PAIN® 154 (2013) 2750–2758

PAIN®

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# Parthenolide inhibits nociception and neurogenic vasodilatation in the trigeminovascular system by targeting the TRPA1 channel

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*Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.*

ARTICLE INFO

*Article history:*
Received 22 May 2013
Received in revised form 23 July 2013
Accepted 5 August 2013

*Keywords:*
CGRP
Migraine
Parthenolide
TRPA1

ABSTRACT

Although feverfew has been used for centuries to treat pain and headaches and is recommended for migraine treatment, the mechanism for its protective action remains unknown. Migraine is triggered by calcitonin gene-related peptide (CGRP) release from trigeminal neurons. Peptidergic sensory neurons express a series of transient receptor potential (TRP) channels, including the ankyrin 1 (TRPA1) channel. Recent findings have identified agents either inhaled from the environment or produced endogenously that are known to trigger migraine or cluster headache attacks, such as TRPA1 simulants. A major constituent of feverfew, parthenolide, may interact with TRPA1 nucleophilic sites, suggesting that feverfew's antimigraine effect derives from its ability to target TRPA1. We found that parthenolide stimulates recombinant (transfected cells) or natively expressed (rat/mouse trigeminal neurons) TRPA1, where it, however, behaves as a partial agonist. Furthermore, in rodents, after initial stimulation, parthenolide desensitizes the TRPA1 channel and renders peptidergic TRPA1-expressing nerve terminals unresponsive to any stimulus. This effect of parthenolide abrogates nociceptive responses evoked by stimulation of peripheral trigeminal endings. TRPA1 targeting and neuronal desensitization by parthenolide inhibits CGRP release from trigeminal neurons and CGRP-mediated meningeal vasodilatation, evoked by either TRPA1 agonists or other unspecific stimuli. TRPA1 partial agonism, together with desensitization and nociceptor defunctionalization, ultimately resulting in inhibition of CGRP release within the trigeminovascular system, may contribute to the antimigraine effect of parthenolide.



## 1. Introduction

Feverfew (*Tanacetum parthenium* L.) has been used for centuries as a remedy for pain, fever, and headaches [26]. Feverfew, alone or in combination with other compounds, has been long evaluated as a prophylactic agent for migraine [24,28,41]. Positive well-powered, randomized clinical trials [14,37] have led to the recommendation of MIG-99, a relatively stable extract manufactured with supercritical $CO_2$ from feverfew, for migraine prevention with a level B of evidence [22]. More recently, 2 similar sublingually administered products containing feverfew and ginger have been tested successfully as an acute treatment for migraine attacks [12,13]. However, the underlying mechanism of the antimigraine action of feverfew remains unknown.

The major constituent of feverfew is the sesquiterpene lactone, parthenolide (Fig. 1A), which has previously been found to exert anti-inflammatory effects [38,44]. Parthenolide may interact with nucleophilic sites of proteins *via* its α-methylene-γ-lactone ring and epoxide moiety [36,42]. This chemical property qualifies a number of reactive molecules as agonists of the transient receptor potential ankyrin 1 (TRPA1), which by covalent modification of

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0304-3959/$36.00 
http://dx.doi.org/10.1016/j.pain.2013.08.002

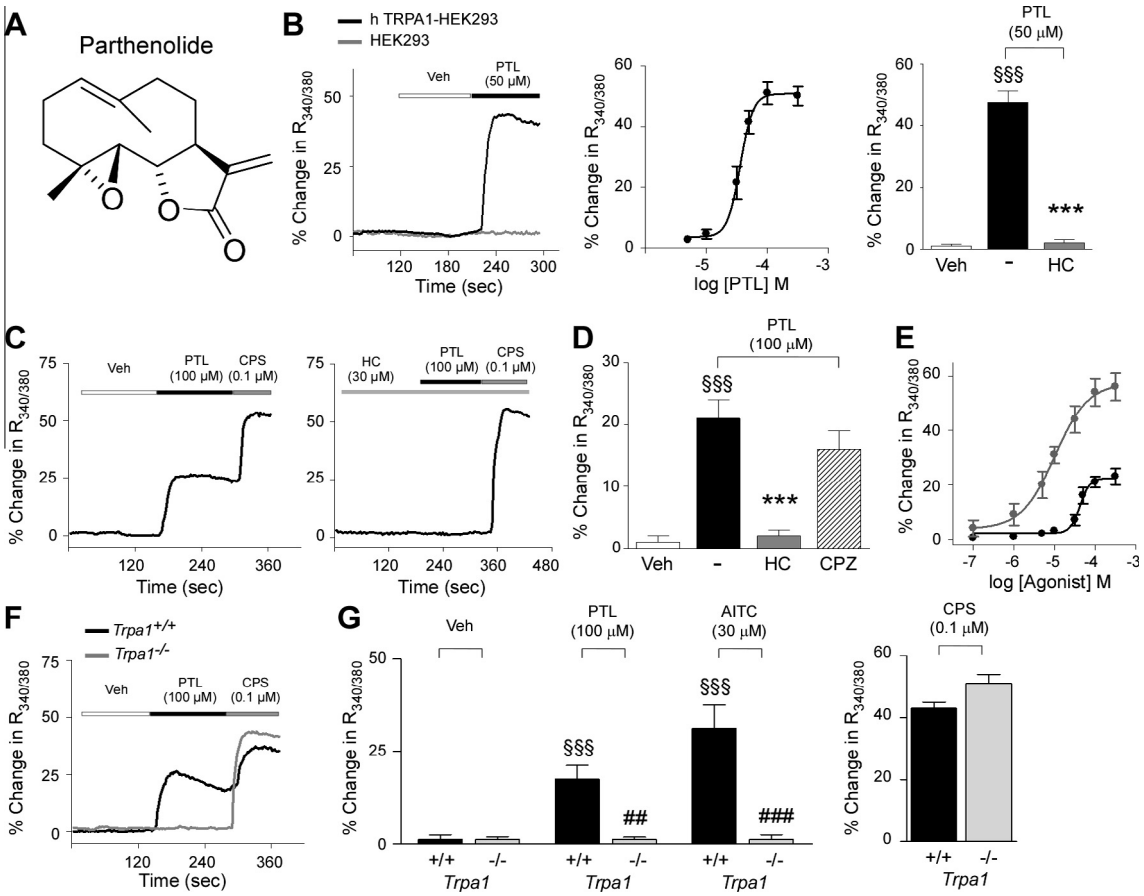


**Fig. 1.** Parthenolide (PTL) selectively activates the TRPA1 channel. (A) Chemical structure of PTL. (B) Representative traces and concentration-response curve (CRC) of intracellular calcium mobilization evoked by PTL in HEK293 cells transfected with the cDNA of human TRPA1 (hTRPA1-HEK293; black line) or in untransfected-HEK293 cells (HEK293; gray line). Calcium response evoked by PTL (50 μM) was abolished by the selective TRPA1 antagonist, HC-030031 (HC; 30 μM). (C) Representative traces, (D) pooled data, and (E) CRC of intracellular calcium mobilization evoked by PTL (black dots and line) or allyl isothiocyanate (AITC; gray dots and line) in cultured rat trigeminal ganglion (TG) neurons. Calcium response induced by PTL was abolished by HC (30 μM) and was unaffected by the selective TRPV1 antagonist, capsazepine (CPZ; 10 μM) (D). (F, G) Calcium response evoked by PTL (100 μM) or AITC (30 μM) in TG neurons from *Trpa1*$^{+/+}$ (black line and columns) was absent in neurons from *Trpa1*$^{-/-}$ mice (gray line and columns). Responses to capsaicin (CPS; 0.1 μM) were unchanged. Veh is the vehicle of PTL. Each trace represents an average of at least 15 responsive cells/neurons; each point or column represents mean ± SEM of n > 25 cells/neurons. Dash indicates combined vehicles of treatments. $^{§§§}P < .001$ vs Veh, $^{***}P < .001$ vs PTL (ANOVA followed by Bonferroni test). $^{##}P < .01$ vs *Trpa1*$^{+/+}$ treated group (Student *t* test).

cysteine residues cause channel activation [19,27,47]. TRPA1 is uniquely stimulated by an unprecedented series of reactive exogenous and endogenous molecules generated by oxidative stress [1,6,7,29,47].

A subset of primary afferents characterized as capsaicin-sensitive because they express the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1) are also enriched with TRPA1 channels and express and release the sensory neuropeptides substance P (SP), neurokinin A (NKA), and calcitonin gene-related peptide (CGRP) [10,23,43]. CGRP release within the trigeminovascular system is now considered to play a key role in the genesis of migraine headaches [16,21,35]. In general, endogenous agents that activate receptors/channels on trigeminal neurons to release CGRP may be considered migraine generators, whereas exogenous compounds that prevent such activation may be considered potential migraine medicines [31].

More recently, the role of TRPA1 in migraine pathophysiology has been suggested by the observation that both the reactive α,β-unsaturated aldehyde and TRPA1 agonist, acrolein [7], and one major volatile component of the headache tree (*U. californica*) scent, umbellulone, selectively target TRPA1 to produce a CGRP-dependent meningeal vasodilatation [15,25,33], which is considered a predictive migraine model. Thus, we hypothesized that parthenolide exerts its antimigraine effect *via* TRPA1 targeting.

Here we found that parthenolide activates both recombinant and neuronal TRPA1. However, parthenolide behaves as a TRPA1 partial agonist, and by targeting TRPA1 causes selective channel desensitization and a nonselective defunctionalization of CGRP-containing sensory neurons. These 3 peculiar features of parthenolide, ultimately resulting in the inhibition of CGRP release from trigeminal neurons, may contribute to the antimigraine effect of feverfew.

## 2. Materials and methods

### 2.1. Animals

Animal experiments were carried out in conformity to the European Communities Council (ECC) guidelines for animal care procedures and the Italian legislation (DL 116/92) application of the ECC directive 86/609/EEC. Studies were conducted under the University of Florence research permits 143/2008-B and 204/2012-B. Male C57BL/6 mice (Harlan Laboratories, Milan, Italy), wild-type (*Trpa1*$^{+/+}$) or TRPA1-deficient (*Trpa1*$^{-/-}$) mice [7], or Sprague-Dawley rats (male, Harlan Laboratories) were used. Animals were killed with intraperitoneal sodium pentobarbital (200 mg/kg).

### 2.2. Parthenolide powder extraction

Flowered aerial parts of feverfew [*Tanacetum parthenium* L. Schultz Bip., 2 kg] were extracted with ethanol. Removal of the solvent left a dark gum, which was partitioned between petroleum ether (2 L) and methanol-water (9:1, 2 L). The defatted methanol phase was evaporated, and the residue (37 g) was suspended in petroleum ether-ethyl acetate 1:1, and purified by vacuum chromatography on neutral alumina (300 g), using petroleum ether-ethyl acetate 1:1 as eluent. The fractions containing parthenolide were pooled and evaporated, and the residue was triturated with ether, with a yield of 6.94 g (0.35% on dried plant material) of a white powder, identified by direct comparison with an authentic sample of parthenolide available from a previous study [3].

### 2.3. Reagents

If not otherwise indicated, all reagents were from Sigma-Aldrich (Milan, Italy). HC-030031 was synthesized as described [2]. Parthenolide extracted powder was dissolved in 100% dimethyl sulfoxide (DMSO) at the final concentration of 100 mM.

### 2.4. Cell culture and isolation of primary sensory neurons

Human embryonic kidney (HEK293) cells stably transfected with the cDNA of human TRPV1 (hTRPV1-HEK293) or with the cDNA of human TRPA1 (hTRPA1-HEK293) and naive HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured as previously described [33]. A tetracycline-regulated system for inducible expression of TRPA1 in Chinese hamster ovary (CHO) cells transfected with the cDNA of the mouse TRPA1 (mTRPA1-CHO) was used and cultured as previously reported [32]. Naive CHO cells were used as control. HumanTRPA1 (hTRPA1-CHO) wild-type or mutant 3C/K-Q (C619S, C639S, C663S, K708Q) cDNAs [19] (1 μg/mL) were expressed in naive CHO cells using a polyethylenimine transfection method (PEI, Sigma-Aldrich, Milan, Italy) [4,45] and cultured as previously reported [19]. All cells were cultured in an atmosphere of 95% air and 5% $CO_2$ at 37°C. Trigeminal ganglia (TG) neurons from rats and *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mice were collected as previously reported [33] (for details see Supplementary methods).

### 2.5. Calcium imaging experiments

Calcium fluorescence was measured in transfected and untransfected HEK293 cells or in trigeminal ganglia neurons, as previously reported [29,33]. Plated cells were loaded with Fura-2AM-ester (5 μM; Alexis Biochemicals, Lausen, Switzerland) added to the buffer solution (37°C) containing the following (in mM): 2 $CaCl_2$; 5.4 KCl; 0.4 $MgSO_4$; 135 NaCl; 10 D-glucose; 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1% bovine serum albumin at pH 7.4. After 40 minutes, cells were washed and transferred to a chamber on the stage of a Nikon Eclipse TE2000U microscope for the recording. Cells were excited alternatively at 340 nM and 380 nM to indicate relative intracellular calcium changes by the $Ratio_{340/380}$ recorded with a dynamic image analysis system (Laboratory Automation 2.0; RCSoftware, Florence, Italy). Cells and neurons were exposed to parthenolide (30 to 1000 μM), allyl isothiocyanate (AITC, 30 μM), or their vehicles (1%, 0.3% DMSO, respectively). Capsaicin (0.1 μM) was used to identify nociceptive neurons. HC-030031 (30 μM), capsazepine (10 μM), or their respective vehicles (3% and 0.1% DMSO) were used. Results are expressed as the percentage of increase of $Ratio_{340/380}$ over the baseline normalized to the maximum effect induced by ionomycin (5 μM) added at the end of the experiment (% change in $R_{340/380}$).

### 2.6. Electrophysiological recordings

Electrophysiological recordings in the whole-cell mode were performed in CHO-transfected cells as previously reported [32] (for details see Supplementary methods). TRPA1-evoked currents were detected upon cell superfusion with parthenolide (100 μM) and AITC (50 to 100 μM).

### 2.7. Eye-wiping assay in mice

Conjunctival application of parthenolide (12.5-25-50 nmol, 5 μL) or its vehicle (5% DMSO) was used to induce an acute nociceptive response in mice [33]. In other experiments, mice received conjunctival AITC (20 nmol) or capsaicin (1 nmol) 40 minutes after a single (4 mg/kg, intraperitoneal) or repeated (once per day for 5 days, 4 mg/kg, intragastric) parthenolide administration. Data are expressed as number of eye movements after drug application.

### 2.8. Dural cannulation and behavior testing

Dural cannulation and behavioral studies were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Arizona and were in accordance with policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for handling and use of laboratory animals. Rat dura cannulation and cutaneous allodynia measurement were performed as described [49]. AITC (10%, 10 μL), capsaicin (1 μM, 10 μL), or their vehicle (mineral oil) were injected through the cannula 40 minutes after parthenolide (4 mg/kg, intraperitoneal) or its vehicle (4% DMSO in phosphate-buffered saline). Calibrated von Frey filaments were applied to the midline of the forehead and to the hind paw at 1-hour intervals after injection of the stimuli. Maximum filament strengths were 8*g* and 15*g* for the face and hind paws, respectively. The withdrawal thresholds were determined by the Dixon up–down method.

### 2.9. CGRP-like immunoreactivity (LI) assay

For CGRP-LI outflow experiments, 0.4-mm slices of rodent tissues (rat spinal cord, trigeminal ganglia and dura mater, and mouse spinal cord) were superfused with an aerated (95% $O_2$ and 5% $CO_2$) Krebs solution with the following composition (in mM): 119 NaCl, 25 $NaHCO_3$, 1.2 $KH_2PO_4$, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 4.7 KCl, 11 D-glucose; maintained at 37°C, containing 0.1% bovine serum albumin, and to minimize peptide degradation, added with the angiotensin-converting enzyme inhibitor captopril (1 μM) and the neutral endopeptidase inhibitor phosphoramidon (1 μM). Tissues were stimulated with parthenolide (10-30-100 μM) or its vehicle (1% DMSO) dissolved in modified Krebs solution. Some tissues were pre-exposed to capsaicin (10 μM, 20 minutes) or superfused with a calcium-free buffer containing EDTA (1 mM). Others tissues were pretreated with HC-030031 (30 μM), capsazepine (10 μM), or pre-exposed to parthenolide (300 μM, 30 minutes) and then, after a prolonged washing, stimulated with parthenolide (100 μM), AITC (100 μM), capsaicin (0.3 μM), or KCl (40 mM). Fractions (4 mL) of superfusate were collected at 10-minute intervals before, during, and after administration of stimulus and then freeze dried, reconstituted with assay buffer, and analyzed for CGRP-LI by using a commercial enzyme-linked immunosorbent assay kit (Bertin Pharma, Montigny le Bretonneux, France). CGRP-LI was calculated by subtracting the mean prestimulus value from those obtained during or after stimulation. Detection limits of the assays were 5 pg/mL. Results are expressed as femtomoles of peptide per gram of tissue. Stimuli did not cross-react with CGRP antiserum.

### 2.10. Meningeal blood flow

Changes in rat middle meningeal artery blood flow were recorded with a laser Doppler flowmeter (Perimed Instruments, Milan, Italy) following the procedure reported elsewhere [33]. Briefly, rats were anesthetized (sodium pentobarbital, 50 mg/kg, intraperitoneal), the head fixed in a stereotaxic frame. A cranial window (4 × 6 mm) was realized into the parietal bone to expose the dura mater. The probe (needle type, tip diameter 0.8 mm) was fixed near a branch of the middle meningeal artery (1 mm from the dural outer layer). The window was filled with a modified synthetic interstitial fluid containing (in mM): 135 NaCl; 5 KCl; 1 $MgCl_2$; 5 $CaCl_2$; 10 D-glucose, and 10 HEPES. Meningeal blood flow was monitored for 30 minutes after administration of parthenolide (4 mg/kg, intraperitoneal) or AITC (1 μmol/kg, intraperitoneal). Meningeal blood flow also was measured after the administration of acrolein (50 nmol, intranasal), ethanol (140 μL/kg, intravenous), sodium nitroprusside (SNP, 1 mM/100 μL, topically to the dura surface) or their vehicles 30 minutes after parthenolide (4 mg/kg, intraperitoneal). Baseline flow was calculated by the mean flow value measured during a 5-minute period prior to stimulus. The increase in blood flow was calculated as percent change over the baseline.

### 2.11. Statistical analysis

All values are the mean ± SEM. Statistical analyses were performed by the unpaired Student *t* test or the 2-way or 1-way analysis of variance (ANOVA), followed by the post-hoc Bonferroni test, or by the Newman–Keuls test. A value of $P < .05$ was considered statistically significant.

## 3. Results

### 3.1. Parthenolide activates recombinant and native TRPA1

Parthenolide is known to react with nucleophiles, in particular with cysteine thiol groups, *via* a Michael addition reaction [42,48], a mechanism through which many compounds activate TRPA1 [2,19,27,47]. To verify if parthenolide targets TRPA1, we exposed hTRPA1-HEK293 cells to parthenolide, which produced a concentration-dependent increase in intracellular calcium, indicating a stimulatory action. This effect of parthenolide was absent in HEK293 untransfected cells (Fig. 1B). In addition, the calcium response evoked by parthenolide was abolished by the selective TRPA1 antagonist, HC-030031 [30] (Fig. 1B), and absent in hTRPV1-HEK293 cells (data not shown). Next, we examined the effect of parthenolide in cultured rat TG neurons. Parthenolide increased intracellular calcium, and the effect was concentration dependent (Fig. 1E), and abrogated by HC-030031, but was unaffected by the TRPV1 antagonist, capsazepine (Fig. 1C and D). Parthenolide, similarly to the selective TRPA1 agonist allyl isothiocyanate (AITC), produced a measurable calcium response in about 40% of TG neurons isolated from *Trpa1*$^{+/+}$ mice, an effect that was completely absent in TG neurons isolated from *Trpa1*$^{-/-}$ mice (Fig. 1F and G). In contrast, calcium response to capsaicin was unchanged in neurons from both *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice (Fig. 1G). All together, these findings indicate selectivity of parthenolide for the TRPA1 channel. Furthermore, in rat trigeminal neurons parthenolide showed, in addition to a lower potency, a much reduced efficacy (maximum response, $E_{max}$: 22% ± 2% of ionomycin percent, n = 30) than that of AITC ($E_{max}$: 58% ± 5% of ionomycin, n = 26, $P < .001$) (Fig. 1E), thus indicating that it behaves as a low-potency partial agonist at the TRPA1 channel.

Whole-cell patch-clamp recordings in both hTRPA1-CHO and mTRPA1-CHO transfected cells confirmed the data obtained with calcium experiments, as extracellular application of parthenolide induced a fast activation of both outward and inward currents, which were blocked by the TRPA1 antagonist HC-030031 and were absent in CHO untransfected cells (Fig. 2A and B and Supplementary Fig. 1). To get better insight into the molecular mechanism of the parthenolide and TRPA1 interaction, mutagenesis studies and desensitization studies were undertaken. hTRPA1 activation by electrophilic agonists is dependent on the presence of 3 key cysteine (C619, C639, C663) and 1 lysine (K708) residues [19,27]. Parthenolide activates the TRPA1 channel through this mechanism, as it was inactive in CHO transfected with the mutant and humanTRPA1 mutant (C619S, C639S, C663S, K708Q) cDNA (3C/K-Q), whereas the nonelectrophilic TRPA1 agonist, menthol [45], activated both mutant and wild-type channels (Fig. 2C). In calcium-containing solutions, application of parthenolide or AITC produced typical inward TRPA1 currents in mTRPA1-CHO cells, followed by a rapid calcium-mediated inactivation. Under these conditions, TRPA1 was refractory to stimulation with a second application of parthenolide or AITC (Fig. 2D and Supplementary Fig. 1).

### 3.2. Parthenolide activates trigeminal nociceptive behavior and allodynia and causes desensitization

Next, to investigate whether parthenolide, *via* TRPA1 targeting, affects the functioning of trigeminal nociceptors, we used the eye wiping assay in mice [33]. Conjunctival parthenolide instillation evoked in a concentration-dependent manner an acute nociceptive behavior in C57/BL6 mice and *Trpa1*$^{+/+}$ mice, whereas a negligible effect, indistinguishable from the response produced by parthenolide vehicle, was observed in *Trpa1*$^{-/-}$ mice (Fig. 3A and B). Of notice, the response evoked by conjunctival instillation of AITC (Fig. 3C) was markedly reduced after systemic administration of parthenolide. AITC was given 40 minutes after 1 single dose of parthenolide (4 mg/kg, intraperitoneal), or 40 minutes after the last of 5 daily treatments with parthenolide (4 mg/kg, intragastric) (Fig. 3D). In contrast, either acute or chronic parthenolide treatment did not affect the eye wiping response evoked by instillation of the TRPV1 agonist capsaicin (Fig. 3C and D). This finding indicates selective desensitization by parthenolide of nociceptive behaviors evoked by TRPA1-specific stimuli.

Prior studies have shown that exposure of the dura to TRPA1 agonists produces time-dependent and reversible cutaneous allodynia of the face and hind paws, which has been proposed as a preclinical rat behavioral model of migraine [15]. Thus, we evaluated whether systemic pretreatment with parthenolide (4 mg/kg intraperitoneal, 40 minutes before dural stimulation) was able to attenuate migraine-related behaviors in rats due to dural TRPA1 activation [15]. The data of the areas-over-time effect curve show significant allodynia after AITC stimulation compared to vehicle (mineral oil). Systemic pretreatment with parthenolide (4 mg/kg intraperitoneal, 40 minutes before dural stimulation), but not its vehicle (4% DMSO, in phosphate-buffered saline), prevented the development of the cutaneous allodynia of the rat face (Fig. 3E) and hind paw (Supplementary Fig. 2) produced by dural application of AITC. However, systemic pretreatment with parthenolide did not alter the cutaneous allodynia evoked by the dural application of capsaicin (Fig. 3E and Supplementary Fig. 2). Again, these findings underline that parthenolide abrogates solely TRPA1-dependent afferent pathways.

### 3.3. Parthenolide releases CGRP from primary afferents and causes nonselective desensitization

There is evidence that TRPA1 is exclusively localized to peptidergic primary sensory neurons [10,43]. Experiments described

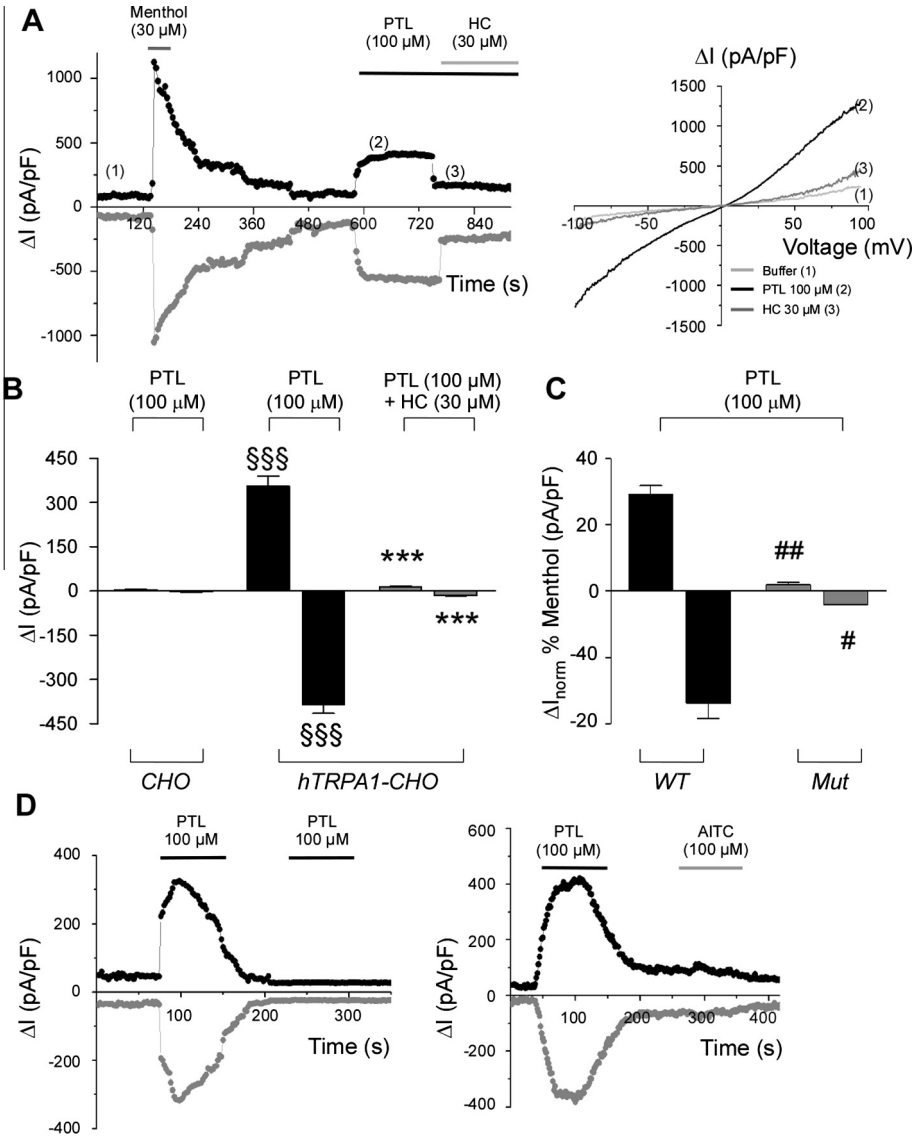


**Fig. 2.** Parthenolide (PTL) elicits characteristic TRPA1 current and causes desensitization. (A) Representative time courses and (B) pooled data of whole-cell currents through humanTRPA1-CHO (hTRPA1-CHO) transfected cells measured at membrane potential of +50 mV (black curve) and −50 mV (gray curve), activated by PTL (100 μM), followed by a fast block by the TRPA1 antagonist, HC-030031 (HC; 30 μM). No response was observed in untransfected CHO cells (CHO). Each column represents mean ± SEM of n > 5 cells. $^{§§§}P < .001$ vs Veh and $^{***}P < .001$ vs PTL (ANOVA followed by Bonferroni test). (C) Average values ± SEM (n = 5 cells) for increased currents (at +50 and −50 mV) evoked by PTL (100 μM) through hTRPA1 wild-type (*WT*) and mutant (3C/K-Q; *Mut*) CHO transfected cells. Values are normalized to activation induced by menthol (30 μM). PTL does not activate *Mut*, whereas menthol, which interacts with TRPA1 in a non covalent manner, does. $^{#}P < .05$, $^{##}P < .01$ vs *WT* (Student *t* test). (D) Representative time course of whole-cell currents through mouseTRPA1-CHO transfected cells measured at membrane potential of +50 mV (black curve) and −50 mV (gray curve) evoked by PTL (100 μM). A second stimulation of TRPA1 by PTL (100 μM) or allyl isothiocyanate (AITC; 100 μM) failed to evoke any current.

in the previous section support this view. To further examine the ability of parthenolide to affect the release of CGRP from both central and peripheral endings of primary sensory neurons, several preparations were used. Parthenolide increased the outflow of CGRP-LI from rat spinal cord slices (Fig. 4A), an effect prevented by pre-exposure to a high concentration of capsaicin [46] and in a calcium-free medium. The effect of parthenolide was abated by HC-030031 but was unaffected by capsazepine (Fig. 4A). In addition, pre-exposure to an elevated concentration of parthenolide prevented parthenolide-evoked CGRP-LI release from rat spinal cord, indicating self-desensitization (Fig. 4A). Similar results were obtained in slices of rat TG (Fig. 4C) or dura mater (Fig. 4D). Exposure to parthenolide increased CGRP-LI outflow from dorsal spinal cord slices obtained from *Trpa1*$^{+/+}$ mice, an effect that was completely absent in tissues taken from *Trpa1*$^{-/-}$ mice (Fig. 4B). Finally, pre-exposure to an elevated concentration of parthenolide abated the increase in CGRP-LI outflow elicited in rat dura mater by either capsaicin or a high $K^+$ medium (Fig. 4E). Thus, parthenolide by targeting TRPA1, after an initial activation, causes nonselective and complete desensitization of CGRP release from trigeminal neurons.

### 3.4. Parthenolide modulates changes in meningeal blood flow by TRP stimulants

A large body of evidence has shown that stimulants of peptidergic trigeminal neurons in rodents cause, among various effects, either extravasation of plasma proteins in the dura mater (an effect mediated by the tachykinins SP and NKA) or vasodilatation of meningeal arterial vessels (a response mediated by CGRP) [31]. However, although tachykinin-dependent neurogenic edema does not

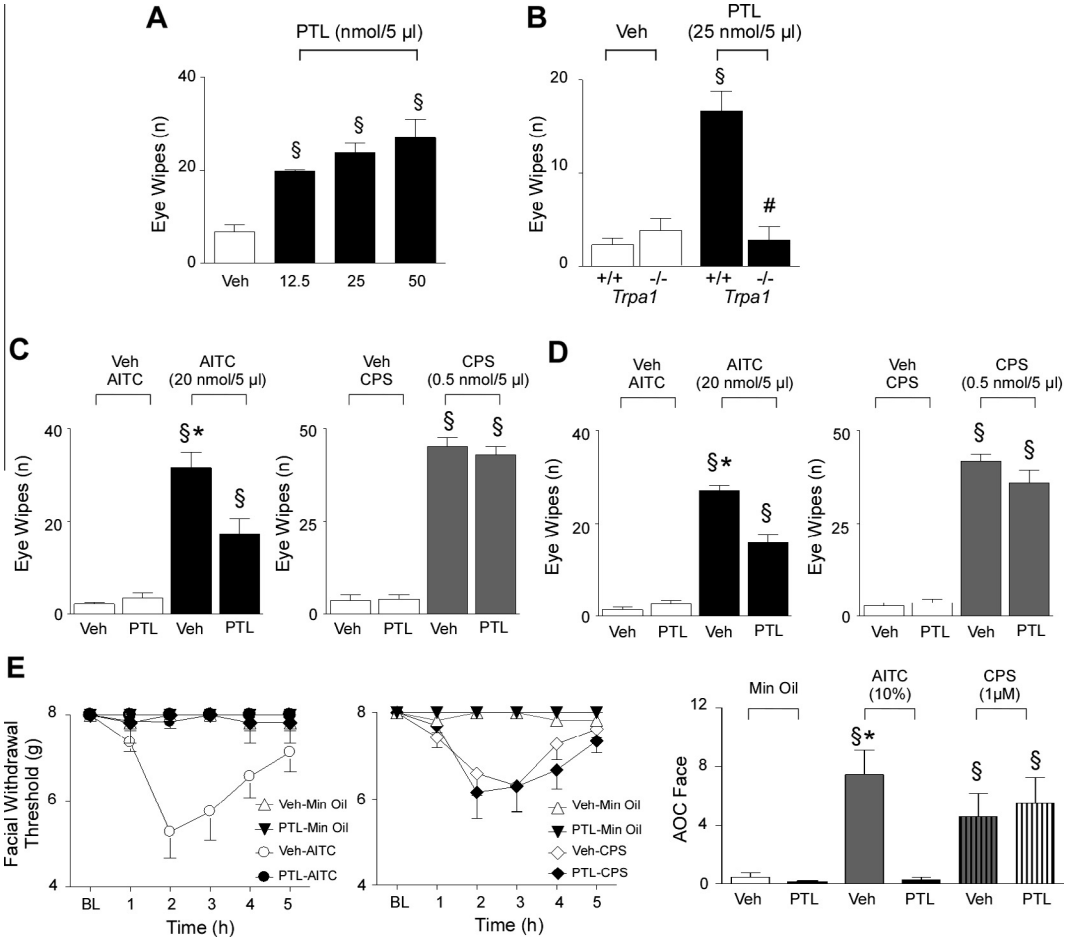


**Fig. 3.** Parthenolide (PTL) activates trigeminal nociceptive behavior in rodents *via* TRPA1 activation and induces desensitization. (A) Ocular instillation of PTL in C57/BL6 mice produced a dose-dependent eye wiping nociceptive response. (B) The response to PTL observed in *Trpa1*$^{+/+}$ mice was completely absent in *Trpa1*$^{-/-}$ mice. Values are mean ± SEM of 5 mice. $^{§§§}P < .001$ vs Veh (ANOVA followed by Bonferroni test). $^{###}P < .001$ vs PTL-*Trpa1*$^{+/+}$ (Student *t* test). (C) The eye wiping response (assessed 40 minutes after PTL) evoked by ocular instillation of allyl isothiocyanate (AITC; 20 nmol/5 µL), was reduced by intraperitoneal PTL pretreatment (4 mg/kg). PTL pretreatment did not modify the effect induced by ocular instillation of capsaicin (CPS; 0.5 nmol/5 µL). (D) The eye wiping response evoked by ocular instillation of AITC (20 nmol, assessed 40 minutes after the last PTL administration) was reduced after repeated treatment with intragastric PTL (4 mg/kg, once per day for 5 days). Veh is the vehicle of AITC or CPS, $^{§§§}P < .001$ vs Veh; $^{**}P < .01$ and $^{***}P < .01$ vs PTL pretreated group (ANOVA followed by Bonferroni). (E) Time course and (F) area over the time-effect curve (AOC; baseline to 5 hours) of the facial allodynia evoked by the application of AITC (10% in mineral oil, Min Oil) or CPS (10 µL) or their vehicle (Min Oil 100%) on rat dura. The generalized allodynia induced by AITC, but not that evoked by topical application of CPS, was prevented by PTL (4 mg/kg, intraperitoneal, given 40 minutes before dural application). Veh is the vehicle of systemic PTL. Data represent the mean ± SEM of 6 rats per group. $^{§}P < .05$ vs Min Oil, $^{**}P < .05$ vs PTL pretreated group. Data were analyzed among groups and across time (baseline to 5 hours) by 2-factor ANOVA followed by the Newman–Keuls test.

seem to play a relevant role in migraine, CGRP release and the resulting neurogenic vasodilatation seems to represent a major underlying migraine mechanism [20,21,35]. Thus, we wondered whether parthenolide affects meningeal blood flow in rats. Whereas administration of AITC (intraperitoneal, 1 µmol/kg) produced a TRPA1-dependent and CGRP-mediated increase in meningeal blood flow (data not shown), the maximum achievable dose of parthenolide (4 mg/kg, intraperitoneal) was ineffective (Fig. 4F). Parthenolide (4 mg/kg, intraperitoneal, 30 minutes before the stimulus) inhibited the meningeal vasodilatation evoked by both intranasal acrolein instillation, a response that has been previously identified as produced by TRPA1 activation [25], and by intranasal capsaicin or intravenous ethanol, responses that are both mediated by TRPV1 stimulation [34]. Parthenolide did not affect meningeal vasodilatation evoked by the direct vasodilator agent sodium nitroprusside (Fig. 4F and G). Finally, pretreatment with HC-030031 prevented the inhibitory effect of parthenolide on ethanol-evoked vasodilatation (Fig. 4F and G). Results suggest that parthenolide produces desensitization of perivascular meningeal sensory fibers, which mediate CGRP-dependent vasodilatation. Because the desensitizing effect of parthenolide is not exclusively confined to TRPA1-agonists, but also to stimuli such as acrolein, capsaicin, or ethanol, which act *via* TRPA1-independent pathways [25,34], it is possible that parthenolide, *via* TRPA1 targeting, causes nonselective defunctionalization of peptidergic sensory nerve terminals.

## 4. Discussion

The first novelty of the present study, supported by unequivocal pharmacological and genetic findings, is that parthenolide targets TRPA1. In particular, deletion of TRPA1 makes mouse neurons unresponsive to parthenolide, and both genetic and pharmacological evidence indicates that parthenolide activates the human TRPA1 variant, suggesting that responses produced by parthenolide in rodents could also be reproduced in humans. Mutagenesis studies of the human TRPA1 indicate that the loss of function of the mutant channel enlists parthenolide into the larger series of electrophilic and reactive molecules, which activate TRPA1 *via*

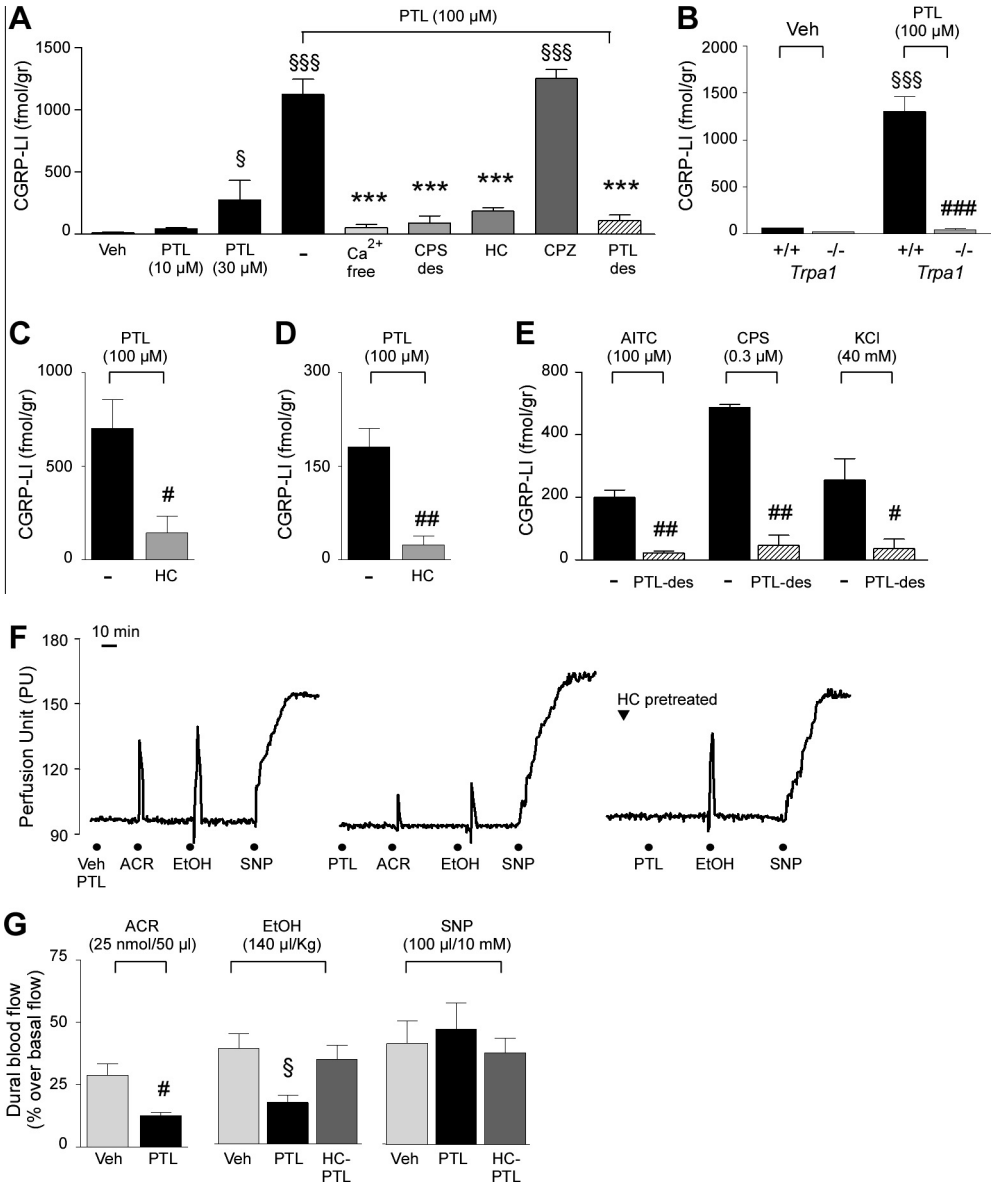


**Fig. 4.** Parthenolide *via* TRPA1 activation releases CGRP from and causes desensitization of rat primary sensory neurons. (A) Parthenolide (PTL) increased the calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) outflow from rat dorsal spinal cord slices in a concentration-dependent manner. Effect of PTL (100 μM) was abolished by calcium removal ($Ca^{2+}$-free), capsaicin desensitization (CPS-des), and the selective TRPA1 antagonist HC-030031 (HC; 30 μM), but not by the selective TRPV1 antagonist capsazepine (CPZ; 10 μM). Pre-exposure to a high concentration of PTL (300 μM, 30 minutes; PTL-des) also abolished PTL-evoked CGRP release. (B) PTL increased CGRP-LI outflow from spinal cord slices from *Trpa1*$^{+/+}$ mice, but not from *Trpa1*$^{-/-}$ mice. PTL elicited a HC-sensitive CGRP-LI release from rat trigeminal ganglia (C) and dura mater (D). (E) Exposure to a high concentration of PTL (300 μM, 30 minutes) abated CGRP-LI release evoked by allyl isothiocyanate (AITC; 100 μM), capsaicin (CPS; 0.3 μM), or KCl (40 mM). Veh is the vehicle of PTL. Values are mean ± SEM of n = 4 experiments. Dash indicates combined vehicles of the treatments. $^{§}P < .05$ and $^{§§§}P < .001$ vs Veh; $^{***}P < .001$ vs PTL (ANOVA followed by Bonferroni test). $^{\#}P < .05$, $^{\#\#}P < .01$, and $^{\#\#\#}P < .001$ vs *Trpa1*$^{+/+}$ or respective vehicle group (Student *t* test). (F) Representative traces and (G) pooled data of the increases in dural blood flow evoked by intranasal acrolein (ACR, 50 nmol/50 μL), intravenous ethanol (EtOH, 140 μL/kg, intravenous) or dural application of sodium nitroprusside (SNP; 10 mM/100 μL). PTL (4 mg/kg, intraperitoneal) treatment (middle panel and bar graph) significantly reduced responses to ACR and EtOH, but not to SNP, and HC (100 mg/kg, intragastric) pretreatment (right hand panel and bar graph) prevented the inhibitory effect evoked by systemic PTL (HC-PTL) on EtOH-evoked response. Veh is the vehicle of PTL. Values are mean ± SEM of n > 5 animals. $^{\#}P < .05$ vs Veh (Student *t* test). $^{§}P < .05$ vs Veh (ANOVA followed by Bonferroni test).

covalent binding to key aminoacidic residues of the protein [19,47].

TRPA1 agonists, including acrolein [25] or umbellulone [8,33], have been proposed to trigger migraine headache attacks *via* their ability to activate TRPA1 and the ensuing release of CGRP from perivascular trigeminal nerve endings. Therefore, the clinical use of feverfew in the treatment of migraine headache appears somehow contradictory to the present observation that parthenolide stimulates TRPA1 and releases CGRP. However, the second important novelty of our study consists in the observation that parthenolide acts as a TRPA1 partial agonist, produces selective channel desensitization, and causes defunctionalization of TRPA1-expressing peptidergic neurons, eg, renders nerve terminals unresponsive to a number of different stimuli. A reduced $E_{max}$ of parthenolide as compared to that of AITC was observed in cultured TG neurons. This finding suggests that parthenolide behaves as a partial agonist at the TRPA1 channel. In the presence of endogenously produced agonists, such as reactive oxygen species [9,40], or their byproducts, including 4-hydroxynonenal [47], oxononenal [45], or acrolein [7], the partial agonist activity of parthenolide may result in TRPA1 inhibition.

Another way by which parthenolide may cause TRPA1 inhibition derives from its ability to produce desensitization of both afferent (nociceptive) and efferent (neuropeptide-mediated and local) responses of primary sensory neurons. In vitro electrophysiological and organ bath studies and in vivo functional experiments showed that parthenolide pretreatment attenuates TRPA1-mediated responses. In particular, after initial activation, parthenolide caused channel desensitization in TRPA1-CHO cells, which quickly became unresponsive to a second application of parthenolide or AITC.

Differences between parthenolide-induced desensitization of afferent and efferent responses seem to depend on the localization of TRPA1 in specific subsets of primary sensory neurons. Studies with TRPA1-deficient mice and pharmacological treatments identified the wiping response to parthenolide and AITC [33] as entirely mediated by activation of TRPA1 expressed in TRPV1-positive neurons. In the case of this purely afferent response, parthenolide pretreatment selectively reduced the wiping response by TRPA1-agonists (AITC or parthenolide), but not that by a TRPV1 agonist (capsaicin) (Fig. 4). Similar results were obtained in experiments of facial allodynia produced by AITC application to the rat dura, an effect that was completely abated by parthenolide, which in contrast did not affect the capsaicin-evoked response. Thus, desensitization to parthenolide seems to be confined to nociceptive responses solely initiated by TRPA1 activation.

A different scenario emerges from neuropeptide release studies in which the efferent function [23] of sensory nerves was investigated. Pre-exposure to parthenolide prevented not only the ability of TRPA1 agonists (AITC or parthenolide) to release CGRP from dural sensory nerve terminals, but also that of a TRPV1 agonist (capsaicin) as well as that induced by high $K^+$, a nonspecific depolarizing agent. The most parsimonious explanation for this finding is that parthenolide causes complete defunctionalization of peptidergic trigeminal nerve endings, which are no longer able to release CGRP and SP/NKA in response to any stimulus. Capsaicin has long been known to produce sensory neuron defunctionalization [23], and this property represents the mechanistic basis for the clinical use of topical capsaicin treatments. The present findings are consistent with the notion that TRPA1 agonists, likewise capsaicin, cause homologous and heterologous desensitization [39].

How can the ability of parthenolide, intimately linked to TRPA1 targeting, to produce selective desensitization of TRPA1-mediated afferent responses and nonselective inhibition of neurogenic (neuropeptide-mediated) responses be clarified? One possible explanation derives from the coexistence of TRPA1 in peptidergic neurons. Although the observation has not been always confirmed [5], TRPA1 seems to colocalize predominantly with neuropeptides (SP/NKA and CGRP) in a specific subset of nociceptive neurons, whereas a substantial portion of TRPV1-positive neurons that responds to capsaicin is not peptidergic and does not express TRPA1 [10,43]. Due to its selective action at TRPA1, parthenolide desensitizes TRPA1 and defunctionalizes exclusively the subset of TRPA1-expressing neurons, which however encompasses the entire peptidergic neuronal subpopulation. Thus, after exposure to parthenolide, peptidergic neurons are no longer able to release CGRP upon exposure to any stimulus, whereas nonpeptidergic, TRPA1-negative, and TRPV1-positive neurons may still produce nociceptive responses when exposed to a variety of stimuli, including capsaicin.

In agreement with this hypothesis, we found that parthenolide pretreatment reduces CGRP-mediated meningeal vasodilatation evoked by either intranasal acrolein, an entirely TRPA1-dependent phenomenon [25], or by intravenous ethanol, an entirely TRPV1-dependent response [34]. In addition, reversal by HC-030031 of parthenolide-evoked inhibition of vasodilatation induced by ethanol robustly supports the role of TRPA1 in this phenomenon. Defunctionalization by parthenolide of neuropeptide-mediated and TRPA1-dependent responses in *in vitro* experiments was complete. However, *in vivo* meningeal vasodilatation experiments, most likely because of poor drug pharmacokinetics, defunctionalization seems to be partial. Poor ability of gaining adequate concentrations for channel activation at perivascular strigeminal endings and/or weak agonism may also be the reasons why intraperitoneal injection of parthenolide failed to produce any measurable meningeal vasodilatation.

Parthenolide and related sesquiterpene lactones have been shown, among other effects, to inhibit the activation of the proinflammatory transcription factor nuclear factor-κB (NF-κB), by different stimuli, such as phorbol esters, tumor necrosis factor-α, and hydrogen peroxide [11,17]. These findings are of relevance in migraine because the nitric oxide donor, glyceryl trinitrate, known to provoke delayed migraine attacks when infused in migraineurs, increased the expression of inducible nitric oxide synthase by an NF-κB–dependent mechanism [38]. The observation that both NF-κB activation and inducible nitric oxide synthase expression were attenuated by parthenolide [38] suggested that the antimigraine action of parthenolide may depend on the inhibition of this key proinflammatory pathway. The underlying pharmacokinetic and/or pharmacodynamic mechanism responsible for the neuronal defunctionalization of TRPA1-expressing neurons by parthenolide remains to be elucidated. Nevertheless, this novel property of parthenolide may add up to previously described anti-inflammatory actions of the compound [18,36,38] to account for the ability of chronically or acutely administered feverfew to prevent migraine attacks [12–14].

## Acknowledgments

This study was supported in part by the Regione Toscana (Programma Operativo Regionale Competitività Regionale e Occupazione (FABER-POR CReO), Fondo Europeo di Sviluppo Regionale (FESR) 2007-2013 1.1.C.) to P.G., in part by Ente Cassa di Risparmio di Firenze to R.N. and M.R.M., as well as the National Institutes of Health/National Institute of Neurological Diseases and Stroke (NS 072204) to G.D. The authors thank Professor David Julius (University of California at San Francisco, CA, USA) for the kind gift of the TRPA1-deficient mice and humanTRPA1 wild-type and humanTRPA1 mutant (C619S, C639S, C663S, K708Q) cDNAs; Professor Bernd Nilius (Katholieke Universiteit, Leuven, Belgium) for providing mouseTRPA1-CHO transfected cells; and Professor Alyn H. Morice (Academic Department of Medicine, Castle Hill Hospital, UK) for providing humanTRPA1-HEK293 stably transfected cells. The authors also thank Dr. Delia Preti and Professor Pier Giovanni Baraldi (University of Ferrara, Italy) for providing HC-030031. P.G. is a member of the editorial boards of *Physiological Reviews*, *Pain*, and *Molecular Pain*, and receives research support from Chiesi Farmaceutici, Merck Sharp & Dohme, Italian Institute of Technology, Regione Toscana, Italian Ministry of University and Research, and Ente Cassa di Risparmio di Firenze. M.R.M. receives research support from MIUR and Ente Cassa di Risparmio di Firenze. All other authors reported no biomedical financial interests or potential conflicts of interest. R.P. is full-time employee at Chiesi Farmaceutici SpA. The other authors declare no competing interests.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain. 2013.08.002.

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