping with the TRPV1 capsaicin-binding site. TRPV1 recovery from desensitization was delayed in the presence of imperatorin. Conversely, imperatorin sensitized TRPV1 to acid activation but did not affect the current amplitude and/or the activation-inactivation properties of $Na_v1.7$, a channel important for transmission of nociceptive information. Thus, our data indicate that furanocoumarins represent a novel group of TRPV1 modulators that may become important lead compounds in the drug discovery process aimed at developing new treatments for pain management.

The *Angelica dahurica* root extracts have been widely used in traditional medicine to treat headache, toothache, and orbital eye pain (1, 2). Thus far, the mechanisms underlying the analgesic properties of the extracts have not been fully elucidated. We hypothesized that furanocoumarin imperatorin, the major active component of the extract (3, 4), may exhibit analgesic properties and identified molecular targets for the pain-relieving action of imperatorin. In this study, we investigated the effects of imperatorin on two ion channels, the transient receptor potential vanilloid type 1 (TRPV1)[3] channel and voltage-gated $Na_v1.7$ channel, which have been implicated in the peripheral detection or transmission of nociceptive stimuli (5, 6).

TRPV1 is a polymodal sensor for noxious stimuli such as low acidic solutions, capsaicin, and temperatures exceeding 42 °C (7). TRPV1 proteins form Ca^{2+}-permeable cation channels expressed in the nerve endings of unmyelinated peripheral C and Aδ fibers conveying pain stimuli to the central nervous system.

When activated by noxious stimuli, the TRPV1 channel opens transiently, allowing the influx of Na^+ and Ca^{2+} ions into nerve endings to induce neuronal depolarization. Local depolarization triggers action potentials that propagate along the axon and results in the release of neurotransmitters, which subsequently activate second order neurons in the spinal cord to convey nociceptive information to higher brain centers. The voltage-gated sodium $Na_v1.7$ channel plays a crucial role in the generation of action potentials in C and Aδ fibers (6). Remarkably, patients with loss of function mutations in the $Na_v1.7$ gene lack the ability to detect pain (8), whereas patients with an increase in $Na_v1.7$ activity experience intense persistent pain (9).

TRPV1 consists of four identical subunits containing six transmembrane domains each (see Fig. 1*A*). The second, third, and fourth transmembrane domains of TRPV1 host the binding site for capsaicin, a selective and potent agonist of the channel. The capsaicin-binding site includes three key residues: Arg^{491}, Tyr^{511}, and Ser^{512} (10, 11). Prolonged treatments with capsaicin result in the complete desensitization of TRPV1 in a Ca^{2+}

* This work was supported, in whole or in part, by National Institutes of Health Grants R01HL083381 and R01HL115140 (to A. G. O.).
[1] These authors contributed equally to the work.
[2] To whom correspondence should be addressed: Indiana University School of Medicine, 635 Barnhill Dr., MS360A, Indianapolis, IN 46202. Tel.: 317-274-8078; Fax: 317-274-3318; E-mail: aobukhov@iupui.edu.

[3] The abbreviations used are: TRPV1, transient receptor potential vanilloid type 1; DRG, dorsal root ganglion.

influx-dependent manner. The pharmacological desensitization of TRPV1 is being evaluated in clinical trials to relieve various pain conditions (12, 13). Indeed, capsaicin has already been approved by the United States Food and Drug Administration for treating back pain and herpes-associated pain in outpatient practice (14).

An adverse effect of topical capsaicin formulations is burning pain upon treatment. Therefore, initial drug discovery has been focused on identifying systemic inhibitors of TRPV1 that could be used as a pain-relieving drug. However, it was soon discovered that potent TRPV1 inhibitors caused severe hyperthermia, thus creating a large obstacle for clinical use (11–13). The second generation of TRPV1 inhibitors was more promising. These inhibitors spared acid activation but blocked all other modes of TRPV1 activation. The second generation TRPV1 inhibitors caused little change in core body temperature and elicited analgesia in a murine knee pain model. However, they were ineffective in TRPV1-related bone cancer pain models, which are associated with the tumor-induced acidification of interstitial fluids (12, 15). In addition, it was established that the potent TRPV1 inhibitors compromised patient recognition of noxious heat stimuli, potentially increasing the probability of severe burns. All of these disadvantages associated with TRPV1 inhibitors merit further research efforts toward identifying novel modulators of the channel.

In this study, we investigated the antinocifensive potential of imperatorin in an *in vivo* rat model and then assessed its ability to modulate the activity of human TRPV1 and $Na_v1.7$ channels. Our results indicate that imperatorin is a partial agonist of TRPV1, sensitizing the channel to acid activation and modulating the rate of TRPV1 recovery from desensitization. Imperatorin did not affect $Na_v1.7$ activity.

EXPERIMENTAL PROCEDURES

Assessment of Nocifensive Behaviors—All animal experiments were performed in accordance with an animal protocol that was approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee and strictly adhered to the guidelines described in the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. All behavioral experiments were conducted with the observers blinded to treatments. All behavioral tests occurred between 9:00 a.m. and 4:00 p.m.

Thermal Withdrawal Thresholds—Paw withdrawal latency to radiant heat was assessed using a high intensity bulb heat source device (Ugo Basile, Comerio, Italy, (16)). Before each test, the animals remained in the test cage for ~30 min to allow acclimation to the behavioral testing environment. The heat source was placed underneath the midplantar surface of the hindpaw. The intensity of the heat source was adjusted to yield baseline latencies ranging from 18 to 20 s, and a cutoff of 30 s was used to avoid tissue damage. The withdrawal latency was taken to be the mean of three trials, with ~45 s between each trial. After baseline testing, imperatorin (2.45 mM; 30 μl) or vehicle (12.3% DMSO in saline; 30 μl) was injected into the plantar surface of the right hindpaw. Thermal thresholds were measured 5, 20, and 35 min after drug administration.

Corneal Nociception Test (Eye Wipe Test)—Imperatorin (400 μM, 40 μl) or vehicle (0.5% DMSO in saline; 40 μl) was pipetted directly into the right eye, and the resulting nocifensive behavior was measured. Nocifensive behavior was defined as time the animal spent (i) holding the eye shut, (ii) actively grooming, or (iii) wiping the treated eye. This nocifensive behavior was recorded for 5 min and followed by capsaicin (1 μM in saline; 40 μl) application onto the pretreated eye. Nocifensive behavior was again observed for 5 min following capsaicin application, as described previously (17).

The Formalin Test—The rat formalin test was used to assess a putative antinocifensive effect of imperatorin. Before each test, the animals remained in the test cage for ~30 min to allow acclimation to the behavioral testing environment. Under light isoflurane sedation, the rat was injected subcutaneously into the plantar surface of the right hindpaw with either imperatorin (2.45 mM; 30 μl) or vehicle (12.3% DMSO in saline; 30 μl). Direct effects of imperatorin and vehicle injections were assessed in the rat by measuring flinching behavior immediately following injection of the test substance for 5 min. Following the 5-min pretreatment, 1% formalin (50 μl) was injected subcutaneously into the plantar surface of the right hindpaw while the rat was restrained manually. The number of flinches following formalin injection was monitored during the two phases of the formalin response (phase 1, 0–10 min; phase 2, 16–40 min).

Cell Culture and Transfection—HEK cells (American Type Culture Collection, Manassas, VA) were cultured in the Eagle's minimum essential medium supplemented with 10% fetal bovine serum as described (18). HEK cells were transfected using the Lipofectamine LTX reagent (Invitrogen) in accordance with the manufacturer's recommendations. The human TRPV1 cDNA clone was purchased from OriGene Technologies (Rockville, MD; transcript variant 1). The accession number of the human TRPV1 cDNA is NM_080704.2. 4 μg of TRPV1 and 0.25 μg of pEYFP-C1 (Clontech) plasmids were diluted in 0.5 ml of Opti-Mem I medium (Invitrogen), and 4.25 μl of Plus reagent (Invitrogen) was added to the solution. After a 5-min incubation, 13 μl of Lipofectamine LTX (Invitrogen) was added to the mixture followed by an additional 30-min incubation. The resulting mixture was added to a 35 mM Petri dish containing HEK cell culture at 70–90% confluency and the Eagle's minimum essential medium supplemented with 10% fetal bovine serum. The cells were cultured for 24–40 h before either the fluorescence imaging or electrophysiological experiments were performed. $Na_v1.7$ currents were studied using a stable HEK cell line (19).

Dorsal Root Ganglion (DRG) Neuron Isolation and Culture—The spinal column was removed from 4–8-week-old wild type or $TRPV1^{-/-}$ knock-out mice and cut open across the ventral and dorsal midlines of the vertebrae for harvesting DRGs. DRGs (T1–L5 vertebral levels) were collected and placed into cold PBS containing no Ca^{2+} or Mg^{2+}. Dorsal root ganglia were digested in collagenase P (1 mg/ml, from *Clostridium histolyticum*; Roche Applied Science) solution in Hanks' balanced salt medium supplemented with 0.2 mM $CaCl_2$, 0.12% BSA, and the trypsin inhibitor from soybean (0.1 mg/ml; Roche Applied Science) for 30–60 min. DRG neurons were dispersed by tritura-

tion and then plated onto growth factor reduced Matrigel (Invitrogen)-coated 15-mm glass coverslips and cultured for 24 h in Eagle's minimum essential medium (Invitrogen) supplemented with 0.2% BSA and 20 ng/ml NGF-2.5S (BD Biosciences, Bedford, MA) at 37 °C in a water-jacketed 5% CO_2 incubator.

Fluorescence Imaging—Fluorescence imaging experiments were performed as described (20). Briefly, transfected HEK cells were loaded with Fura-2AM (4 μM) or Fura-FF-AM (4 μM) in PBS containing Ca^{2+} and Mg^{2+} (Ca/Mg-PBS) for 1 h, followed by an additional 30-min incubation in Ca/Mg-PBS containing no fluorescence dye. DRG neurons were loaded with Fura-2AM (4 μM) in Ca/Mg-PBS for 1 h followed by an additional 30-min incubation in Ca/Mg-PBS without Fura-2AM. A Till Photonics single-cell fluorescence imaging system equipped with an Andor DU885 charge-coupled device camera (Andor Technology PLC, South Windsor, CT) was used to monitor intracellular Ca^{2+} changes in single Fura-2-loaded HEK cells. The standard extracellular solution contained 145 mM NaCl, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5.5 mM glucose (pH 7.2). The experiments to assess the TRPV1 recovery rate from desensitization were performed using the standard extracellular solution with the concentration of $CaCl_2$ increased up to 2 mM. To induce voltage-gated calcium channel activity in DRG neurons, we used a modified standard extracellular solution that contained 80 mM NaCl and 70 mM KCl rather than 145 mM NaCl and 2.5 mM KCl. The cells were superfused continuously with the test solutions at a rate of 1.5 ml/min.

Electrophysiological Experiments—Electrophysiological whole cell patch clamp experiments were performed as described elsewhere (18). Briefly, TRPV1 and $Na_v1.7$ currents were amplified using an Axopatch 200B amplifier (Molecular Devices) and digitized using an analog-digital converter Digidata 1440a at a sampling rate of 1 kHz. The currents were filtered at 3 kHz. Series resistance compensation was set to 50%. The standard external solution contained 145 mM NaCl, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5.5 mM glucose (pH 7.2). The intracellular solution contained 125 mM $CsMeSO_3$, 3.77 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA (100 nM free Ca^{2+}), and 10 mM HEPES (pH 7.2). The $NMDG^+$ solution contained 150 mM NMDG-Cl, 10 mM HEPES, and 5.5 mM glucose (pH 7.2). The pCLAMP 10 software package was used for acquisition control and data analyses. We used an episodic stimulation voltage protocol to record TRPV1 currents. Cells were voltage-clamped at a potential of −60 mV, and voltage ramps from −100 to +100 mV were applied at 2-s intervals. The current density was calculated by dividing the peak current by the cell capacitance. To record $Na_v1.7$ current-voltage relationships (*I-V*), HEK cells stably expressing $Na_v1.7$ were voltage-clamped at −80 mV and stimulated with trains of 150-ms depolarizing pulses. During the train, the potential was stepped from −100 to +40 mV with 10-mV increments at 2-s intervals between episodes. To determine the voltage dependence of the steady-state inactivation for $Na_v1.7$ channels, cells were voltage-clamped at −90 mV, and 500-ms voltage prepulses to various potentials from −120 to −10 mV with 10-mV increments followed by a 10-ms test pulse to −10 mV were applied with a 5-s interval between episodes. The data were fitted to a Boltzmann-charge-voltage function $f(V) = C + I_{max}/(1 + e[(V_{0.5} - V)/V_C])$, where $V_{0.5}$ is the voltage of half-maximum inactivation, using the Levenberg-Marquardt search method and the sum of squared errors as the minimization approach (pCLAMP 10 software). All of the electrophysiological recordings were performed at room temperature (22–24 °C).

Statistics—The SigmaPlot 12 software (Systat Software, Inc.) was used for the statistical analyses. The *t* test followed by the Mann-Whitney rank sum test was used to determine whether there is a statistically significant difference between two groups, whereas the one-way analysis of variance on Ranks test followed by Dunn's post hoc test was used for comparison of multiple groups. The significance level was set to <0.05. All of the data were presented as means ± S.E.

RESULTS

Imperatorin Does Not Alter Thermal Withdrawal Thresholds—To determine whether imperatorin has an *in vivo* effect on thermal nociception, we first measured whether the compound would elicit direct nocifensive behaviors upon subcutaneous injection into the hindpaw. We observed that imperatorin did not induce nocifensive behavior in this test; animals injected with imperatorin flinched an average of 10.4 ± 1.2 times over 5 min, which was unchanged from the 9.9 ± 1.4 times upon injection with vehicle alone. We next determined paw withdrawal thresholds upon stimulation with a radiant light heat source (16), as a measure of thermal sensitivity. Baseline thermal withdrawal thresholds were established and then intraplantar injections of either vehicle or imperatorin were administered into the right hindpaw of the rats. Local injection of imperatorin had no effect on the thermal withdrawal latency when compared with baseline at 5, 20, and 35 min postinjection (Fig. 1*B*). These results suggest that a local application of imperatorin does not alter nociceptive sensitivity to a ramped increase in heat.

Imperatorin Attenuates Capsaicin-induced Nociceptive Behaviors in the Eye Wipe Test—We used the corneal wipe test to determine whether imperatorin may alter chemically induced activation of TRPV1 and subsequently alter nocifensive behavior. The cornea is a specialized tissue innervated by trigeminal afferent nerves, of which ~25% express TRPV1 (21, 22). Application of noxious substances to the eye induces a transient nocifensive response in conscious animals, which can be reversed by either peripherally or systemically administered antinociceptives (23–25). Thus, it is a good model for determining the antinociceptive effect of peripherally administered imperatorin on acute trigeminally mediated nociception. Application of imperatorin alone to the cornea did not induce nocifensive behavior (Fig. 1*C*), which is consistent with our finding that injection into the hindpaw did not induce flinching behaviors. However, a 5-min pretreatment with imperatorin (400 μM) significantly attenuated the nocifensive behavior induced by capsaicin (1 μM) instillation (Fig. 1*C*; $p < 0.05$). This finding suggests that although imperatorin did not modulate nocifensive behavior or alter thermal hyperalgesia, the compound decreased the *in vivo* nocifensive response induced by capsaicin. A plausible mechanism for this decrease in behavior is that imperatorin desensitized, at least to some extent, the TRPV1 receptor to stimulation with capsaicin.

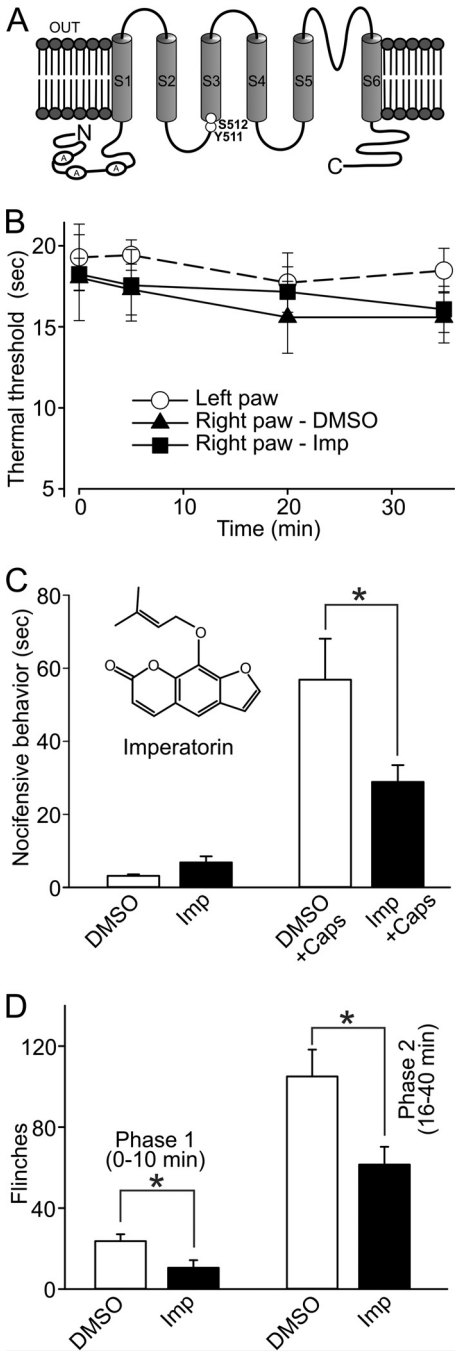

FIGURE 1. **Imperatorin (*Imp*) inhibited *in vivo* nocifensive behavior induced by capsaicin.** *A*, the topology of TRPV1. Ser^{512} and Tyr^{511} are critical residues within the TRPV1 capsaicin-binding site located between the S2 and S3 transmembrane domains. *B*, thermal threshold responses of rats following injection of DMSO ($n = 8$) or imperatorin ($n = 8$). *C*, nocifensive behavioral response following instillation of DMSO ($n = 8$) or imperatorin ($n = 8$) and subsequent instillation of capsaicin onto the eye. *, $p < 0.001$; determined by *t* test comparing the imperatorin group to the vehicle group. *D*, formalin induced flinching following intraplantar injection of DMSO ($n = 6$) or imperatorin ($n = 6$) and subsequent intraplantar injection of 1% formalin. *, $p < 0.05$ determined by *t* test comparing the imperatorin group to the vehicle group. The results are presented as means ± S.E.

Imperatorin Attenuates Nociceptive Behaviors in the Formalin Test—To determine whether imperatorin may inhibit acute and inflammatory nociceptive responses, we performed the formalin test. Subcutaneous injection of formalin into the rat hindpaw results in a biphasic behavioral reaction (26). The first phase is a result of direct stimulation of nociceptors and persists for 5–10 min, whereas the second phase involves sensitization of nociceptors and can be observed for ~15–40 min following injection (27). We injected imperatorin (2.45 mM; 30 μl) or vehicle (12.3% DMSO in saline; 30 μl) and first observed whether a direct effect of these injections elicits nociceptive responses. As observed previously, there was no direct effect of the vehicle or drug. Five minutes following the injection of vehicle or imperatorin, the rats were injected with 1% formalin. Pretreatment with imperatorin significantly attenuated both phases of the formalin test (Fig. 1*D*). These findings suggest that imperatorin attenuates both acute and inflammatory nociceptive responses.

Imperatorin Is a Weak Agonist of the TRPV1 Channel—Our *in vivo* data suggested that imperatorin may target TRPV1 channels. Therefore, we next investigated whether the compound modulates TRPV1 activity. During these experiments, we employed HEK cells transiently expressing human TRPV1 (TRPV1-HEK cells). We first performed fluorescence imaging experiments and found that imperatorin stimulated intracellular Ca^{2+} transients in TRPV1-HEK cells (Fig. 2*A*). The effective concentration that elicited 50% maximum effect (EC_{50}) was 12.3 ± 3.28 μM, suggesting that imperatorin is a weak agonist of the TRPV1 channel. Another furanocoumarin isoimperatorin, a close structural analog of imperatorin, also stimulated Ca^{2+} transients in TRPV1-HEK cells, indicating that other furanocoumarins may also modulate TRPV1 activity. Isoimperatorin was less potent than imperatorin (Fig. 2*E*), leading us to focus our subsequent investigations on imperatorin.

To confirm that imperatorin-induced Ca^{2+} transients were due to TRPV1 activation, we tested whether JNJ-17203212, a specific blocker of TRPV1, inhibited the Ca^{2+} transients. Fig. 2*B* shows that 500 nM JNJ-17203212 was very effective in inhibiting 20 nM capsaicin-induced Ca^{2+} increases in TRPV1-expressing HEK cells, whereas Fig. 2*C* demonstrates that the same concentration of JNJ-17203212 completely inhibited 10 μM imperatorin-induced responses. Capsazepine (10 μM), another blocker of TRPV1, also inhibited imperatorin induced responses, further corroborating that imperatorin effects were due to activation of TRPV1 (Fig. 2*D*). Notably, both JNJ-17203212 and capsazepine decreased the basal level fluorescence in TRPV1-expressing cells, evidencing the presence of TRPV1 spontaneous activity in our TRPV1 overexpression HEK model. 100 μM Imperatorin-elicited Ca^{2+} transients were significantly smaller ($p < 0.05$) than Ca^{2+} increases stimulated by 1 μM capsaicin, suggesting that imperatorin is a partial agonist for TRPV1 channel.

Imperatorin Activates Ca^{2+} Influx Exclusively in TRPV1-expressing Mouse DRG Neurons—Next we performed fluorescence imaging experiments to investigate whether imperatorin may activate native TRPV1 in freshly isolated DRG neurons from wild type mice. Fig. 2*F* shows that imperatorin (50 μM) stimulated Ca^{2+} influx exclusively in capsaicin-sensitive DRG neurons from $TRPV1^{+/+}$ wild type mice and had no effect on the neurons that lacked capsaicin responses. This observation supported our hypothesis that imperatorin is a partial agonist of TRPV1. To further confirm this, we next determined whether imperatorin can activate intracellular Ca^{2+}

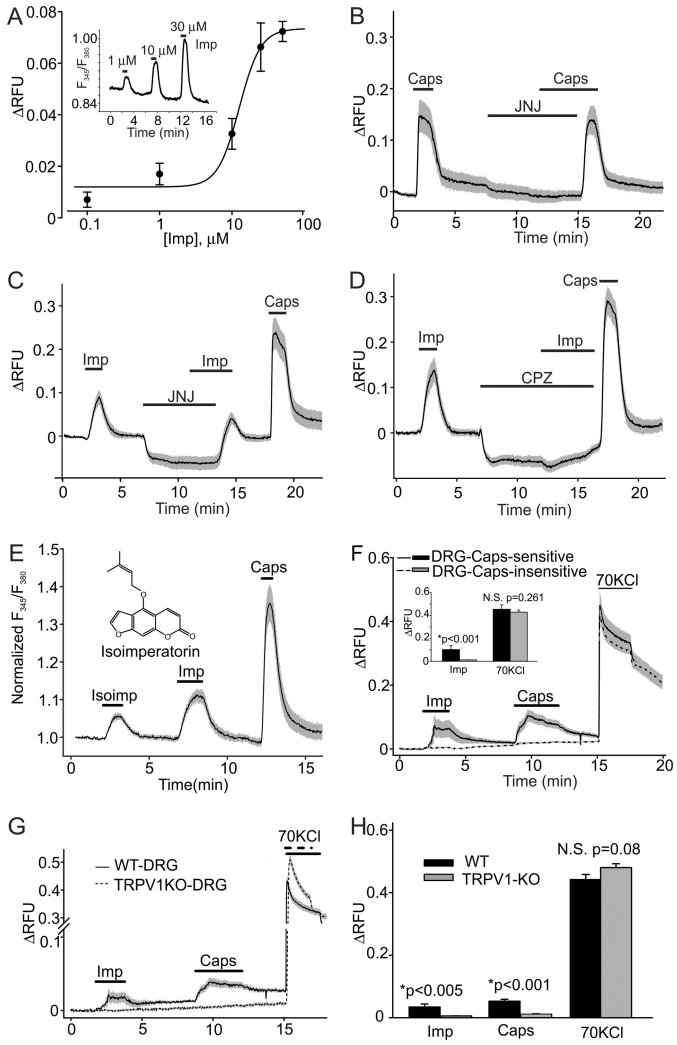

FIGURE 2. **Furanocoumarin imperatorin stimulated intracellular Ca^{2+} increases by activating the TRPV1 channel.** *A*, imperatorin (*Imp*) concentration-response relationship for TRPV1. *RFU,* ratio of fluorescence units. The *inset* shows a sample trace of imperatorin-stimulated Ca^{2+} increases. *B*, JNJ-17203212 (*JNJ*), a competitive and potent TRPV1 antagonist, inhibited the capsaicin (*Caps*)-induced intracellular Ca^{2+} increases. *C*, imperatorin does not activate Ca^{2+} transients in the presence of JNJ-17203212. *D*, capsazepine (*CPZ*), another TRPV1 antagonist, has a similar effect as JNJ-17203212. *E*, isoimperatorin (*Isoimp*)-stimulated intracellular Ca^{2+} increases in TRPV1-HEK cells. The *inset* shows the chemical structure of isoimperatorin. Isoimperatorin was less potent than imperatorin to activate TRPV1. The *solid lines* are averaged intracellular [Ca^{2+}] changes in 14–20 cells. The *vertical lines* represent S.E. The compounds were applied at the times indicated with the *horizontal bars*. *F*, 50 μM imperatorin induced Ca^{2+} influx in capsaicin-sensitive wild type TRPV1$^{+/+}$ DRG neurons ($n = 24$) from four wild type mice, whereas capsaicin-insensitive DRG neurons did not respond to imperatorin ($p < 0.001$, $n = 87$), although all neurons exhibited a similar response to 70 mM KCl solution ($p = 0.261$). Capsaicin (1 μM) application was used to identify TRPV1-expressing DRG neurons. *G*, neither imperatorin (50 μM) nor capsaicin (1 μM) induced Ca^{2+} transients in DRG neurons from three TRPV1$^{-/-}$ knock-out mice (TRPV1-KO). *H*, summary data for *F* and *G*. Capsaicin-sensitive WT-DRG neurons responded to both imperatorin and capsaicin. Conversely, TRPV1$^{-/-}$-KO-DRG neurons ($p < 0.001$, $n = 111$ *versus* $n = 171$) were insensitive to either imperatorin or capsaicin. WT and TRPV1-KO DRG neurons had similar responses to 70 mM KCl solution ($p = 0.08$).

increases in DRG neurons isolated from TRPV1$^{-/-}$ knock-out mice. We observed that none of TRPV1$^{-/-}$ DRG neurons responded to imperatorin (50 μM) or capsaicin (1 μM; Fig. 2, *G* and *H*). On the other hand, depolarization of TRPV1$^{-/-}$ DRG neurons induced by an application of the high potassium extracellular solution containing 70 mM KCl and 80 mM NaCl stimulated robust intracellular Ca^{2+} increases similar to that observed in wild type DRG neurons ($p = 0.08$).

Imperatorin Delays the Recovery of TRPV1 from Desensitization—The imperatorin pretreatment significantly attenuated the nocifensive behavior induced by capsaicin instillation. To identify the mechanisms of action of imperatorin, we determined whether this compound affects the TRPV1 recovery rate from capsaicin-induced desensitization. During these experiments, we used Fura-FF dye with a lower affinity (~5.5 μM) for Ca^{2+} to avoid signal saturation. HEK cells transfected with the empty vector were used as negative control. We preincubated TRPV1-expressing HEK cells with either imperatorin (100 μM) or DMSO and then challenged the cells twice with 1 μM capsaicin. Fig. 3 (*A* and *C*) shows that DMSO had no effect on resting intracellular Ca^{2+} levels in TRPV1-HEK cells. In contrast, 100 μM imperatorin induced large, sustained Ca^{2+} transients showing fluorescence ratio increases of 0.33 ± 0.03 ratio units. Brief removal of extracellular Ca^{2+} reversibly eliminated imperatorin-induced Ca^{2+} increases, indicating that the compound stimulated Ca^{2+} influx in TRPV1-HEK cells. The first application of capsaicin elicited peak amplitudes of Ca^{2+} transients that were not significantly altered by the presence or absence of imperatorin ($p = 0.07$; Fig. 3*C*). Conversely, the peak amplitudes of the Ca^{2+} transients stimulated by the second application of capsaicin were significantly decreased in the presence of imperatorin as compared with DMSO (Fig. 3*D*). The normalized mean peak amplitudes amounted to 0.49 ± 0.04 in the DMSO-treated cells and 0.31 ± 0.02 in imperatorin-treated cells ($p < 0.01$, normalized to the peak amplitude obtained with the first capsaicin application), suggesting that imperatorin delays the recovery of TRPV1 from desensitization. The current amplitudes at the end of the first capsaicin application normalized to the peak values were also significantly smaller ($p < 0.05$; Fig. 3*D*) in imperatorin-pretreated cells, indicating that imperatorin sped up TRPV1 desensitization. We also tested whether lower doses of imperatorin (1 μM), producing a very weak activation of TRPV1, modulate the rate of TRPV1-mediated Ca^{2+} influx decay (Fig. 3*E*) to exclude the possibility that the faster decay rate of TRPV1-mediated Ca^{2+} influx in the presence of 100 μM imperatorin is simply due to the agonist effect of imperatorin. Fig. 3 (*E* and *F*) show that 1 μM imperatorin induced very small or no activation of intracellular Ca^{2+} transients in HEK cells expressing TRPV1 loaded with Fura-FF. Again, the peak amplitudes of the first capsaicin-induced transients in the presence of DMSO or 1 μM imperatorin were not significantly different ($p = 0.196$), yet the second response to capsaicin in the present of 1 μM imperatorin was significantly decreased as compared with the DMSO group ($p < 0.05$; Fig. 3*F*). This suggests that even lower concentrations of imperatorin may modulate the desensitization rate of TRPV1. Our data reveal that imperatorin did indeed speed up the desensitization rate of TRPV1 and delayed its recovery from the desensitization state. This may be a possible mechanism for the imperatorin nocifensive action observed in our *in vivo* studies.

Imperatorin Does Not Potentiate Capsaicin-activated TRPV1 Currents—We next performed whole cell patch clamp experiments to investigate the ability of imperatorin to modulate cap-

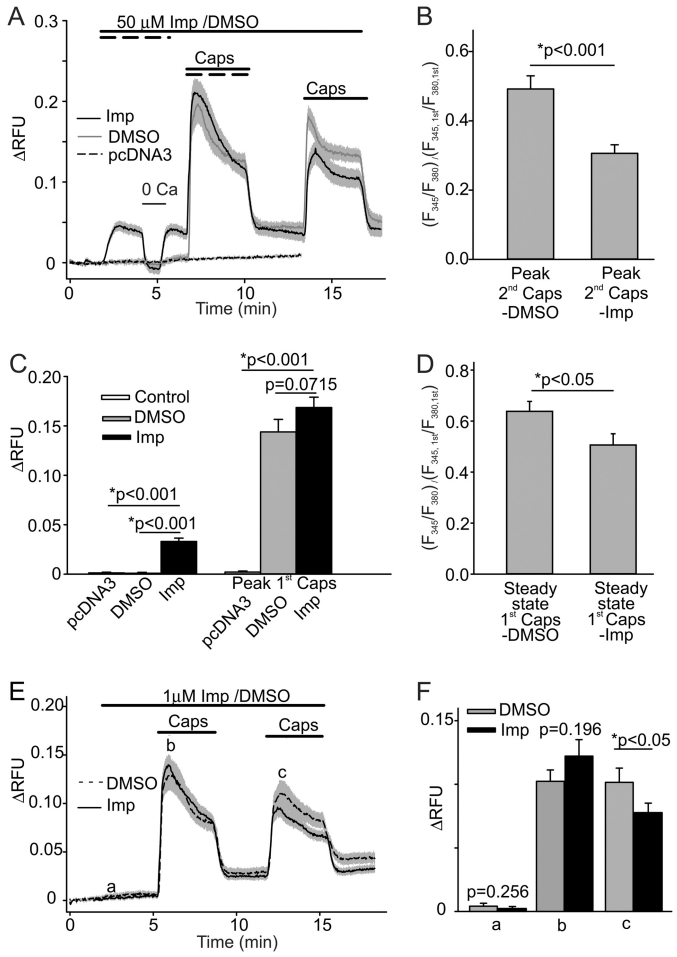

FIGURE 3. **Imperatorin (*Imp*) speeds up TRPV1 desensitization and delays the recovery from TRPV1 desensitization.** *A*, averaged capsaicin (*Caps*)-induced F_{345}/F_{380} fluorescence ratio changes (Fura-FF) in the presence of the vehicle (DMSO, $n = 85$) or 100 μM imperatorin in TRPV1 ($n = 49$) or empty vector ($n = 54$)-expressing cells are shown. *RFU*, ratio of fluorescence units. The compounds were applied at the times indicated with the *horizontal bars*. *B–D*, summary data are shown. *B*, second capsaicin application induced a significantly smaller response in the presence of imperatorin as compared with DMSO. The peak amplitude of the second capsaicin-induced Ca^{2+} increase was normalized to the peak amplitude of the first capsaicin-induced Ca^{2+} increase. *C*, imperatorin-induced intracellular Ca^{2+} increases were significantly greater in TRPV1-expressing HEK cells as compared with empty vector expressing HEK cells. DMSO induced no significant intracellular Ca^{2+} increases in TRPV1-expressing HEK cells. During the double application protocol, the peak amplitudes of the first capsaicin-induced Ca^{2+} transients (*1st Caps*) were not significantly different in the presence of either DMSO or imperatorin. *D*, the current amplitude at the end of the capsaicin-induced first Ca^{2+} transient was significantly smaller in the presence of imperatorin as compared with DMSO ($p < 0.05$). *E*, shown are averaged capsaicin-induced F_{345}/F_{380} fluorescence ratio changes in the presence of the vehicle (DMSO) or 1 μM imperatorin. *F*, summary data of *E* are shown. The peak amplitudes of the first capsaicin-induced transients were not significantly different in the presence of DMSO or 1 μM imperatorin. The second response to capsaicin in the present of 1 μM imperatorin was significantly decreased as compared with the DMSO group. The data are presented as means ± S.E.

saicin-activated TRPV1 currents. Using the 1.2 mM standard extracellular solution to reduce Ca^{2+}-dependent desensitization of TRPV1, we found that in TRPV1-expressing HEK cells, 40 μM imperatorin induced only small outward currents as compared with capsaicin-induced TRPV1 currents (Fig. 4*A*). Surprisingly, we observed little or no inward currents in TRPV1-HEK cells stimulated with imperatorin. These data are consistent with our previous conclusion that the compound is a

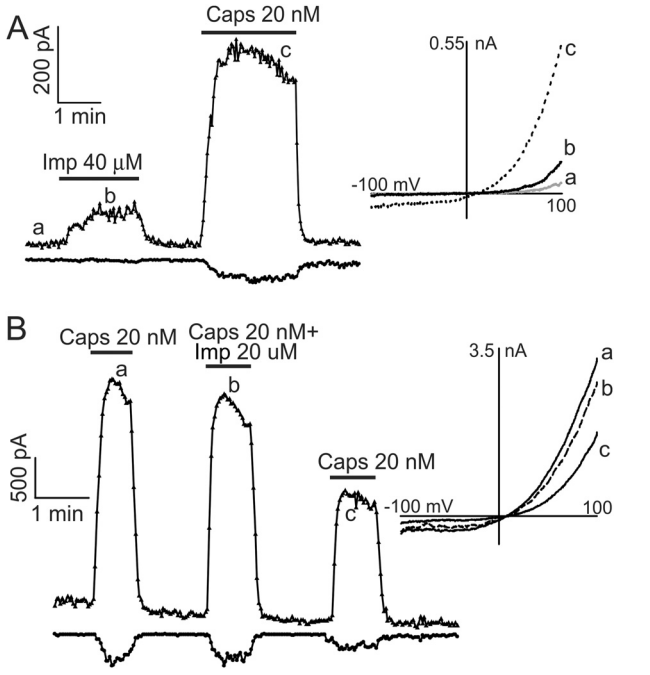

FIGURE 4. **Imperatorin-induced smaller TRPV1 currents as compared with capsaicin-stimulated currents.** *A*, current traces obtained during the applications of 40 μM imperatorin (*Imp*) followed by 20 nM capsaicin (*Caps*) in TRPV1-HEK cells ($n = 7$). The *inset* shows current-voltage relationships acquired at the indicated times. *B*, current traces of TRPV1 currents activated by capsaicin (20 nM) or the mixture of capsaicin and imperatorin. The inward and outward current amplitudes were measured at holding potentials of −100 mV (*lower trace*) and +100 mV (*upper trace*).

partial agonist of TRPV1. We observed no imperatorin-dependent potentiation of capsaicin-induced current when we compared TRPV1 currents activated by capsaicin alone with those induced by a mixture of imperatorin and capsaicin (Fig. 4*B*).

Imperatorin May Interact with a Site Overlapping with the Capsaicin-binding Site on TRPV1—One plausible explanation for the submaximal effects of imperatorin may be that imperatorin acts on the same binding site within the TRPV1 protein as capsaicin, but with much lower affinity. To test this hypothesis, we constructed a mutant of TRPV1 in which tyrosine 511 and serine 512, two essential residues within the human TRPV1 capsaicin-binding site, were substituted by alanines ($TRPV1_{Y511A/S512A}$). This mutant was functional, as determined by its sensitivity to pH, but was insensitive to capsaicin and imperatorin activation (Fig. 5, *A* and *B*). The current density of imperatorin-induced outward currents was significantly smaller in the capsaicin-insensitive TRPV1 mutant (5.65 ± 3.63 pA/pF, $n = 8$) compared with the wild type TRPV1 (91.6 ± 26.9 pA/pF, $n = 14$; Fig. 5*C*), indicating that indeed the imperatorin-binding site may overlap with the TRPV1 capsaicin-binding site.

Imperatorin Potentiates Acid-induced TRPV1 Currents—Low pH solutions (pH < 6) activate TRPV1 channels at room temperature, whereas capsaicin is known to potentiate the acid-induced TRPV1 currents. Because our mutagenesis data indicated that imperatorin acts on the capsaicin-binding site, we hypothesized that imperatorin can modulate acid-induced TRPV1 currents. To test the hypothesis, we employed a protocol including triple applications of acidic extracellular solution to establish TRPV1 current rundown (Fig. 6*A*). We then deter-

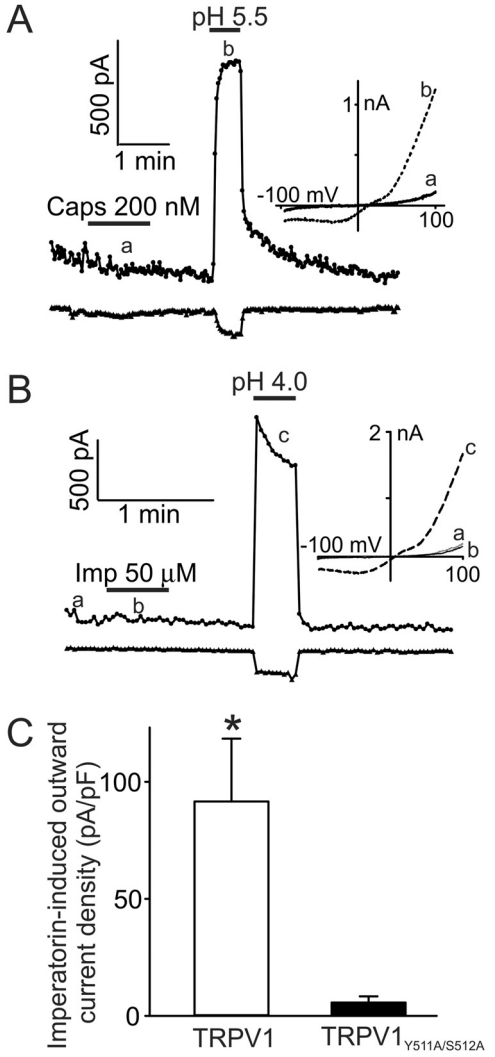

FIGURE 5. **Imperatorin activated no currents in TRPV1$_{Y511A/S512A}$-expressing HEK cells.** *A* and *B*, current traces and current-voltage relationships are shown. Current-voltage relationships were obtained at the indicated times. Acidic extracellular solutions were used to confirm the functional activity of TRPV1$_{Y511A/S512A}$. *C*, shown is a comparison of current densities of imperatorin-induced outward currents recorded in 14 WT-TRPV1-HEK and 8 TRPV1$_{Y511A/S512A}$-HEK cells. *, $p < 0.002$. Summary data are presented as means ± S.E. The inward and outward current amplitudes were measured at −100 mV (*lower trace*) and +100 mV (*upper trace*), respectively. The drugs were applied at the times indicated by the *horizontal bars*. *Caps*, capsaicin; *Imp*, imperatorin.

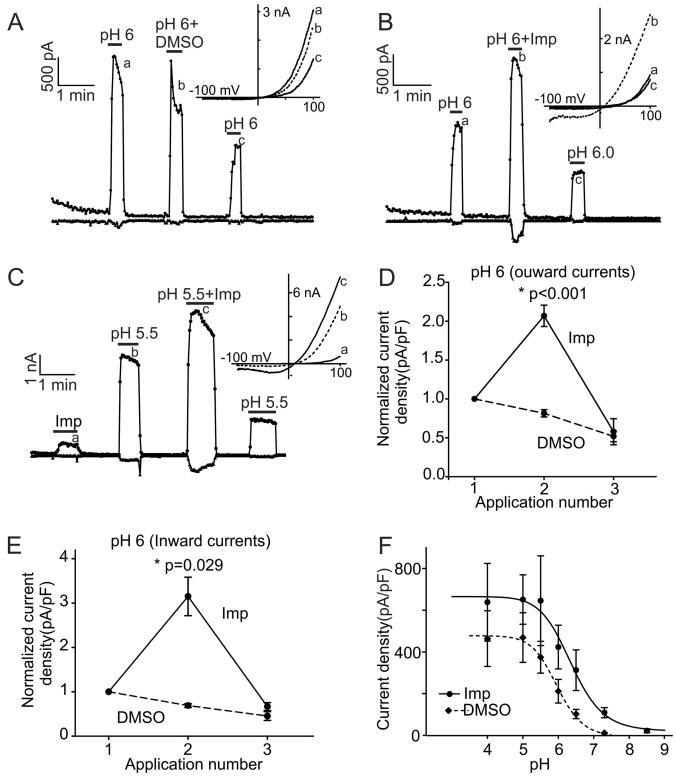

FIGURE 6. **Imperatorin (*Imp*) potentiates the pH 6 evoked TRPV1 currents.** *A–C*, current traces of pH-evoked TRPV1 currents are shown. The *insets* show current-voltage relationships recorded at the times as indicated. The inward and outward current amplitudes were measured at −100 mV (*lower trace*) and +100 mV (*upper trace*), respectively. During the second application, the acidic solution was supplemented either with DMSO or imperatorin (50 μM). *D* and *E*, summary data for the effects of DMSO ($n = 4$) and imperatorin (50 μM, $n = 4$) on pH 6 induced TRPV1 outward and inward currents. The current amplitudes were normalized to the peak amplitudes of the first application of low pH solution. The results are presented as means ± S.E. *F*, pH response curves for TRPV1 obtained in the presence of imperatorin or DMSO ($n = 4–10$).

mined whether imperatorin-induced TRPV1 currents were additive with H^+-activated TRPV1 currents. We found that imperatorin significantly potentiated acid-activated TRPV1 currents as compared with the vehicle (DMSO; Fig. 6, *A* and *B*). The peak amplitude of acid-activated TRPV1 currents measured in the presence of imperatorin was clearly greater than the sum of the approximated acid- and imperatorin-stimulated TRPV1 currents (Fig. 6*C*). The summary data for outward and inward current potentiation by the pH 6 extracellular solutions are shown in Fig. 6 (*D* and *E*, respectively). Noticeably, the potentiation of inward currents (DMSO, 0.69 ± 0.04 pA/pF *versus* imperatorin, 3.15 ± 0.4 pA/pF; $n = 4$; $p < 0.001$) was much greater than that of outward currents (DMSO, 0.82 ± 0.05 pA/pF *versus* imperatorin, 2.10 ± 0.14 pA/pF; $n = 4$; $p < 0.001$). The pH response curve for TRPV1 was slightly shifted to the right with pH_{50} of 6.37 ± 0.14 for imperatorin as compared with the vehicle (DMSO, 5.97 ± 0.08; Fig. 6*F*). Thus, in contrast to the capsaicin-induced currents, the pH-induced currents were sensitive to imperatorin modulation.

Acid-induced TRPV1$_{Y511A/S512A}$ Currents Are Not Potentiated by Imperatorin—To further support the hypothesis that imperatorin modulates TRPV1 activity via the capsaicin binding site, we next investigated whether the compound is capable of modulating acid-activated currents through TRPV1$_{Y511A/S512A}$. The double mutant had a lower expression rate than WT TRPV1; therefore, to reliably measure the currents through the mutated TRPV1, we used acidic solutions with a pH of 4. We first demonstrated that imperatorin significantly potentiated the pH 4 induced WT-TRPV1 inward currents (Fig. 7*A*; imperatorin, 1.50 ± 0.07, $n = 4$, *versus* DMSO, 0.64 ± 0.07 pA/pF; $n = 4$; $p < 0.001$). However, we found that pH 4 stimulated outward currents were not affected by imperatorin ($p = 0.11$; Fig. 7*B*), suggesting that the pH 4 stimulated outward current amplitude already reached the saturation point in this case. We next investigated the effects of imperatorin on pH 4 induced currents through TRPV1$_{Y511A/S512A}$. Fig. 7 (*C* and *D*) demonstrates that neither inward nor outward currents through TRPV1$_{Y511A/S512A}$ were sensitive to imperatorin. Thus, these find-

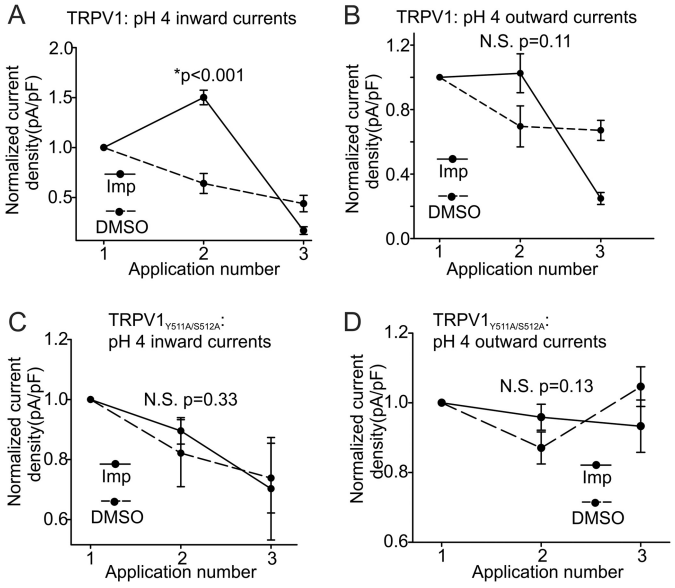

FIGURE 7. **Imperatorin (*Imp*) does not potentiate the pH 4 evoked inward currents in TRPV1$_{Y511A,S512A}$–HEK cells.** *A* and *B*, summary data for DMSO ($n = 4$) and imperatorin (50 μM, $n = 4$) effects on pH 4 induced currents in WT-TRPV1 cells. *C* and *D*, summary data for DMSO ($n = 3$) and imperatorin (50 μM, $n = 6$) effects on pH 4 induced currents in TRPV1$_{Y511A,S512A}$–HEK cells. A protocol including triple applications of acidic extracellular solution was used. DMSO or imperatorin was added during the second H^+ application. The peak current amplitudes obtained during the second and third H^+ application were normalized to the peak current amplitudes obtained during the first H^+ application. The results are presented as means ± S.E.

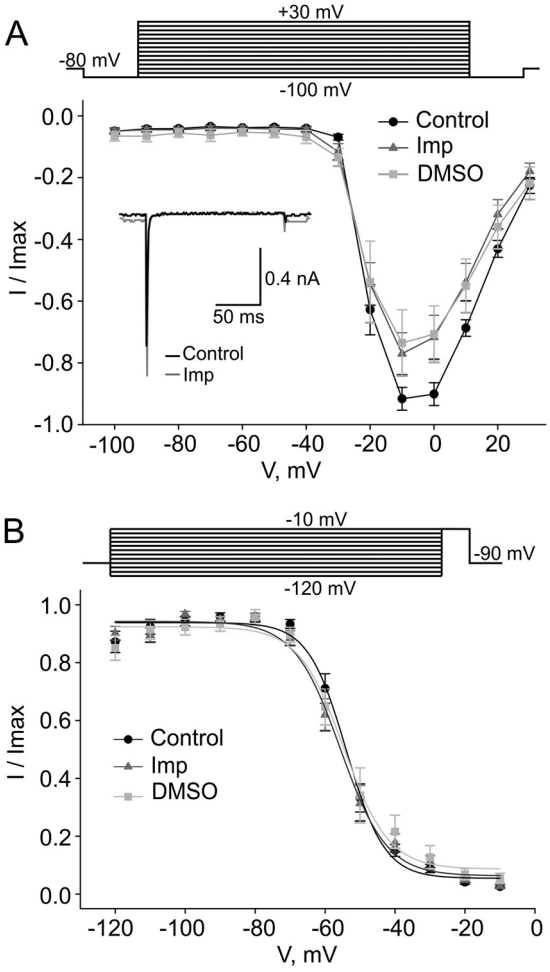

FIGURE 8. **Imperatorin (*Imp*) does not affect the activity of the Na$_v$1.7 channel.** *A*, shown are step pulse current-voltage relationships. The peak amplitudes of Na$_v$1.7 currents evoked by depolarizing pulses to −10 mV from a holding potential of −80 mV in the presence of imperatorin ($n = 13$) or DMSO ($n = 8$) were not significantly different ($p > 0.05$). *B*, imperatorin did not shift the steady-state inactivation curve for Na$_v$1.7 currents. Current amplitudes were normalized to the maximal values. The voltage protocols used to investigate Na$_v$1.7 currents are shown in the *upper part* of each panel. The data are expressed as means ± S.E.

ings were consistent with the hypothesis that imperatorin modulates acid-activated TRPV1 currents through the capsaicin-binding site.

*Imperatorin Has No Effect on Na$_v$1.7 Currents*—The voltage-gated sodium Na$_v$1.7 channel plays a crucial role in neuronal excitability and transmission of nociceptive information. We employed HEK cells stably expressing the human Na$_v$1.7 channel to test whether imperatorin affects Na$_v$1.7 channel activity. The peak amplitudes of Na$_v$1.7 currents evoked by stepwise depolarization from a holding potential of −100 mV to 0 mV were not significantly different ($p = 0.47$) in the presence of 50 μM imperatorin (0.72 ± 0.07, $n = 13$; normalized to the peak amplitude of Na$_v$1.7 currents acquired before the treatment) or DMSO (0.71 ± 0.09, $n = 8$; Fig. 8*A*). Also, the steady-state inactivation of the voltage-gated Na$_v$1.7 currents was not affected by 50 μM imperatorin (Fig. 8*B*). The values for the voltages of half-maximum inactivation amounted to −53.41 ± 3.11 for DMSO ($n = 8$) and −55.07 ± 1.86 for imperatorin ($n = 13$). Thus, imperatorin does not significantly affect Na$_v$1.7 activity.

DISCUSSION

Here we present evidence that imperatorin, a furanocoumarin from *A. dahurica*, is a partial agonist of human TRPV1, a channel implicated in the pain pathway. *A. dahurica* extracts are widely used in traditional medicine for treating headache, toothache, and orbital pain. Interestingly, we found that imperatorin attenuates acute and inflammatory nociceptive behaviors in the formalin test in rats. Furthermore, we found that imperatorin was effective in attenuating TRPV1-dependent, trigeminally mediated nocifensive behavior in an *in vivo* eye wipe test assessing nociception in rats (24). We established that imperatorin likely acts on a site overlapping with the capsaicin-binding site on the TRPV1 channel. In addition, we ascertained that imperatorin significantly potentiated acid-dependent activation of TRPV1 but had no effect on the activity of the human Na$_v$1.7 channel. The chemical structure of imperatorin differs from chemical structures of all known agonists and antagonists of TRPV1 (Fig. 9). We also showed that isoimperatorin, another furanocoumarin, also activated TRPV1 currents in a concentration-dependent manner (Fig. 2*E*).

Although our study identified a plausible mechanism for imperatorin effects on TRPV1, we do not know why only a very small or no imperatorin-induced inward sodium current was observed during our patch clamp experiments, whereas we recorded large imperatorin-stimulated intracellular Ca^{2+} transients using the fluorescence imaging approach. It was reported that the diameter and Ca^{2+} permeability (P_{Ca}/P_{Na}) of the TRPV1 pore dynamically changes during capsaicin-dependent TRPV1 activation, "pore dilation" (28). We speculate that the

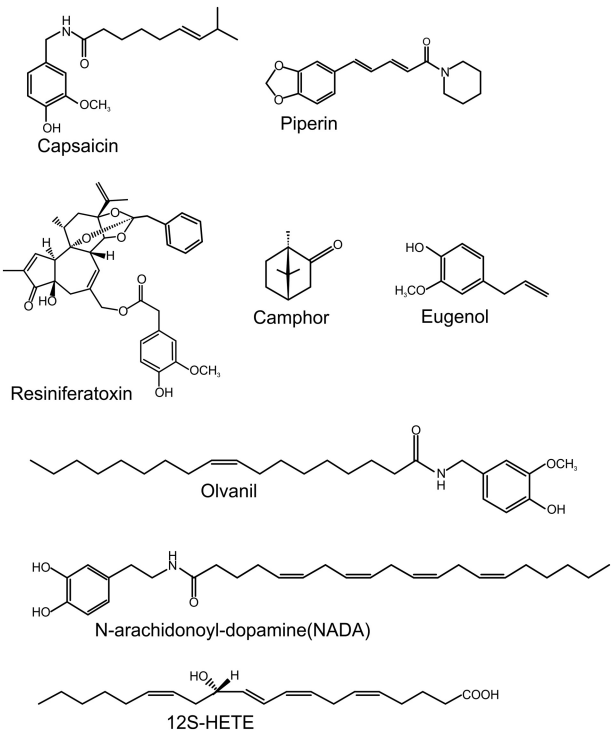

FIGURE 9. **Chemical structures of known TRPV1 agonists.**

partial agonist imperatorin is not capable of causing TRPV1 pore dilation. It is possible that imperatorin opens only a narrow TRPV1 cation permeation pathway with higher Ca^{2+} selectivity resulting in the failure of recording sodium inward currents. Interestingly, highly Ca^{2+} selective store-operated channels, such as calcium release-activated channels, have a smaller pore diameter of ~0.6 nm (29) compared with the predicted 1–1.2-nm pore diameter of TRPV1 (28), a nonselective cation channel. However, further work will be needed to prove or refute this hypothesis. Importantly, imperatorin-induced intracellular Ca^{2+} increases were not observed in Ca^{2+}-free extracellular solutions, suggesting that imperatorin stimulates Ca^{2+} influx through TRPV1 rather than Ca^{2+} release from the intracellular stores.

Each TRPV1 channel subunit has six transmembrane domains (S1–S6) with a short pore-forming stretch between S5 and S6 and the intracellular N- and C-terminal regions. Phosphatidylinositol 4,5-bisphosphate (PIP_2), Ca^{2+}, calmodulin, and phosphorylation of several cytosolic N- and C-terminal sites may regulate TRPV1 activity and/or desensitization rate (30–33). It is possible that imperatorin can modulate calmodulin/PIP_2 binding affinity to TRPV1 or channel phosphorylation. However, imperatorin does not modulate the activity of the $TRPV1_{Y511A/S512A}$ mutant, suggesting that direct interaction of the compound with TRPV1 is necessary for TRPV1 modulation.

TRPV1 activation is implicated in the pathogenesis of dull pain, including but not limited to, acute thermal pain, inflammatory thermal hypersensitivity, trigeminal neuralgia, postherpetic neuralgia, diabetic peripheral neuropathy, constriction type nerve injury, and bone cancer pain (34–36). Specifically, pain associated with bone cancers is a longstanding clinical problem affecting scores of patients. Advanced cancer patients cope with chronic pain that, in part, results from tumor-induced acidosis. Bone cancer pain is often opioid-resistant, requiring alternative treatment. Indeed, the treatments targeted at TRPV1 inhibition are considered promising alternatives to traditional opioid drugs. However, potent TRPV1 antagonists have been shown to cause severe hyperthermia (12, 13), making usage of these compounds impossible. Subsequent development of activation-mode specific TRPV1 antagonists eliminated the untoward development of hyperthermia (15), although these antagonists are incapable of attenuating the acid-induced activation of TRPV1, which is an integral mediator of bone cancer pain.

Our data suggest that imperatorin speeds up capsaicin-induced TRPV1 channel desensitization and delays the channel recovery from desensitization (Fig. 3). This effect may or may not be due to the agonistic effect of imperatorin on TRPV1, resulting in stronger desensitization of TRPV1 in the presence of the compound. The ability of imperatorin to modulate TRPV1 desensitization rates may underlie its capacity to decrease the nocifensive responses induced by capsaicin. In addition, we found that imperatorin potentiated the acid activation mode of TRPV1, indicating that desensitizing/agonistic effects of the compound or its more potent analogs may be effective in preventing bone cancer pain originating in part from tumor-induced acidosis or increased TRPV1 expression in nerve terminals. It is possible that potentiation of TRPV1 acid activation mode by imperatorin may lead to Ca^{2+} overload of the sensory nerve terminals innervating tumor-bearing bones with consequent TRPV1 desensitization and probably the retraction of sensory nerve ending, hence bringing pain relief. Thus, we propose that furanocoumarins may be effective for treating bone cancer pain.

Importantly, imperatorin is a partial agonist of the human TRPV1 channel. Therefore, it is unlikely that this compound would reduce core body temperature *in vivo*. In this report, we show that imperatorin did not alter baseline nocifensive behavior induced by noxious heat in our rat model. Thus, imperatorin does not compromise the ability to recognize noxious heat stimuli in the treated subjects, a problem observed with potent TRPV1 antagonists.

Also noteworthy is that the pain relieving potential of imperatorin is not limited by its ability to modulate TRPV1 activity. Imperatorin attenuated formalin-induced acute behavior, which was somewhat surprising because the acute phase of the formalin test has been shown to be mediated through the TRPA1 channels (37). There is evidence to suggest, however, that heterologous desensitization between the TRP channels can occur *in vivo*: capsaicin, injected into the hindpaw to activate the TRPV1 channel and induce nociceptive behavior, can desensitize the TRPA1 channel so that challenge with a TRPA1 agonist induces an attenuated nociceptive behavioral response (38). Therefore, imperatorin, a partial agonist of TRPV1, may be heterologously desensitizing the TRPA1 response to formalin. However, our studies here cannot rule out that imperatorin may directly or indirectly modulate the activity of TRPA1 channels. However, importantly, imperatorin also attenuated behaviors during the second phase of the formalin test, which is

attributed to the release of inflammatory mediators and subsequent sensitization, in part, of TRPV1 and TRPA1 channels (37, 39), indicating that imperatorin may inhibit TRPV1-mediated inflammatory pain.

In addition to its TRP-related actions, imperatorin was also shown to reduce the lipopolysaccharide-induced protein expression of cyclooxygenase-2 (40, 41), a target of several analgesic medications. In addition to its analgesic properties, imperatorin is known to exhibit anti-inflammatory, antibacterial, and anti-proliferative/antitumor properties (40–42). In addition, imperatorin was reported to modulate the activity of the L-type of voltage-gated Ca^{2+} channels (43) and voltage-gated Na^{+} channels endogenously expressed in differentiated neuronal NG108–15 cells (44). Interestingly, we did not find any imperatorin modulatory effects on $Na_v1.7$ channels stably expressed in HEK cells. A likely explanation for such a discrepancy may be that $Na_v1.7$ channels are not expressed in differentiated neuronal NG108–15 cells.

CONCLUSIONS

We here report that furanocoumarin imperatorin represents a novel class of TRPV1 partial agonists that modulate TRPV1 desensitization rate and potentiate acid activation of TRPV1. Ca^{2+} overload-induced desensitization of TRPV1 in nerve terminals innervating tumor-bearing bones may have analgesic potential. Thus, furanocoumarins emerge as important lead compounds for discovery of novel TRPV1 modulators with analgesic properties.

Acknowledgments—We thank Dr. Gerry Oxford for valuable discussions and the members of the Obukhov lab for technical assistance. We also thank James Jackson for culturing $Na_v1.7$-HEK cells.

REFERENCES

1. Sarker, S. D., and Nahar, L. (2004) Natural medicine: the genus *Angelica*. *Curr. Med. Chem.* **11,** 1479–1500
2. Lu, H. C. (2005) *Chinese Natural Cures*, Black Dog & Leventhal Publishers, Inc., New York
3. Liu, R., Li, A., and Sun, A. (2004) Preparative isolation and purification of coumarins from *Angelica dahurica* (Fisch. ex Hoffn) Benth, et Hook. f (Chinese traditional medicinal herb) by high-speed counter-current chromatography. *J. Chromatogr. A* **1052,** 223–227
4. Zhang, H., Gong, C., Lv, L., Xu, Y., Zhao, L., Zhu, Z., Chai, Y., and Zhang, G. (2009) Rapid separation and identification of furocoumarins in *Angelica dahurica* by high-performance liquid chromatography with diode-array detection, time-of-flight mass spectrometry and quadrupole ion trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **23,** 2167–2175
5. Salat, K., Moniczewski, A., and Librowski, T. (2013) Transient receptor potential channels: emerging novel drug targets for the treatment of pain. *Curr. Med. Chem.* **20,** 1409–1436
6. Dib-Hajj, S. D., Yang, Y., Black, J. A., and Waxman, S. G. (2013) The $Na_v1.7$ sodium channel: from molecule to man. *Nat. Rev. Neurosci.* **14,** 49–62
7. Planells-Cases, R., Valente, P., Ferrer-Montiel, A., Qin, F., and Szallasi, A. (2011) Complex regulation of TRPV1 and related thermo-TRPs: implications for therapeutic intervention. *Adv. Exp. Med. Biol.* **704,** 491–515
8. Cox, J. J., Reimann, F., Nicholas, A. K., Thornton, G., Roberts, E., Springell, K., Karbani, G., Jafri, H., Mannan, J., Raashid, Y., Al-Gazali, L., Hamamy, H., Valente, E. M., Gorman, S., Williams, R., McHale, D. P., Wood, J. N., Gribble, F. M., and Woods, C. G. (2006) An SCN9A channelopathy causes congenital inability to experience pain. *Nature* **444,** 894–898
9. Dib-Hajj, S. D., Cummins, T. R., Black, J. A., and Waxman, S. G. (2010) Sodium channels in normal and pathological pain. *Annu. Rev. Neurosci.* **33,** 325–347
10. Jordt, S. E., and Julius, D. (2002) Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell* **108,** 421–430
11. Szallasi, A., Cortright, D. N., Blum, C. A., and Eid, S. R. (2007) The vanilloid receptor TRPV1: 10 years from channel cloning to antagonist proof-of-concept. *Nat. Rev. Drug Discov.* **6,** 357–372
12. Brederson, J. D., Kym, P. R., and Szallasi, A. (2013) Targeting TRP channels for pain relief. *Eur. J. Pharmacol.* **716,** 61–76
13. Cortright, D. N., and Szallasi, A. (2009) TRP channels and pain. *Curr. Pharm. Des* **15,** 1736–1749
14. (2011) Capsaicin patch (Qutenza) for postherpetic neuralgia. *Med. Lett. Drugs Ther.* **53,** 42–43
15. Reilly, R. M., McDonald, H. A., Puttfarcken, P. S., Joshi, S. K., Lewis, L., Pai, M., Franklin, P. H., Segreti, J. A., Neelands, T. R., Han, P., Chen, J., Mantyh, P. W., Ghilardi, J. R., Turner, T. M., Voight, E. A., Daanen, J. F., Schmidt, R. G., Gomtsyan, A., Kort, M. E., Faltynek, C. R., and Kym, P. R. (2012) Pharmacology of modality-specific transient receptor potential vanilloid-1 antagonists that do not alter body temperature. *J. Pharmacol. Exp. Ther.* **342,** 416–428
16. Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J. (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32,** 77–88
17. Brittain, J. M., Duarte, D. B., Wilson, S. M., Zhu, W., Ballard, C., Johnson, P. L., Liu, N., Xiong, W., Ripsch, M. S., Wang, Y., Fehrenbacher, J. C., Fitz, S. D., Khanna, M., Park, C. K., Schmutzler, B. S., Cheon, B. M., Due, M. R., Brustovetsky, T., Ashpole, N. M., Hudmon, A., Meroueh, S. O., Hingtgen, C. M., Brustovetsky, N., Ji, R. R., Hurley, J. H., Jin, X., Shekhar, A., Xu, X. M., Oxford, G. S., Vasko, M. R., White, F. A., and Khanna, R. (2011) Suppression of inflammatory and neuropathic pain by uncoupling CRMP-2 from the presynaptic Ca^{2+} channel complex. *Nat. Med.* **17,** 822–829
18. Chakraborty, S., Berwick, Z. C., Bartlett, P. J., Kumar, S., Thomas, A. P., Sturek, M., Tune, J. D., and Obukhov, A. G. (2011) Bromoenol lactone inhibits voltage-gated Ca^{2+} and transient receptor potential canonical channels. *J. Pharmacol. Exp. Ther.* **339,** 329–340
19. Xiao, Y., Bingham, J. P., Zhu, W., Moczydlowski, E., Liang, S., and Cummins, T. R. (2008) Tarantula huwentoxin-IV inhibits neuronal sodium channels by binding to receptor site 4 and trapping the domain ii voltage sensor in the closed configuration. *J. Biol. Chem.* **283,** 27300–27313
20. Kumar, S., Chakraborty, S., Barbosa, C., Brustovetsky, T., Brustovetsky, N., and Obukhov, A. G. (2012) Mechanisms controlling neurite outgrowth in a pheochromocytoma cell line: The role of TRPC channels. *J. Cell. Physiol.* **227,** 1408–1419
21. Guo, A., Vulchanova, L., Wang, J., Li, X., and Elde, R. (1999) Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4-binding sites. *Eur. J. Neurosci.* **11,** 946–958
22. Nakamura, A., Hayakawa, T., Kuwahara, S., Maeda, S., Tanaka, K., Seki, M., and Mimura, O. (2007) Morphological and immunohistochemical characterization of the trigeminal ganglion neurons innervating the cornea and upper eyelid of the rat. *J. Chem. Neuroanat.* **34,** 95–101
23. Diogenes, A., Patwardhan, A. M., Jeske, N. A., Ruparel, N. B., Goffin, V., Akopian, A. N., and Hargreaves, K. M. (2006) Prolactin modulates TRPV1 in female rat trigeminal sensory neurons. *J. Neurosci.* **26,** 8126–8136
24. Farazifard, R., Safarpour, F., Sheibani, V., and Javan, M. (2005) Eye-wiping test: a sensitive animal model for acute trigeminal pain studies. *Brain Res. Brain Res. Protoc.* **16,** 44–49
25. Szolcsányi, J., and Jancsó-Gábor, A. (1975) Sensory effects of capsaicin congeners I. Relationship between chemical structure and pain-producing potency of pungent agents. *Arzneimittelforschung* **25,** 1877–1881
26. Dubuisson, D., and Dennis, S. G. (1977) The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* **4,** 161–174
27. Hunskaar, S., and Hole, K. (1987) The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* **30,** 103–114
28. Chung, M. K., Güler, A. D., and Caterina, M. J. (2008) TRPV1 shows dynamic ionic selectivity during agonist stimulation. *Nat. Neurosci.* **11,**

555–564
29. Hou, X., Pedi, L., Diver, M. M., and Long, S. B. (2012) Crystal structure of the calcium release-activated calcium channel Orai. *Science* **338,** 1308–1313
30. Rohacs, T. (2013) Regulation of transient receptor potential channels by the phospholipase C pathway. *Adv. Biol. Regul.* **53,** 341–355
31. Lukacs, V., Rives, J. M., Sun, X., Zakharian, E., and Rohacs, T. (2013) Promiscuous activation of transient receptor potential vanilloid 1 channels by negatively charged intracellular lipids, the key role of endogenous phosphoinositides in maintaining channel activity. *J. Biol. Chem.* **288,** 35003–35013
32. Chuang, H. H., Prescott, E. D., Kong, H., Shields, S., Jordt, S. E., Basbaum, A. I., Chao, M. V., and Julius, D. (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P_2-mediated inhibition. *Nature* **411,** 957–962
33. Premkumar, L. S., and Ahern, G. P. (2000) Induction of vanilloid receptor channel activity by protein kinase C. *Nature* **408,** 985–990
34. Pabbidi, R. M., Yu, S. Q., Peng, S., Khardori, R., Pauza, M. E., and Premkumar, L. S. (2008) Influence of TRPV1 on diabetes-induced alterations in thermal pain sensitivity. *Mol. Pain* **4,** 9
35. Premkumar, L. S., and Abooj, M. (2013) TRP channels and analgesia. *Life Sci.* **92,** 415–424
36. Mathie, A. (2010) Ion channels as novel therapeutic targets in the treatment of pain. *J. Pharm. Pharmacol.* **62,** 1089–1095
37. McNamara, C. R., Mandel-Brehm, J., Bautista, D. M., Siemens, J., Deranian, K. L., Zhao, M., Hayward, N. J., Chong, J. A., Julius, D., Moran, M. M., and Fanger, C. M. (2007) TRPA1 mediates formalin-induced pain. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 13525–13530
38. Ruparel, N. B., Patwardhan, A. M., Akopian, A. N., and Hargreaves, K. M. (2008) Homologous and heterologous desensitization of capsaicin and mustard oil responses utilize different cellular pathways in nociceptors. *Pain* **135,** 271–279
39. Btesh, J., Fischer, M. J., Stott, K., and McNaughton, P. A. (2013) Mapping the-binding site of TRPV1 on AKAP79: implications for inflammatory hyperalgesia. *J. Neurosci.* **33,** 9184–9193
40. Huang, G. J., Deng, J. S., Liao, J. C., Hou, W. C., Wang, S. Y., Sung, P. J., and Kuo, Y. H. (2012) Inducible nitric oxide synthase and cyclooxygenase-2 participate in anti-inflammatory activity of imperatorin from *Glehnia littoralis*. *J. Agric. Food Chem.* **60,** 1673–1681
41. Ban, H. S., Lim, S. S., Suzuki, K., Jung, S. H., Lee, S., Lee, Y. S., Shin, K. H., and Ohuchi, K. (2003) Inhibitory effects of furanocoumarins isolated from the roots of *Angelica dahurica* on prostaglandin E2 production. *Planta Med.* **69,** 408–412
42. Luszczki, J. J., Wojda, E., Andres-Mach, M., Cisowski, W., Glensk, M., Glowniak, K., and Czuczwar, S. J. (2009) Anticonvulsant and acute neurotoxic effects of imperatorin, osthole and valproate in the maximal electroshock seizure and chimney tests in mice: a comparative study. *Epilepsy Res.* **85,** 293–299
43. Lu, W., Zhang, T., Li, P., Wang, F., Pan, X., Wang, C., Hu, Z., and Zhang, J. (2013) Structural modeling and identification of imperatorin as novel L-type calcium channel blocker. *Med. Chem.* **9,** 993–998
44. Wu, K. C., Chen, Y. H., Cheng, K. S., Kuo, Y. H., Yang, C. T., Wong, K. L., Tu, Y. K., Chan, P., and Leung, Y. M. (2013) Suppression of voltage-gated Na channels and neuronal excitability by imperatorin. *Eur. J. Pharmacol.* **721,** 49–55