Cox-dependent fatty acid metabolites cause pain through activation of the irritant receptor TRPA1

Serena Materazzi*, Romina Nassini*, Eunice André†, Barbara Campi†, Silvia Amadesi‡, Marcello Trevisanif†, Nigel W. Bunnett†‡, Riccardo Patachinni§, and Pierangelo Geppettii†¶

*Department of Preclinical and Clinical Pharmacology, University of Florence, Florence, Italy; †Center of Excellence for the Study of Inflammation, University of Ferrara, Ferrara, Italy; ‡Departments of Surgery and Physiology, University of California, San Francisco, CA 94143; and ¶Department of Pharmacology, Chiesi Pharmaceuticals, Parma, Italy

Edited by Susan E. Leeman, Boston University School of Medicine, Boston, MA, and approved June 6, 2008 (received for review March 8, 2008)

Prostaglandins (PG) are known to induce pain perception indirectly by sensitizing nociceptors. According, the analgesic action of nonsteroidal anti-inflammatory drugs (NSAIDs) results from inhibition of cyclooxygenases and blockade of PG biosynthesis. Cyclopentenone PGs, 15-d-PGJ2, PGA2, and PAG1, formed by dehydration of their respective parent PGs, PGD2, PGE2, and PGE1, possess a highly reactive α,β-unsaturated carbonyl group that has been proposed to gate the irritant transient receptor potential A1 (TRPA1) channel. Here, by using TRPA1 wild-type (TRPA1+/+) or deficient (TRPA1−/−) mice, we show that cyclopentenone PGs produce pain by direct stimulation of nociceptors via TRPA1 activation. Cyclopentenone PGs caused a robust calcium response in dorsal root ganglion (DRG) neurons of TRPA1+/+, but not of TRPA1−/− mice, and a calcium-dependent release of sensory neu- ropeptides from the rat dorsal spinal cord. Intraperitoneal injection of cyclopentenone PGs stimulated c-fos expression in spinal neurons of the dorsal horn and evoked an instantaneous, robust, and transient nociceptive response in TRPA1+/+ but not in TRPA1−/− mice. The classical proalgesic PG, PGE2, caused a slight calcium response in DRG neurons, increased c-fos expression in spinal neu-rons, and induced a delayed and sustained nociceptive response in both TRPA1+/+ and TRPA1−/− mice. These results expand the mechan-ism of NSAID analgesia from blockade of indirect nociceptor sen-sitization by classical PGs to inhibition of direct TRPA1-dependent nociceptor activation by cyclopentenone PGs. Thus, TRPA1 antago-nism may contribute to suppress pain evoked by PG metabolites without the adverse effects of inhibiting cyclooxygenases.

cyclopentenone isoprostane | cyclopentenone prostaglandins

Prostanoids are a group of bioactive compounds that include prostaglandins (PGD2, PGE2, PGF2a), prostacyclin (PGI2), and thromboxane A2. They originate from arachidonic acid, which is released intracellularly from plasma membrane phospholipids upon tissue damage and inflammation. Constitutively expressed cyclooxygenase (COX)-1 and inducible COX-2 convert arachidonic acid to the precursors PGG2 and PGH2, from which all prostanoids are generated by tissue-specific synthases. The C20 polyunsaturated fatty acid, dithromo-γ-linolenic acid, is also a substrate of COX for eicosanoid production, from which prostanoids of the 1-series, including PGE1, derive (1). Inhibition of COX activity is the main mechanism of action of aspirin and related nonsteroidal anti-inflammatory drugs (NSAIDs), as discovered by the pioneering work of Vane and colleagues (2). At least eight G protein-coupled receptor subtypes for prostanoids have been identified, which differ in tissue distribution, signal transduction pathways, and sensitivity to the various agonists (3, 4).

Pain relief, by far, is the most frequent therapeutic indication of COX inhibitors, and the contribution of prostanoids and their receptors to pain has been an area of intense investigation (5, 6). PGs do not directly excite primary sensory neurons but rather indirectly sensitize the peripheral terminals of nociceptors, with PGE2 and PGI2 inducing a robust sensitization by activating EP1,4 and IP receptors, respectively (7–9). Accordingly, the analgesic property of NSAIDs and selective COX-2 inhibitors (Coxibs) has been assigned to their ability to suppress nociceptor sensitization by COX metabolites (7–9).

Transient receptor potential A1 (TRPA1) is a recently identified excitatory ion channel (10, 11) that is coexpressed with the “capsaicin receptor” TRPV1 by a subpopulation of primary afferent neurons containing substance P (SP) and calcitonin gene-related peptide (CGRP), which mediate pain and neurogenic inflammation (12–15). Agonists of TRPA1 include the pungent ingredients of various spices, including mustard, garlic, and cinnamon (11, 16), environmental irritants such as acrolein (14), formaldehyde (17), and 4-hydroxy-2-nonenal (4-HNE), an endogenous α,β-unsaturated aldehyde generated in response to oxidative stress after inflammation and tissue injury (18, 19).

Cyclopentenone prostaglandins, including PAG2, PGA1, and PGI2, are PG metabolites formed by dehydration within the cyclopentane ring of PGE2, PGE1, and PGD2, respectively. The biological actions of cyclopentenone PGs are not mediated by activation of the classical G protein-coupled prostanoid receptors, but rather through interaction with other target proteins (20). For example, 15-deoxy-Δ12,14-PGJ2 (15-d-PGJ2), a PGI3 metabolite, is a potent natural activator of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)-γ (20). Cyclopentenone PGs are characterized by the presence of a highly reactive α,β-unsaturated carbonyl group within their cyclopentenone ring, a moiety that confers the ability to adduct thiol-containing compounds, via Michael addition (20), and through this mechanism they have been proposed to stimulate TRPA1 (21).

We hypothesized that cyclopentenone PGs stimulate TRPA1 and thereby directly activate nociceptors to cause pain, in contrast to the classical PGs that indirectly sensitize nociceptors. We have obtained genetic evidence that cyclopentenone PGs excite nociceptors, release sensory neuropeptides, activate spinal nociceptive neurons, and induce acute pain by a mechanism that is completely and exclusively dependent on TRPA1. Cyclopentenone PGs may represent a new class of COX-dependent algesic agents, and their novel mechanism of action adds a new dimen-sion to our understanding of the mechanisms of the analgesic effect of NSAIDs and Coxibs.

Results

Cyclopentenone PGs Activate TRPA1 in Dorsal Root Ganglion (DRG) Neurons. To assess whether cyclopentenone PGs directly excite DRG neurons by a TRPA1-dependent mechanism, we tested the

Author contributions: N.W.B., R.P., and P.G. designed research; S.M., R.N., E.A., B.C., S.A., M.T., performed research; S.M., R.N., E.A., B.C., S.A., M.T., N.W.B., R.P., and P.G. analyzed data; and N.W.B., R.P., and P.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

To whom correspondence should be addressed at: Department of Preclinical and Clinical Pharmacology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy. E-mail: pierangelo.geppetti@unifi.it.

This article contains supporting information online at www.pnas.org/cgi/content/full/0802354105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA
C57BL6 mice (21). These findings are consistent with the report that cyclopentenone PGs and isoprostane. These findings are consistent with the report that cyclopentenone PGs and isoprostane increase calcium ([Ca²⁺]) in DRG neurons from Swiss mice and from TRPA1 wild-type (TRPA1+/−) and deficient (TRPA1−/−) mice. We also evaluated the effects of the isoprostane, 8-iso-PGA2, which can also stimulate TRPA1 (21). Isoprostanes are PG-like substances whose generation does not require COX activation (22). A cyclopentenone isoprostane isomer of PGA2, 8-iso-PGA2, like cyclopentenone PGs, is produced by dehydration of E-isoprostane within the cyclopentane ring. It (8-iso-PGA2) contains an electrophilic α,β-unsaturated carbonyl moiety that rapidly adds cellular thiols by Michael addition (23).

15-d-PGJ2, PGA2, PGA1, and 8-iso-PGA2 evoked a concentration-dependent increase in [Ca²⁺] in DRG neurons of Swiss mice. EC₅₀ (confidence interval, C.I.) were 8.9 (7.3–10.7); 24.0 (20.4–27.9); 15.1 (9.4–24.2); 22.4 (10.8–46.7) μM, respectively (Fig. 1A). All neurons activated by cyclopentenone PGs or 8-iso-PGA2 also responded to capsaicin, while ~40% of the capsaicin-sensitive neurons (n = 437) responded to the various cyclopentenone PGs or isoprostane. These findings are consistent with the report that cyclopentenone PGs and isoprostane excite nociceptive, capsaicin-sensitive trigeminal neurons of C57BL6 mice (21).

In DRG neurons from TRPA1+/− mice (C57 background), 15-d-PGJ2, PGA2, PGA1, and 8-iso-PGA2 (all 30 μM) increased [Ca²⁺], comparable to responses of neurons from Swiss mice (Fig. 1 B and C). The ratio between the number of neurons responding to cyclopentenone compounds and neurons responding to capsaicin was: PGA2 (23/47, 49%), PGA1 (28/72, 38%), 15-d-PGJ2 (36/76, 53%), and 8-iso-PGA2 (19/43, 44%). These percentages are entirely consistent with the report that TRPA1-expressing, mustard oil-responsive neurons represent a subset (~50%) of TRPV1-expressing, capsaicin-responsive DRG neurons (16, 24). In contrast, neurons from TRPA1−/− mice were almost completely unresponsive to 15-d-PGJ2, PGA2, PGA1, and 8-iso-PGA2 (Fig. 1 B and C), and the ratio between the number of neurons responding to cyclopentenone compounds (30 μM) and neurons responding to capsaicin was: PGA2 (2/34, 5%), PGA1 (3/46, 2%), 15-d-PGJ2 (2/30, 6%), and 8-iso-PGA2 (3/48, 6%). Moreover, the magnitude of the increase in [Ca²⁺] in the few neurons from TRPA1−/− mice that responded to cyclopentenone compounds was always <5% of that of ionomycin, whereas responses of neurons from TRPA1+/− mice were 30–50% of the ionomycin response (Fig. 1C). No response to PGD2 was observed in neurons from either TRPA1+/− or TRPA1−/− mice (Fig. 1C). In cultured DRG neurons from Swiss mice PGE2 caused a small response (maximal response was 10.9 ± 1.2% of ionomycin, n = 84 cells), with an EC₅₀ of 7.6 (C.I., 2.7–21.4) μM. The small calcium response to PGE2 was identical in neurons from TRPA1+/− mice and TRPA1−/− mice (Fig. 1C). As expected, the TRPV1 agonist capsaicin produced similar responses in neurons from TRPA1+/− and TRPA1−/− mice (Fig. 1B and C), and the proportion of neurons responding to both PGE2 and capsaicin was identical in TRPA1+/− (8/25, 32%) and TRPA1−/− (12/40 30%) mice. As expected, the TRPV1 agonist capsaicin produced similar responses in neurons from TRPA1+/− and TRPA1−/− mice (Fig. 1B and C), whereas the selective TRPA1 agonist, cinnamaldehyde (25), evoked calcium responses exclusively in neurons from TRPA1+/− mice (Fig. 1C). Thus, cyclopentenone PGs and isoprostane strongly excite nociceptive neurons by a mechanism that is entirely and exclusively dependent on TRPA1, whereas the minor response to PGE2 is unrelated to TRPA1.

Cyclopentenone PGs Release Neuropeptides from Central Endings of Nociceptors. To test whether TRPA1-evoked calcium mobilization by cyclopentenone compounds stimulated the release of neuropeptides from central endings of nociceptors, we challenged slices of rat dorsal spinal cord with cyclopentenone PGs...
and measured release of CGRP and SP. 15-d-PGJ2, PGA2, PGA1, and 8-iso-PGA2 (30 μM) strongly stimulated CGRP and SP release (Fig. 2). Desensitization of nociceptors by prolonged exposure to a high concentration of capsaicin (10 μM, 20 min) abolished these responses. This procedure renders capsaicin-sensitive nerve terminals unresponsive to substances that excite nociceptors through TRPV1 and other mechanisms (26, 27). Depletion of extracellular calcium also abolished responses to cyclopentenone PGs (Fig. 2). Thus, cyclopentenone PGs or isoprostane release CGRP and SP, two major neuropeptides associated with pain transmission by capsaicin-sensitive nociceptive nerve terminals in the dorsal spinal cord.

Cyclopentenone PGs Activate Spinal Nociceptive Neurons by a TRPA1-Dependent Mechanism. We examined whether cyclopentenone PGs and 8-iso-PGA2 activate a nociceptive pathway in the spinal cord by stimulating TRPA1. PGs were administered to the mouse paw and c-fos was localized in neurons of the dorsal horn of the spinal cord as an indicator of neuronal activation. Intraplantar injection (20 μl/paw) of vehicle stimulated a modest increase in the number of c-fos containing neurons in the lumbar spinal cord that was similar in TRPA1+/− or TRPA1−/− mice (Fig. 3B). Intraplantar injection of capsaicin (0.1 nmol) or cinnamaldehyde (30 nmol) produced a 2- to 3-fold increase in c-fos expression in TRPA1+/− mice, respectively (Fig. 3B) [see supporting information (SI) Fig. S1]. While the effect of capsaicin was maintained, the response to cinnamaldehyde was completely absent in TRPA1−/− mice (Fig. 3B and Fig. S1). Intraplantar injection of 15-d-PGJ2, PGA2, PGA1, and 8-iso-PGA2 (15 nmol) produced a robust increase in c-fos expression in TRPA1+/− mice, but not in TRPA1−/− mice. Intraplantar PGE2 (15 nmol) increased c-fos expression in a similar manner in both TRPA1+/− and TRPA1−/− mice (Fig. 3A and B). Thus, cyclopentenone PGs and isoprostane activate a spinal nociceptive pathway by a mechanism that requires TRPA1, but PGE2 activates that pathway by a TRPA1-independent mechanism.

Cyclopentenone PGs Induce Pain by a TRPA1-Dependent Mechanism. We investigated whether cyclopentenone PG- and isoprostane-evoked nociceptor stimulation, release of sensory neuropeptides, and c-fos expression in the spinal cord were associated with a nocicefensive response and examined the contribution of TRPA1 to this response. Intraplantar injection (20 μl/paw) of capsaicin (0.1 nmol) or cinnamaldehyde (30 nmol) caused a rapid and intense nociceptive response that terminated after 2–4 min in TRPA1+/− mice (Fig. 4). In TRPA1−/− mice the response to capsaicin was unaltered, whereas that to cinnamaldehyde was completely absent (Fig. 4). Intraplantar injection of 15-d-PGJ2 (15 nmol) caused a robust nociceptive behavior in TRPA1+/− mice that was maximal at 1 min and sustained for 5–7 min (Fig. 4). 15-d-PGJ2 failed to produce any significant increase in nociceptive behavior in TRPA1−/− mice (Fig. 4). PGE2, PGA2, PGA1, and 8-iso-PGA2 (15 nmol) evoked nociceptive behaviors similar to that of 15-d-PGJ2 in TRPA1+/− mice (Fig. 4). Responses were all rapid in onset and lasted <10 min. These responses were practically absent in TRPA1−/− mice (Fig. 4).

Intraplantar injection of PGD2 (15 nmol) did not evoke detectable nociceptive behavior either in TRPA1+/− or TRPA1−/− mice (Fig. 4). PGE2 (15 nmol) produced a slowly developing and mild-to-moderate nociceptive behavior in TRPA1+/− mice. The response to PGE2, that was absent or minimal during the first 4–5 min, increased with time, becoming more evident after 8–10 min and was still present at 15 min. Nociceptive behavior evoked by PGE2 was indistinguishable in TRPA1+/− and TRPA1−/− mice (Fig. 4). The vehicle did not produce nociceptive behavior. Thus, 15-d-PGJ2, PGE2, PGA1, and 8-iso-PGA2 cause nociception by TRPA1 activation. The time course of cyclopentenone PGs- and isoprostane-evoked nociception is similar to that caused by other agents that directly activate nociceptors, such as capsaicin or cinnamaldehyde, and different from that evoked by agents that indirectly sensitize nociceptors, such as PGE2.

Discussion

Injury and inflammation are associated with increased prostanooid synthesis and pain hypersensitivity. Prostanoids mediate inflammation and immune responses, as their administration reproduces the major signs of inflammation, including hyperalgesia (7–9). Prostanoids induce pain by indirect mechanisms of nociceptor sensitization. Thus, prostanoids activate neuronal PG receptors, mainly of the EP subtype, that couple to downstream mediators including cAMP and protein kinases A and C (28, 29),
which activate voltage-dependent sodium channels (30), or inhibit voltage-dependent potassium channels (31), inducing neuronal hypersensitivity. Prostanoids similarly sensitize other channels on sensory neurons to cause hyperalgesia, as is the case for TRPV1, where PGs lowered the threshold temperature for channel activation (32). There is no evidence that PGs cause pain by direct stimulation of nociceptors (7–9). However, nonenzymatic pathways of PG degradation produce metabolites, including cyclopentenone PGs, whose known biological functions are not mediated by G protein-coupled prostanoid receptors. For example, 15-d-PGJ2, which has been extensively investigated, exerts delayed anti-inflammatory and antihyperalgesic effects via stimulation of the PPAR-γ receptor (33, 34). We investigated whether these metabolites cause pain by directly activating nociceptors.

Recently, because of the presence of a highly reactive α,β-unsaturated bond in their structure, cyclopentenone PGs and isoprostane have been proposed as TRPA1 agonists (21). The TRPA1 channel, expressed in TRPV1-positive somatosensory neurons, is targeted by metabolic irritants generated during inflammation. In this manner, bradykinin-dependent activation of the B2 receptor stimulates a phospholipase C pathway that activates TRPA1 and causes pain (14). Alternatively, bioactive aldehydes including acrolein and 4-HNE, which originate from peroxidation of plasma membrane phospholip-
In contrast to their cyclopentenone metabolites, PGD2 (15-d-PGJ2 precursor) and PGE2 (PGA2 precursor), either did not produce any nociceptive effect (PGD2) or evoked neuronal responses and nociceptive effects (PGE2) that were completely independent from TRPA1 activation. PGD2 is the most abundant PG released from inflammatory cells at sites of inflammation (37). Although PGD2 can inhibit postspike hyperpolarization of nociceptors in vagal afferents (38), we and others (39, 40) reported that it is unable to directly excite DRG or trigeminal neurons. Thus, although its relevance in nociception remains per se questionable, the role of all PGD2-derived cyclopentenone metabolites (20) should be considered in pain transmission. It is well established that PGE2 indirectly sensitizes nociceptors through activating prostanoid receptors that regulate ion channels and lower pain thresholds (7–9). We observed that the time course of the nociceptive behavior evoked by intraplantar PGE2 was dissimilar from that produced by its cyclopentenone metabolite, PGA2. PGE2-evoked pain was delayed and protracted, suggesting indirect sensitization, whereas PGA2-evoked pain was immediate and transient, indicating direct nociceptor stimulation. Indeed, all cyclopentenone PGs and isoprostane evoked similar immediate and transient nociceptive responses, comparable to those elicited by other direct algesic compounds, such as cinnamaldehyde and capsaicin. Thus, our results indicate that the cyclopentenone PGs, derived from PGs that either sensitize or do not target nociceptors, elicit pain responses by directly stimulating TRPA1.

An important question is whether cyclopentenone PGs or isoprostane are produced in sufficient amount to stimulate the TRPA1 at sites of inflammation or injury. Although formation of cyclopentenone PGs from their parent compounds has been demonstrated both in vitro and in vivo (34, 37, 41), the extent to which cyclopentenone PGs are formed in vivo is unclear. The unsaturated carbonyl groups of cyclopentenone PGs rapidly react with thiol-containing proteins and peptides, and measurements of 15-d-PGJ2 (or other cyclopentenone PGs) in vivo underestimate true concentrations. Thus, 15-d-PGJ2 is detectable in vivo primarily as a Michael conjugate to glutathione (42). The same consideration applies to the measurement of 8-iso-PGJ2 at sites of inflammation or tissue injury (22). Thus, more appropriate methods are required to assess actual levels of PGA1, PGA2, 15-d-PGJ2, and 8-iso-PGJ2 and thereby assess their contribution to inflammatory pain.

NSAIDs and Coxibs are considered to produce analgesia via their anti-inflammatory action because of inhibition of PG generation. Our results extend the mechanism of the antinociceptive action of NSAIDs/Coxibs from preventing indirect nociceptor sensitization by classical PGs, to inhibiting direct nociceptor activation by cyclopentenone PGs and TRPA1. The beneficial actions of NSAIDs and Coxibs are offset by their well known and severe adverse effects in the gastrointestinal (7–9) and cardiovascular systems (43). TRPA1-mediated pain is also induced by other irritant COX-independent fatty acid metabolites, such as 8-iso-PGJ2 or 4-HNE (18, 22, 23, 44). Should these compounds play a significant role in inflammatory pain, their action would be clearly unaffected by NSAIDs or Coxibs, whereas it could be blocked by TRPA1 antagonists. Thus, TRPA1 inhibitors could represent a novel therapeutic strategy for reducing inflammatory pain, with consistent advantages at the level of both safety and efficacy. Compared with NSAIDs and Coxibs, their use should not be associated with adverse effects in the gastrointestinal (7–9) and cardiovascular systems (43), where PG biosynthesis would be preserved. Moreover, TRPA1 antagonists could reduce pain evoked by COX-independent nociceptive fatty acid metabolites.

**Materials and Methods**

**Reagents.** PGD2, PGA1, PGA2, 15-d-PGJ2, and 8-iso-PGJ2 were from Cayman Chemicals; PGE2, cinnamaldehyde, and capsaicin from Sigma; rabbit anti-c-fos from Santa Cruz Biotechnology; goat anti-rabbit biotinylated ABC and DAB kits from Vector Laboratories; and Fura-2-AM-ester from Alexis Biochemicals or Invitrogen.

**Animals.** Institutional Animal Care and Use Committees of the University of Florence and the University of California, San Francisco approved all procedures. Sprague–Dawley rats (male, 250 g), Swiss mice, TRPA1−/− mice (14), and wild-type littermates (male, 30 g) were housed in a temperature- and humidity-controlled vivarium (12 h dark/ light cycle, free access to food and water). The TRPA1 heterozygote male mice had been backcrossed with C3H/BlL female mice for two generations to obtain the C57BL/6 genetic background. TRPA1 wild-type and -deficient mice used in the present experiments derived from breeding heterozygous animals. Experiments with TRPA1 wild-type and -deficient littermates were blinded to the genotype. Animals were killed with sodium pentobarbital (200 mg/kg i.p.) and thoracotomy.

**DRG Neuron Isolation and Culture.** DRG from thoracic and lumbar spinal cord of mice were removed and minced in cold HBSS as described in ref. 45. Ganglia were digested using 1 mg/ml of collagenase type 1A and 1 mg/ml of papain in HBSS (25 min, 37°C). Neurons were pelleted and resuspended in Ham’s-F12 containing 10% FBS, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, and 2 mM glutamate, dissociated by gentle trituration, and plated on glass coverslips coated with poly-L-lysine (8.3 μM) and laminin (5 μM). Neurons were cultured for 3–4 d.

**Calcium Imaging.** DRG neurons were loaded with Fura-2-AM (5 μM) in HBSS containing CaCl2 1.6 mM, KCl 1.4 mM, MgSO4 0.4 mM, NaCl 135 mM, D-glucose 5 mM, Hepes 10 mM, BSA 0.1%, pH 7.4 (40 min, 37°C). Cells were washed and transferred to a chamber for recording changes by the F340/F380 ratio in individual neurons using a dynamic image analysis system as described in ref. 45. DRG were challenged with capsaicin (1 μM) and by KC1 (50 mM) to identify nociceptive neurons and at the end of each experiment with ionomycin (5 μM). Results were expressed as the percentage of the maximum response to ionomycin.

**Neuropeptide Release.** Slices (0.4 mm) of the lumbar enlargements of the dorsal spinal cord from rats were stabilized for 60 min in an oxygenated Krebs solution at 37°C. Fractions (4 ml) were collected at 10-min intervals into ethanolic acid (final concentration 2 N) before, during, and after administration of cyclopentenone PGs or isoprostane. Fractions were freeze-dried, reconstituted with assay buffer, and analyzed for CGRP and SP immunoreactivities as described in ref. 45. Neither cyclopentenone PGs nor cyclopentenone isoprostane (30 μM) cross-reacted with SP or CGRP antisera. Exposure to capsaicin (10 μM, 20 min) was used to produce a specific and complete desensitization of TRPV1-expressing sensory nerve terminals. Further details are reported in a SI Text section.

**C-fos Localization.** The following agents were injected into the plantar surface of one hind paw (20 μl/paw) of mice: cyclopentenone PGs or isoprostane (15 nmol), cinnamaldehyde (30 nmol), capsaicin (0.1 nmol) or vehicle (7.5% DMSO in PBS). After 90 min, animals were transcardially perfused with 0.1 M of PBS followed by 4% paraformaldehyde in PBS. Serial sections (L4–L5) were immersion fixed in parafomaldehyde overnight, and 20-μm coronal sections prepared using a freezing microtome. Serial sections were incubated with c-fos antibody (1:5000, overnight, 4°C), washed, incubated with goat anti-rabbit biotinylated antibody (1:500, 1 h, room temperature), and stained using avidin-biotin peroxidase complex and chromogen nickel/3,3′-diaminobenzidine solution. Ten sections for each mouse were randomly selected and the expression of c-fos immunoreactivity was evaluated by counting the number of positive neurons in the superficial dorsal horn region (lamina I–II).

**Nocifensive Responses.** Agonists were injected into the mouse paw as described (46, 47). Immediately after injection, mice were placed in a Plexiglas chamber. The total time spent licking and lifting of the injected hind paw was recorded for 15 min. Further details are reported in a SI Text section.

**Statistical Analysis.** Data are expressed as mean ± SEM. Statistical significance was determined using Student’s t test for unpaired data or the two-way ANOVA, followed by Bonferroni’s post hoc analysis. P < 0.05 was considered statistically significant.
significant. Agonist potency was expressed as the molar concentration producing 50% of the maximal effect (EC50).

NOTE. Contemporary to the submission of the present study a paper has been published (48) reporting that reactive oxygen species and 15-d-PGJ2 stimulated TRPA1 and that the nociceptive behavior evoked by 15-d-PGJ2 was absent in TRPA1-deficient mice. However, this study failed to detect a calcium response by PGJ2 in Chinese hamster ovary cells expressing the mouse TRPA1. This observation is at variance with previous (21) and present (Fig. 1) data obtained in HEK293 cells expressing the human TRPA1 and mouse DRG neurons, respectively.

ACKNOWLEDGMENTS. We thank David Julius (University of California San Francisco, San Francisco, CA) for providing TRPA1-deficient mice. This work was supported by grants from Ente Cassa di Risparmio di Firenze (Florence); Ministry for University and Scientific Research (MiUR) Rome, Italy; Grants FISR RB10107Y012 and PRIN 2006089824; (P.G. and R.P.); and National Institutes of Health Grants DK 57840, DK 39597, and DK 43207 (N.W.B.).

Materials and Methods

Neuropeptide Release. The spinal cords of rats were removed and slices (0.4 mm) from the dorsal part of the cervical and lumbar enlargements were prepared at 4°C using a tissue slicer (McIlwain tissue chopper; Mickle Laboratory Engineering). Slices (100 mg) were placed in 2 ml chambers and superfused with oxygenated (95% O₂, 5% CO₂) Krebs solution (NaCl 119, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.5, CaCl₂ 2.5, KCl 4.7 and d-glucose 11 mM) maintained at 37°C and containing 0.1% BSA, 1 μM phosphoramidon and 1 μM captopril. After a 90 min stabilization period, slices of rat spinal cord were stimulated with cyclopentenone PGs and cyclopentenone isoprostane (all 30 μM) or their vehicle for 10 min. In some experiments, slices were perfused with Ca²⁺-free medium, containing 1 mM EDTA, or afferent nerves were depleted of neuropeptides by preincubation with 10 μM capsaicin for 20 min before stimulation. Fractions (4 ml) of superfusate were collected at 10-min intervals into ethanoic acid (final concentration 2 N) before, during, and after administration of stimulus and then freeze-dried, reconstituted with assay buffer, and analyzed for CGRP and SP immunoreactivities as described (1–3). Peptide release was determined by subtracting basal levels from concentrations measured during and immediately after exposure to the treatment. Detection limits of the assays were 5 pg/ml for CGRP and 2 pg/ml for SP. The results are expressed as femtomoles of peptide per g of tissue per 20 min. Neither cyclopentenone PGs nor cyclopentenone isoprostane (30 μM) crossreacted with SP or CGRP antisera.

Nocifensive Responses. Intraplantar (i.pl.) injection (20 μl/paw) of capsaicin (0.1 nmol), cinnamaldehyde (30 nmol), cyclopentenone PGs (15 nmol each), or isoprostane (15 nmol) was used to induce nociceptive responses as described previously (4, 5). Control animals received the same volume of vehicle (7.5% DMSO in PBS). Animals were placed individually in chambers (transparent glass cylinders of 20 cm in diameter) and were adapted for 20 min before injection of test compounds or their vehicle. The amount of time spent licking, raising, and shaking the injected paw (indicative of nociception) was timed each minute with a chronometer for a 15-min time period.

Fig. S1. Cinnamaldehyde and capsaicin evoke c-fos expression in L4–L5 spinal cord lumbar sections. Photomicrographs of transverse sections of the lumbar spinal cord showing the effects of intraplantar injection of cinnamaldehyde (30 nmol) and capsaicin (0.1 nmol) on the expression of c-fos-like immunoreactivity (LI) in TRPA1+/+ or TRPA1−/− mice 90 min after injection in the hind paw. Each photomicrograph is a representative example of one section (L4–L5) of dorsal horn ipsilateral to the injection.