Accession numbers. The human and mouse *GPR120* cDNA sequences were deposited in GenBank under numbers AB115768 and AB115769, respectively.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank S. Mine, T. Tanaka, Y. Kitagawa and S. Suzuki for their technical assistance. This study was performed through Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government (G.T.). S.K. and T.A. are supported by the 21st Century Center of Excellence Program "Knowledge Information Infrastructure for Genome Science."

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 8 September; accepted 22 November 2004
Published online at http://www.nature.com/naturemedicine/

1. Zimmet, P., Alberti, K.G. & Shaw, J. Global and societal implications of the diabetes epidemic. *Nature* **414**, 782–787 (2001).
2. Gadsby, R. Epidemiology of diabetes. *Adv. Drug. Deliv. Rev.* **54**, 1165–1172 (2002).
3. Jovanovic, L. & Gondos, B. Type 2 diabetes: the epidemic of the new millennium. *Ann. Clin. Lab. Sci.* **29**, 33–42 (1999).
4. Creutzfeldt, W. & Ebert, R. New developments in the incretin concept. *Diabetologia* **28**, 565–573 (1985).
5. Kieffer, T.J. & Habener, J.F. The glucagon-like peptides. *Endocr. Rev.* **20**, 876–913 (1999).
6. Kreymann, B., Williams, G., Ghatei, M.A. & Bloom, S.R. Glucagon-like peptide-1 7-36: a physiological incretin in man. *Lancet* **2**, 1300–1304 (1987).
7. Nunez, E.A. Biological complexity is under the 'strange attraction' of non-esterified fatty acids. *Prostaglandins. Leukot. Essent. Fatty Acids* **57**, 107–110 (1997).
8. Thomsen, C., Storm, H., Holst, J.J. & Hermansen, K. Differential effects of saturated and monounsaturated fats on postprandial lipemia and glucagon-like peptide 1 responses in patients with type 2 diabetes. *Am. J. Clin. Nutr.* **77**, 605–611 (2003).
9. MacDonald, P.E. *et al.* The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes* **51** Suppl 3, S434–S442 (2002).
10. Drucker, D.J. Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis. *Mol. Endocrinol.* **17**, 161–171 (2003).
11. Fredriksson, R., *et al.* Seven evolutionarily conserved human rhodopsin G protein-coupled receptors lacking close relatives. *FEBS Lett.* **554**, 381–388 (2003).
12. Tsao, P., Cao, T. & von Zastrow, M. Role of endocytosis in mediating downregulation of G-protein-coupled receptors. *Trends. Pharmacol. Sci.* **22**, 91–96 (2001).
13. Ding, G.J., *et al.* Characterization and quantitation of NF-κB nuclear translocation induced by interleukin-1 and tumor necrosis factor-α. Development and use of a high capacity fluorescence cytometric system. *J. Biol. Chem.* **273**, 28897–28905 (1998).
14. Milligan, G., Marshall, F. & Rees, S. G16 as a universal G protein adapter: implications for agonist screening strategies. *Trends. Pharmacol. Sci.* **17**, 235–237 (1996).
15. Vilsboll, T., *et al.* Incretin secretion in relation to meal size and body weight in healthy subjects and people with type 1 and type 2 diabetes mellitus. *J. Clin. Endocrinol. Metab.* **88**, 2706–2713. (2003).
16. Holst, J.J. & Orskov, C. Incretin hormones--an update. *Scand. J. Clin. Lab. Invest.* Suppl.**234**, 75–85 (2001).
17. Guimbaud, R., *et al.* Intraduodenal free fatty acids rather than triglycerides are responsible for the release of CCK in humans. *Pancreas.* **14**, 76–82 (1997).
18. Sidhu, S.S., Thompson, D.G., Warhurst, G., Case, R.M. & Benson, R.S. Fatty acid-induced cholecystokinin secretion and changes in intracellular Ca^{2+} in two enteroendocrine cell lines, STC-1 and GLUTag. *J. Physiol.* **528**, 165–176 (2000).
19 Itoh, Y., *et al.* Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* **422**, 173–176 (2003).
20. Cao, X., *et al.* Aberrant regulation of human intestinal proglucagon gene expression in the NCI-H716 cell line. *Endocrinology* **144**, 2025–2033 (2003).
21. Reimer, R.A., *et al.* A human cellular model for studying the regulation of glucagon-like peptide-1 secretion. *Endocrinology* **142**, 4522–4528 (2001).
22. Trogan, E., *et al.* Laser capture microdissection analysis of gene expression in macrophages from atherosclerotic lesions of apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. USA* **99**, 2234–2239 (2002).
23. Briscoe, C.P., *et al.* The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *J. Biol. Chem.* **278**, 11303–11311 (2003).
24. Brown, A.J., *et al.* The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J. Biol. Chem.* **278**, 11312–11319 (2003).
25. Nilsson, N.E., Kotarsky, K., Owman, C. & Olde, B. Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. *Biochem. Biophys. Res. Commun.* **303**, 1047–1052 (2003).
26. Hirasawa, A., *et al.* Subtype-specific differences in subcellular localization of α1-adrenoceptors: chlorethylclonidine preferentially alkylates the accessible cell surface α1-adrenoceptors irrespective of the subtype. *Mol. Pharmacol.* **52**, 764–770 (1997)
27. Awaji, T., *et al.* Real-time optical monitoring of ligand-mediated internalization of alpha1b-adrenoceptor with green fluorescent protein. *Mol. Endocrinol.* **12**, 1099–1111 (1998).
28. Horie, K., Hirasawa, A. & Tsujimoto, G. The pharmacological profile of cloned and stably expressed alpha 1b-adrenoceptor in CHO cells. *Eur. J. Pharmacol.* **268**, 399–407 (1994).
29. Awaji, T., Hirasawa, A., Shirakawa, H., Tsujimoto, G., Miyazaki, S. Novel green fluorescent protein-based ratiometric indicators for monitoring pH in defined intracellular microdomains. *Biochem. Biophys. Res. Commun.* **289**, 457–462 (2001).
30. Gevrey, J.C., Cordier-Bussat, M., Nemoz-Gaillard, E., Chayvialle, J.A. & Abello, J. Co-requirement of cyclic AMP- and calcium-dependent protein kinases for transcriptional activation of cholecystokinin gene by protein hydrolysates. *J. Biol. Chem.* **277**, 22407–22413 (2002).

Table 1 pEC_{50}s of fatty acids tested on HEK 293 cells stably expressing GPR120-Gα16

Fatty acid	pEC50 ± s.e.m
n-Caproic acid (C6)	inactive
Octanoic acid (C8)	inactive
Nonanoic acid (C9)	inactive
n-Capric acid (C10)	inactive
Lauric acid (C12)	inactive
Myristic acid (C14)	4.53 ± 0.17
Palmitic acid (C16)	4.28 ± 0.1
Palmitoleic acid (C16:1)	5.49 ± 0.17
Stearic acid (C18)	4.74 ± 0.12
Elaidic acid (C18:1)	4.48 ± 0.23
Oleic acid (C18:1)	4.51 ± 0.19
α-Linolenic acid (C18:3)	6.37 ± 0.13
α-Linolenic acid methyl ester	inactive
γ-Linolenic acid (C18:3)	5.98 ± 0.12
Arachidic acid (C20)	inactive
cis-8,11,14-Eicosatrienoic acid (C20:3)	4.84 ± 0.03
cis-11,14,17-Eicosatrienoic acid (C20:3)	5.85 ± 0.03
cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5)	5.55 ± 0.03
Behenic acid (C22)	inactive
cis-13,16,19-Docosatrienoic acid (C22:3)	inactive
cis-7,10,13,16-Docosatetraenoic acid (C22:4)	4.79 ± 0.11
cis-7,10,13,16,19-Docosapentaenoic acid (C22:5)	4.58 ± 0.22
cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA, C22:6)	5.41 ± 0.11

Receptor activation was measured from dose-dependent changes in $[Ca^{2+}]_i$ levels using a fluorometric imaging plate reader. Inactive, no response at 100 μM.

Molecular Pain

BioMed Central

Research

Open Access

Sensitization of TRPV1 by EP_1 and IP reveals peripheral nociceptive mechanism of prostaglandins

Tomoko Moriyama[†1,2], Tomohiro Higashi[†2,3], Kazuya Togashi[2,3], Tohko Iida[1], Eri Segi[4], Yukihiko Sugimoto[5], Tomoko Tominaga[2,3], Shuh Narumiya[4] and Makoto Tominaga[*1,2,3]

Address: [1]Dept.of Cellular and Molecular Physiology, Mie University School of Medicine, Mie 514-8507, Japan, [2]Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki 444-8787, Japan, [3]Department of Physiological Sciences, The Graduate University for Advanced Studies, Okazaki 444-8787, Japan, [4]Dept. ofPharmacology, Kyoto University Faculty of Medicine, Kyoto 606-8501, Japan and [5]Dept. of Physiological Chemistry, Graduated School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan

Email: Tomoko Moriyama - tomokom@nips.ac.jp; Tomohiro Higashi - tomohiro@nips.ac.jp; Kazuya Togashi - togashi@nips.ac.jp; Tohko Iida - iida@doc.medic.mie-u.ac.jp; Eri Segi - e-segi@mfour.med.kyoto-u.ac.jp; Yukihiko Sugimoto - ysugimot@pharmsun.pharm.kyoto-u.ac.jp; Tomoko Tominaga - tomokoto@nips.ac.jp; Shuh Narumiya - snaru@mfour.med.kyoto-u.ac.jp; Makoto Tominaga* - tominaga@nips.ac.jp

* Corresponding author †Equal contributors

Published: 17 January 2005

Molecular Pain 2005, 1:3 doi:10.1186/1744-8069-1-3

Received: 06 January 2005
Accepted: 17 January 2005

This article is available from: http://www.molecularpain.com/content/1/1/3

Abstract

Prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) are major inflammatory mediators that play important roles in pain sensation and hyperalgesia. The role of their receptors (EP and IP, respectively) in inflammation has been well documented, although the EP receptor subtypes involved in this process and the underlying cellular mechanisms remain to be elucidated. The capsaicin receptor TRPV1 is a nonselective cation channel expressed in sensory neurons and activated by various noxious stimuli. TRPV1 has been reported to be critical for inflammatory pain mediated through PKA- and PKC-dependent pathways. PGE_2 or PGI_2increased or sensitized TRPV1 responses through EP_1 or IP receptors, respectively predominantly in a PKC-dependent manner in both HEK293 cells expressing TRPV1 and mouse DRG neurons. In the presence of PGE_2 or PGI_2, the temperature threshold for TRPV1 activation was reduced below 35°C, so that temperatures near body temperature are sufficient to activate TRPV1. A PKA-dependent pathway was also involved in the potentiation of TRPV1 through EP_4 and IP receptors upon exposure to PGE_2 and PGI_2, respectively. Both PGE_2-induced thermal hyperalgesia and inflammatory nociceptive responses were diminished in TRPV1-deficient mice and EP_1-deficient mice. IP receptor involvement was also demonstrated using TRPV1-deficient mice and IP-deficient mice. Thus, the potentiation or sensitization of TRPV1 activity through EP_1 or IP activation might be one important mechanism underlying the peripheral nociceptive actions of PGE_2 or PGI_2.

Background

Tissue damage and inflammation produce an array of chemical mediators such as ATP, bradykinin, prostanoids, protons, cytokines and peptides including substance P that can excite or sensitize nociceptors to elicit pain at the site of injury. Among them prostanoids were shown to influence inflammation, and their administration was found to reproduce the major signs of inflammation

including augmented pain [1]. Prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) are the products of arachidonic acid metabolism through the cyclooxygenase pathway. In addition to numerous other physiological actions *in vivo*, previous studies have indicated important roles for PGE_2 in nociception and inflammation [2,3]. PGE_2 is generated in most cells in response to mechanical, thermal or chemical injury and inflammatory insult, resulting in sensitization or direct activation of nearby sensory nerve endings. Analgesic effects of non-steroidal anti-inflammatory drugs (NSAIDs) are attributed predominantly to inhibition of prostaglandin synthesis. Prostaglandins act upon a family of pharmacologically distinct prostanoid receptors including EP_1, EP_2, EP_3, EP_4 and IP that activate several different G protein-coupled signaling pathways [2,4,5]. Primary sensory neurons in dorsal root ganglion (DRG) are known to express mRNAs encoding several prostanoid receptor subtypes, IP, EP_1, EP_3 and EP_4 [6,7]. The role of IP in inflammation has been clearly shown by the analysis of IP-deficient mice, although the underlying cellular mechanisms still remain to be elucidated [8]. In contrast, the potential involvement of EP receptors other than IP in inflammation and pain generation has not been well studied, although some earlier studies have suggested that prostanoids contribute to the development of pain through EP receptors [9,10].

The capsaicin receptor TRPV1 is a non-selective cation channel expressed predominantly in unmyelinated C-fibers [11]. TRPV1 is activated not only by capsaicin, but also by protons or heat (with a threshold > ~43 °C), both of which cause pain *in vivo* [11-13]. A prominent role of TRPV1 in nociception has been demonstrated in studies of TRPV1-deficient mice [14,15].

Recently, we reported that inflammatory mediators such as ATP, bradykinin and trypsin or tryptase potentiate TRPV1 activity in a PKC-dependent manner [16-18], and identified two target serine residues in TRPV1 as substrates for PKC-dependent phosphorylation [19]. On the other hand, there are several reports showing that a PKA signaling pathway mediates PGE_2-induced potentiation of capsaicin-evoked responses in rat sensory neurons [20-22]. Therefore, we examined the effects of PGE_2 and PGI_2 on TRPV1 activity. Surprisingly, we found the functional interaction of TRPV1 with PGE_2 or PGI_2 occurs mainly through a PKC-dependent pathway at both cellular and behavioral levels.

Results

Functional interaction between TRPV1 and PGE_2

In order to examine the possibility that TRPV1 is involved in PGE_2-induced hyperalgesia *in vivo*, we performed a behavioral analysis using wild type and TRPV1-deficient ($TRPV1^{-/-}$) mice. PGE_2 (500 pmol/20 μL) produced a significant reduction in paw withdrawal latency in response to radiant heat (thermal hyperalgesia) at 5 to 90 min following intraplantar injection in wild type mice (Figure 1A). On the other hand, the PGE_2-induced thermal hyperalgesia was almost completely abolished in $TRPV1^{-/-}$ mice, suggesting a functional interaction between PGE_2 and TRPV1 (Figure 1A), consistent with a previous report that capsaicin-ablation of primary afferent neurons prevents PGE_2-induced thermal hyperalgesia [23]. We next examined the interaction between PGE_2 and TRPV1 in mouse DRG neurons using the patch-clamp technique. Capsaicin (100 nM) evoked small inward currents in DRG neurons. The capsaicin-evoked currents were significantly potentiated by 1.5 min pretreatment with PGE_2 (1 μM) in 19 of 23 cells as previously reported [21] (Figure 1C) (3.36 ± 0.55 fold increase, n = 23 for PGE_2 (+); 0.78 ± 0.08 fold for PGE_2 (-) (Cont.), n = 5, $p < 0.05$). Because it has been suggested that a PKA-dependent pathway is predominantly involved in the PGE_2-induced potentiation of capsaicin-activated currents in rat DRG neurons [21], we examined the potential involvement of such a mechanism both in mouse DRG neurons and human embryonic kidney-derived HEK293 cells expressing TRPV1. No potentiation of the capsaicin-activated current responses was observed in DRG neurons treated with a mixture of forskolin (FSK, 10 μM), 3-isobutyl-1-methylxanthine (IBMX, 1 mM) and dibutyryl-cAMP (dbcAMP, 3 mM) for the same time period (1.5 min) (1.15 ± 0.20 fold increase, n = 9) although a significant increase in cAMP level was confirmed during such the treatment (Figures 1B and 1C). When we treated cells longer (6.5 min), 7 out of 14 cells showed increase of capsaicin-activated currents (2.15 ± 0.77 fold increase, n = 14, p = 0.28) (Figure 1C). In HEK293 cells, two different short (1.5 min) treatments to activate PKA produced no potentiation (Figure 1D) (treatment with a mixture of FSK, IBMX and dbcAMP in cells expressing TRPV1, 1.20 ± 0.19 fold increase, n = 11, and treatment with isoproterenol (Isop.) in cells expressing both TRPV1 and mouse β_1-adrenergic receptors (β_1-ADR), 0.83 ± 0.12 fold increase, n = 4) although a significant increase in cAMP level was confirmed following both treatments in HEK293 cells (Figure 1B). We also examined the effects of long treatment (6.5 min) with a mixture of FSK, IBMX and dbcAMP. This treatment caused significant potentiation of capsaicin-activated currents (2.39 ± 0.60 fold increase, n = 7, $p < 0.05$) (Figure 1D). These results suggest that both PKA-dependent and -independent pathways are involved in the potentiation of the capsaicin-activated currents by PGE_2, that it takes longer to cause potentiation of capsaicin-activated currents through a PKA-dependent pathway, and that the PKA-independent pathway is predominantly involved under the short treatment conditions. Indeed, it has been reported that capsaicin-activated currents were not increased upon FSK/IBMX

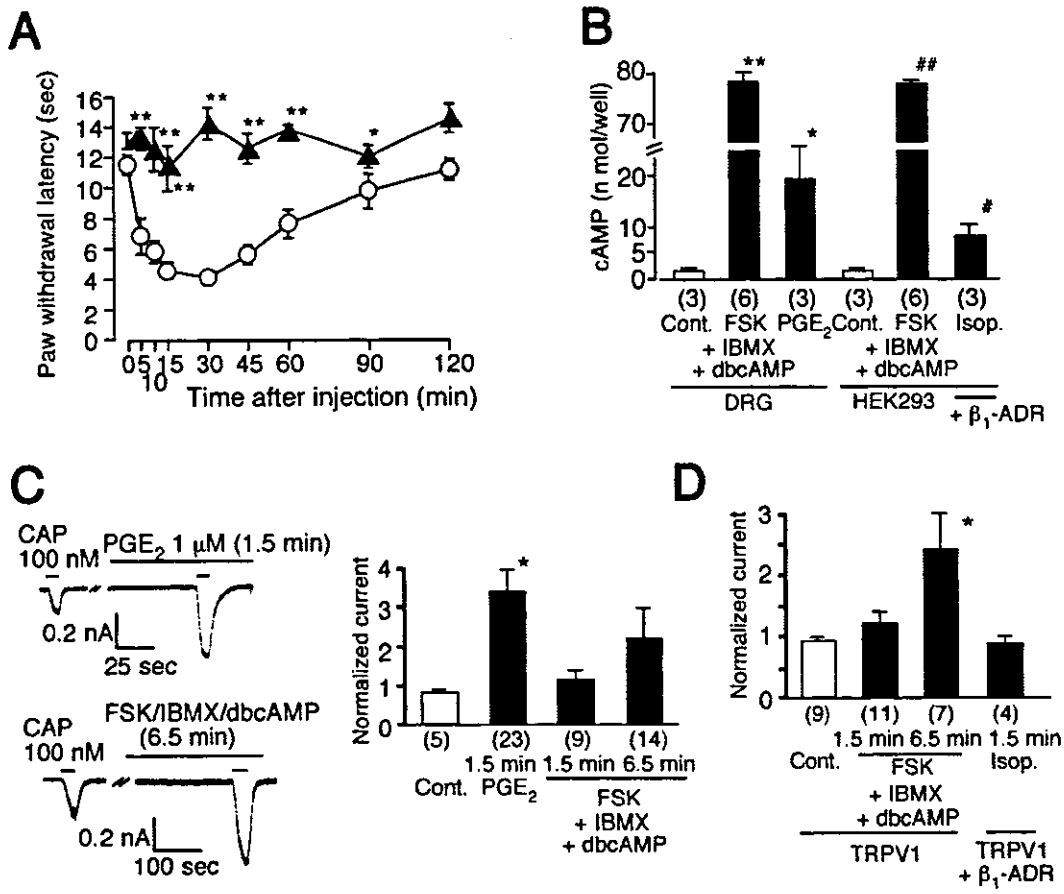

Figure 1

Physiological interaction of PGE_2 with TRPV1 in mice. **(A)** PGE_2-induced thermal hyperalgesia in $TRPV1^{+/+}$ mice (○, n = 6) or $TRPV1^{-/-}$ mice (▲, n = 6). Reduction of paw withdrawal latency (thermal hyperalgesia) by intraplantar PGE_2 (500 pmol/ 20 μL) injection was significantly diminished in $TRPV1^{-/-}$ mice. * $p < 0.05$, ** $p < 0.01$ vs. $TRPV1^{+/+}$ mice. **(B)** Intracellular cAMP levels in mouse DRG neurons or HEK293 cells treated with a mixture of forskolin (FSK, 10 μM), IBMX (1 mM) and dibutyryl cAMP (dbcAMP, 3 mM), or PGE_2 (1 μM) or isoproterenol (Isop., 10 μM). *, # $p < 0.05$ vs. Cont., **, ## $p < 0.01$ vs. Cont. **(C)** Representative traces of potentiation of capsaicin (100 nM)-activated current by extracellular PGE_2 (1 μM, 1.5 min) or a mixture of FSK(10 μM), IBMX (1 mM) and dbcAMP (3 mM) (6.5 min) in mouse DRG neurons. Currents were normalized to values induced by first capsaicin application in the absence of additives (bar graph). Capsaicin was reapplied 1.5 or 6.5 min after exposure to bath solution with additives. Numbers in parenthesis indicate cells tested. * $p < 0.05$ vs. Cont. Holding potential (V_h): -60 mV. **(D)** Long (6.5 min) but not short (1.5 min) activation of PKA pathway has effect on TRPV1 responses in HEK293 cells. FSK (10 μM), IBMX (1 mM) and dbcAMP (3 mM) were applied to cells expressing rat TRPV1. Isop. (10 μM) was applied to cells expressing both rat TRPV1 and β_1-adrenergic receptors (β_1-ADR). Numbers in parenthesis indicate cells tested. V_h: -60 mV. * $p < 0.05$ vs Cont.

or 8-bromo-cAMP (8-Br-cAMP)/IBMX treatment in *Xenopus* oocytes expressing TRPV1, or treatment with isoproterenol in oocytes expressing both TRPV1 and β_1-ADR [24].

PGE_2 increases TRPV1 activity through EP_1 receptors

To explore the mechanism underlying the PKA-independent PGE_2 (1.5 min)-induced potentiation of the capsaicin-evoked responses observed in DRG neurons, we first examine the effects of PGE_2 on capsaicin-activated currents in HEK293 cells expressing TRPV1 and each EP receptor. PGE_2 (1 μM, 1.5 min) caused a robust increase in the magnitude of low dose (20 nM) capsaicin-activated currents in HEK293 cells co-expressing TRPV1 with EP_1 (0.90 ± 0.04 fold increase, n = 9 for control (Cont.); 4.60 ± 1.03 fold, n = 17 for PGE_2, $p < 0.05$) (Figures 2A and 2B). This increase lasted more than three minutes, as we previously reported for PAR-2 (proteinase activated receptor 2)-mediated potentiation of TRPV1 activity [16]. In contrast, no such potentiation was detected in cells expressing TRPV1 with other EP receptor subtypes (0.91 ± 0.09 fold increase, n = 7; 0.77 ± 0.13, n = 9; 0.72 ± 0.24, n = 5; 0.98 ± 0.18, n = 7; 0.89 ± 0.15, n = 9 for EP_2, $EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$ or EP_4, respectively) (Figure 2B). Protracted (6.5 min) treatment with PGE_2 caused a significant increase in capsaicin-activated currents in cells expressing TRPV1 and EP_4, a phenomenon like that observed following treatment with a mixture of FSK, IBMX and dbcAMP (3.03 ± 0.48 fold increase, n = 6, $p < 0.05$ vs. Cont.) (Figure 2B), suggesting that the EP_4 receptor, known to be expressed in DRG and coupled to Gs protein, is the receptor that activates a PKA-dependent signaling pathway upon prostaglandin exposure. All cells exhibiting an increase of capsaicin-activated currents upon treatment with a mixture of FSK, IBMX and dbcAMP also showed an increase in current in the presence of PMA (data not shown), suggesting that both PKA- and PKC-dependent pathways work in the same cells. To examine how PGE_2 changes TRPV1 responsiveness, we measured TRPV1 current in single cells by applying a range of concentrations of capsaicin in the absence or presence of PGE_2. The currents were normalized to the maximal current produced by 1 μM capsaicin in each cell. Maximal current in the presence of PGE_2 was almost the same as that in the absence of PGE_2. The resultant dose-response curves clearly demonstrate that PGE_2 enhances capsaicin action on TRPV1 by lowering EC_{50} values without altering maximal responses (EC_{50} from 81.0 nM to 27.6 nM) (Figure 2C). We next examined the effects of PGE_2 on the thermal sensitivity of TRPV1. When temperature ramps were applied to HEK293 cells expressing both TRPV1 and EP_1 in the absence of PGE_2, heat-evoked currents developed at 40.7 ± 0.3°C (n = 8) (Figure 2D). In contrast, the temperature threshold for TRPV1 activation was significantly reduced to 30.6 ± 1.1°C in the presence of PGE_2 (n = 8, $p < 0.05$) (Figure 2D) implying that under these conditions, TRPV1 could be activated at normal body temperature. A similar potentiating effect of PGE_2 was observed for proton (pH 6.2)-evoked TRPV1 current responses (0.91 ± 0.06 fold increase, n = 3 for control; 4.47 ± 1.09 fold, n = 7 for PGE_2, $p < 0.01$) (Figure 2E). These data clearly show that TRPV1 currents evoked by any of three different stimuli (capsaicin, proton, or heat) are potentiated or sensitized by PGE_2 through EP_1 receptor activation. On the other hand, the temperature threshold for TRPV1 activation was not changed upon treatment with a mixture of FSK, IBMX and dbcAMP in HEK293 cells expressing TRPV1 (40.8 ± 0.8°C, n = 4), suggesting different actions on TRPV1 by PKA and PKC.

The signaling pathway downstream of EP_1 remains to be clarified. We have reported that $G_{q/11}$-coupled metabotropic receptor activation such as ATP (P2Y), bradykinin (B2) and proteinase-activated receptor 2 (PAR2) receptors causes potentiation or sensitization of TRPV1 through the PKC-dependent phosphorylation of TRPV1 [16-18,25]. Therefore, we examined whether a similar signal transduction pathway is involved in the regulation of TRPV1 responses through EP_1. When calphostin C (Calp.C), a specific PKC inhibitor, was added to the pipette solution, the effect of PGE_2 was almost completely inhibited (0.92 ± 0.15 fold increase, n = 10) (Figure 2F). Similarly, a PKCε translocation inhibitor (PKCε-I) abolished the potentiation of TRPV1 response by PGE_2 (1.11 ± 0.25 fold increase, n = 11) (Figure 2F). These data suggest that PGE_2-induced potentiation of TRPV1 responsiveness develops through activation of PKCε. To further confirm the involvement of PKC-dependent phosphorylation, PGE_2 effects were examined using cells expressing a TRPV1 mutant, S502A/S800A which is insensitive to PKC-dependent phosphorylation [19]. No potentiation of capsaicin-activated currents was observed upon PGE_2 treatment of cells expressing S502A/S800A (0.85 ± 0.15 fold increase, n = 5) (Figure 2F), further indicating the involvement of PKC-dependent phosphorylation. Since S502 is a PKA-phosphorylation site as well [26], we examined the effects of treatment with a mixture of FSK, IBMX and dbcAMP on the capsaicin-activated currents in cells expressing S502A/S800A. Such treatment failed to potentiate the capsaicin-activated currents (1.13 ± 0.07 fold increase, n = 10), suggesting that S502 is a substrate for PKA-dependent phosphorylation of TRPV1 as well.

Sensitization of TRPV1 by EP_1 receptors in mouse

To examine the involvement of EP_1 in PGE_2 (1.5 min)-induced potentiation of capsaicin-evoked response in native neurons, we used a specific EP_1 agonist, ONO-DI-004 [27], and a specific EP_1 antagonist, ONO-8713 [28], in mouse DRG neurons. ONO-DI-004 was found to significantly increase the capsaicin-activated currents to an extent similar to that observed with PGE_2 (3.36 ± 0.68 fold

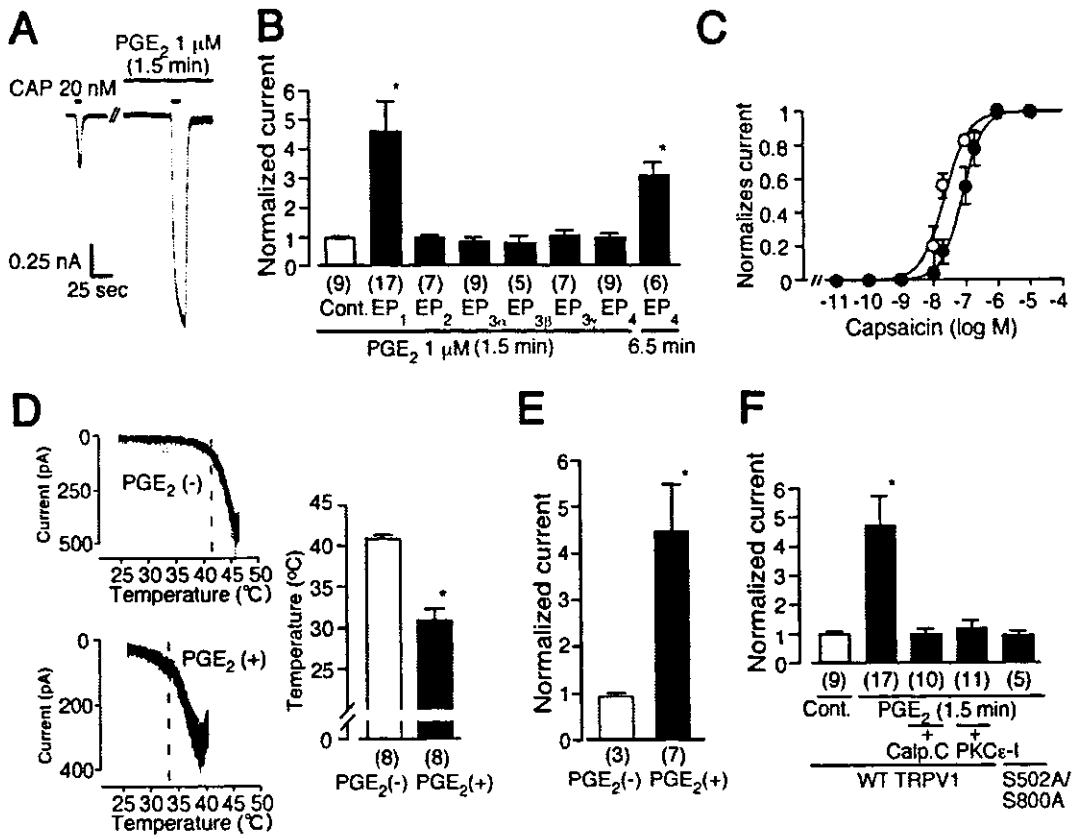

Figure 2

PGE_2 increases TRPV1 activity through EP_1 receptors in a PKC-dependent manner in HEK293 cells. **(A)** and **(B)** Treatment with PGE_2 (1.5 min) potentiates capsaicin-evoked responses in cells expressing rat TRPV1 with mouse EP_1 receptors, but not with other mouse EP receptors. Cells were pretreated with PGE_2 (1 μM) for 1.5 or 6.5 min before second capsaicin (20 nM) application. V_h: -60 mV. Currents were normalized as described in Figure 1. * $p < 0.05$ vs. control (Cont.). Numbers in parenthesis indicate cells tested. **(C)** Capsaicin dose-response curves for TRPV1 activation in the absence (•) and presence (○) of extracellular 1 μM PGE_2. Currents were normalized to the current maximally activated by 1 μM capsaicin in the absence of PGE_2. Figure shows averaged data fitted with the Hill equation. EC_{50} = 81.0 nM and Hill coefficient = 1.33 in the absence of PGE_2. EC_{50} = 27.6 nM and Hill coefficient = 1.01 in the presence of PGE_2. Data were obtained from 54 different cells. **(D)** Temperature threshold for TRPV1 activation was reduced in the presence of extracellular PGE_2 (1 μM). Representative temperature-response profiles in the absence (upper) and presence (lower) of PGE_2 (left). Temperature threshold for TRPV1 activation in the presence of PGE_2 (30.6 ± 1.1°C) was significantly lower than that in the absence of PGE_2 (40.7 ± 0.3°C) (right). * $p < 0.05$ vs. PGE_2 (-). Numbers in parenthesis indicate cells tested. **(E)** Proton-evoked TRPV1 responses were significantly potentiated by PGE_2 (1 μM). * $p < 0.01$ vs. PGE_2 (-). **(F)** PKC-dependent pathway is involved in the PGE_2 (1 μM, 1.5 min)-induced potentiation of capsaicin-activated currents. In some experiments, calphostin C (Calp. C) (1 μM) or PKCε translocation inhibitor (PKCε-I) (200 μM) was included in the pipette solution. Currents were normalized as described in Figure 1. Numbers in parenthesis indicate cells tested. * $p < 0.05$ vs. Cont. V_h: -60 mV.

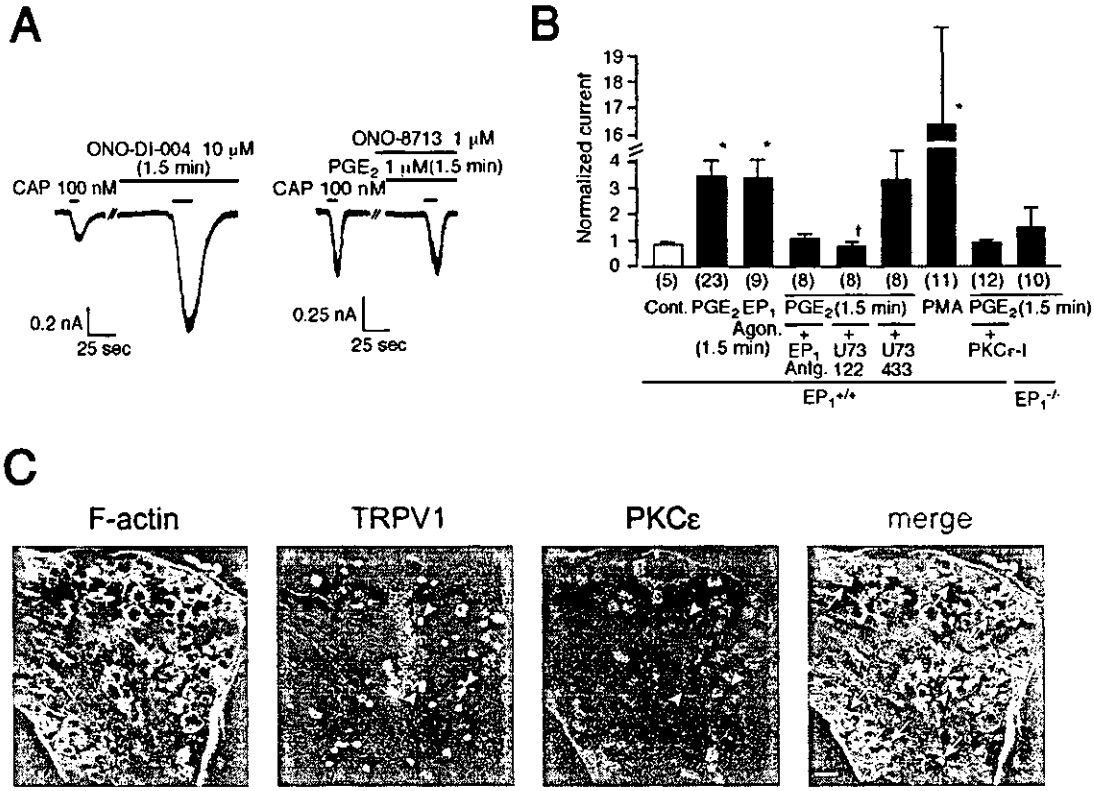

Figure 3

EP_1 receptor involvement in PGE_2 (1.5 min)-induced potentiation of capsaicin-activated currents in mouse DRG neurons. (A) Representative traces of potentiation of capsaicin-activated currents by a specific EP_1 agonist, ONO-DI-004 (10 μM, 1.5 min), and reverse of the PGE_2 (1.5 min)-induced potentiation by a specific EP_1 antagonist, ONO-8713 (1 μM). V_h: -60 mV. **(B)** Effects of PGE_2 (1 μM), ONO-DI-004 (EP_1 Agon., 10 μM), PGE_2 plus ONO-8713 (EP_1 Antg., 1 μM), PGE_2 plus U73122 (3 μM), PGE_2 plus U73343 (3 μM), phorbol 12-myristate 13-acetate (PMA, 100 nM) or PGE_2 plus PKCε-I (200 μM) on capsaicin-activated currents in DRG neurons from wild type ($EP_1^{+/+}$) mice, and effects of PGE_2 on capsaicin-activated currents in DRG neurons from $EP_1^{-/-}$ mice. Currents are normalized as described in Fig. 1. * $p < 0.05$ vs. Cont., + $p < 0.05$ vs. U73343. Numbers in parenthesis indicate cells tested. **(C)** Co-expression of TRPV1 (green) and PKCε (blue) in mouse DRG. Arrowheads indicate neurons positive for TRPV1 but not for PKCε. Arrows indicate neurons positive for both TRPV1 and PKCε (light blue). Bar, 100 μm.

increase for PGE_2, n = 23, $p < 0.05$ vs. control (Cont.); 3.30 ± 0.68 fold for ONO-DI-004 (EP_1 Agon.), n = 9, $p < 0.05$ vs. Cont.) (Figures 3A left and 3B). Furthermore, potentiation of the capsaicin-activated currents by PGE_2 was inhibited in the presence of ONO-8713 (EP_1 Antg., 1.00 ± 0.17 fold increase, n = 8) (Figures 3A right and 3B). These results indicate that PGE_2 (1.5 min)-induced potentiation of the capsaicin-activated current responses occurs through EP_1 receptors in DRG neurons. To confirm the involvement of PKC-dependent events downstream of PGE_2 effects in DRG neurons, we first examined the effects of a specific phospholipase C (PLC) inhibitor, U73122 (3 μM). PGE_2-induced potentiation of capsaicin-activated current was significantly diminished in the presence of U73122 while control U73343 exhibited no such effects (0.73 ± 0.11 fold increase, n = 8 for U73343; 3.40 ± 1.11

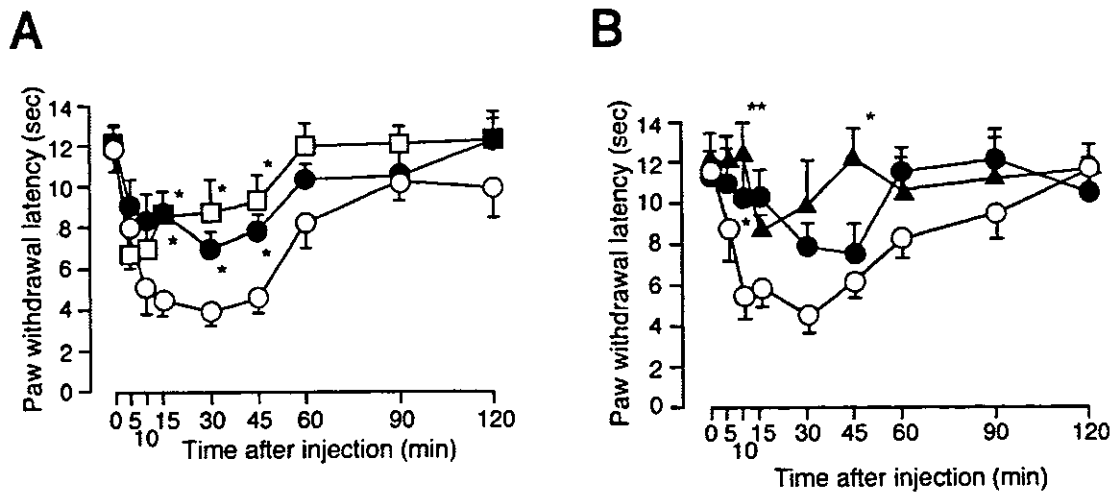

Figure 4
Interaction between TRPV1 and EP_1 receptors in a behavioral level. **(A)** PGE_2-induced thermal hyperalgesia in wild type mice with (, n = 6) or without (○, n = 6) pretreatment (ONO-8713, 500 pmol/ 20 μL), or in $EP_1^{-/-}$ mice (•, n = 6). * $p < 0.05$ vs. wild type mice. **(B)** 10% Mustard oil-induced thermal hyperalgesia in wild type mice (○, n = 12), $TRPV1^{-/-}$ mice (▲, n = 6) or $EP_1^{-/-}$ mice (•, n = 6). * $p < 0.05$, ** $p < 0.01$ vs. wild type mice.

fold, n = 8 for U73433, $p < 0.05$) (Figure 3B). Furthermore, PGE_2 failed to potentiate the capsaicin-activated currents when PKCε-I was included in the pipette solution (0.86 ± 0.09 fold increase, n = 12) (Figure 3B). A robust potentiating effect of phorbol 12-myristate 13-acetate (PMA, 100 nM) also supported the involvement of PKC-dependent events (16.36 ± 3.68 fold increase, n = 11, $p < 0.05$) (Figure 3B). To further confirm the involvement of EP_1 receptors, DRG neurons of EP_1 deficient mice ($EP_1^{-/-}$) were subjected to patch-clamp analysis. PGE_2 failed to potentiate capsaicin-activated currents in the DRG neurons from $EP_1^{-/-}$ mice (1.45 ± 0.70 fold increase, n = 10) (Figure 3B). Functional interaction of PKCε with TRPV1 prompted us to examine the expression of the two proteins in mouse DRG. Three hundred seventy eight out of 541 TRPV1 positive neurons (69.9 %) were stained with anti-PKCε antibody, supporting the TRPV1 activation pathway through PKCε (Figure 3C).

We next investigated the involvement of EP_1 receptors in PGE_2-induced thermal hyperalgesia at the behavioral level. PGE_2-induced thermal hyperalgesia was significantly diminished at 15 to 45 min after injection in $EP_1^{-/-}$ mice (Figure 4A), relative to that observed in wild type mice. The involvement of EP_1 receptors in the PGE_2-induced hypersensitivity was supported by another behavioral analysis in which PGE_2 caused less reduction of paw withdrawal latency in wild type mice pretreated with a specific EP_1 antagonist (500 pmol/ 20 μL) than in vehicle control (Figure 4A). These results suggest that a PKC-dependent pathway downstream of EP_1 activation is mainly involved in PGE_2-induced thermal hyperalgesia. We have hypothesized that the potentiation of TRPV1 activity by several inflammatory mediators could represent one important mechanism underlying acute inflammatory pain sensation. To prove the accuracy of this hypothesis, we investigated the involvement of EP_1 in inflammatory pain-related responses using mustard oil which is known to cause inflammation [29,30]. Topical application of mustard oil induced clear thermal hyperalgesia (Figure 4B). The mustard oil-induced thermal hyperalgesia was significantly reduced both in $TRPV1^{-/-}$ mice and $EP_1^{-/-}$ mice. Thus, these data show that EP_1 contributes to inflammatory nociception in mice and support the hypothesis.

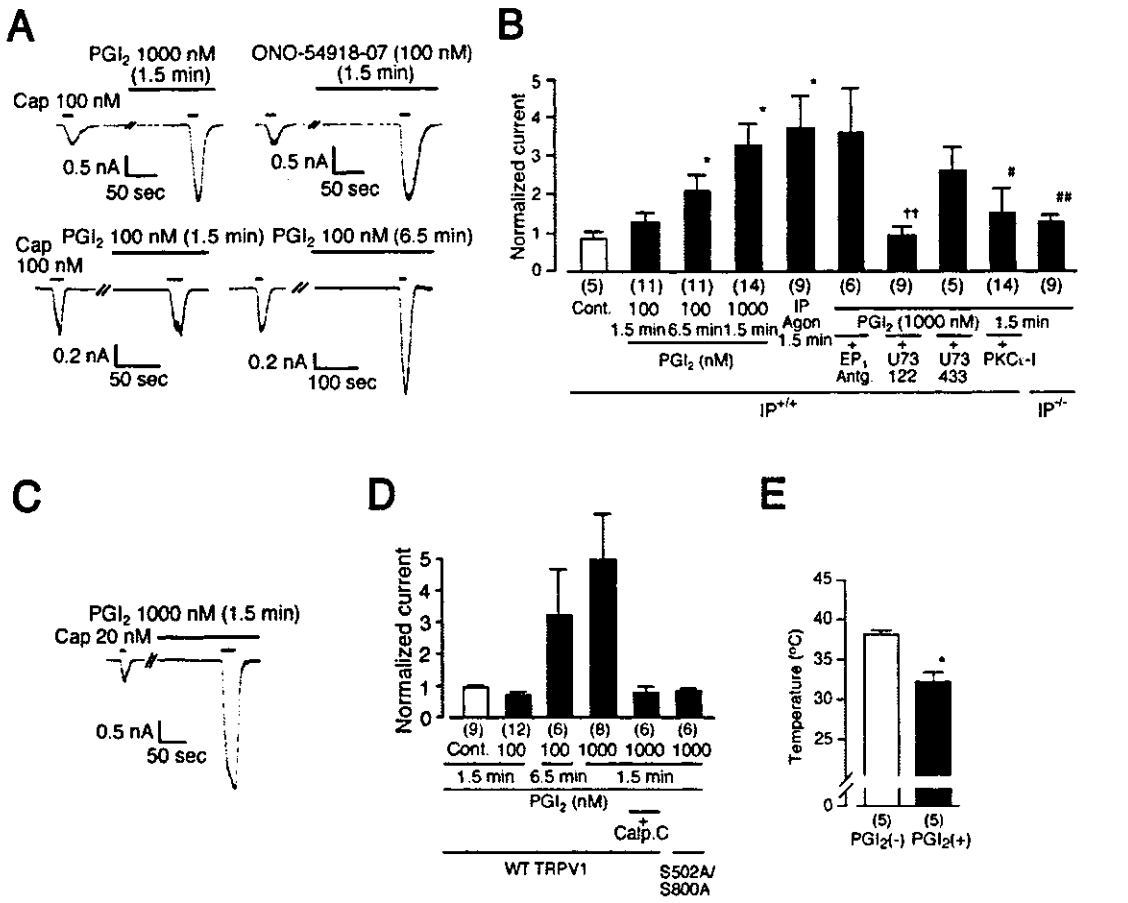

Figure 5

PGI_2 causes potentiation or sensitization of TRPV1 through mainly through PKC activation. (**A**) Representative traces of potentiation of capsaicin-activated currents by PGI_2 (1000 nM, 1.5 min), a specific IP agonist, ONO-54918-07 (1.5 min) or PGI_2 (100 nM, 6.5 min), but not by PGI_2 (100 nM, 1.5 min) in mouse DRG neurons. V_h: -60 mV. (**B**) Effects of treatments (1.5 or 6.5 min) with PGI_2 (100 or 1000 nM), ONO-54918-07 (IP Agon., 100 nM), PGI_2 (1000 nM) plus ONO-8713 (EP_1 Antg., 1 μM), PGI_2 (1000 nM) plus U73122 (3 μM), PGI_2 (1000 nM) plus U73343 (3 μM) or PGI_2 (1000 nM) plus PKCε-I (200 μM) on capsaicin-activated currents in DRG neurons from wild type ($IP^{+/+}$) mice, and effects of PGI_2 on capsaicin-activated currents in DRG neurons from IP-deficient ($IP^{-/-}$) mice. Currents are normalized as described in Figure 1. * $p < 0.05$ vs. Cont. ++ $p < 0.01$ vs. U73343, # $p < 0.05$, ## $p < 0.01$ vs. PGI_2 (1000 nM, 1.5 min) in DRG neurons from $IP^{+/+}$ mice. Numbers in parenthesis indicate cells tested. (**C**) A representative trace of potentiation of capsaicin-activated currents by PGI_2 (1000 nM, 1.5 min) in HEK293 cells expressing both TRPV1 and IP. V_h: -60 mV. (**D**) Effects of treatments (1.5 or 6.5 min) with PGI_2 (100 or 1000 nM) or PGI_2 (1000 nM) plus calphostin C (Calp. C, 1 μM) on capsaicin-activated currents in HEK293 cells expressing rat wild type TRPV1 or S502A/S800A mutant with IP. Currents are normalized as described in Figure 1. * $p < 0.05$ vs. Cont. (**E**) Temperature threshold for TRPV1 activation in the presence of PGI_2 (32.2 ± 1.2°C) was significantly lower than that in the absence of PGI_2 (38.2 ± 0.5°C) in HEK293 cells expressing rat TRPV1 and IP. * $p < 0.01$ vs. PGI_2 (-).

Sensitization of TRPV1 by IP receptors

In order to determine whether the observed responses are specific to PGE_2, we extended our analysis to PGI_2 whose receptor has been reported to be involved in nociception [8]. We first examined the effects of PGI_2 on capsaicin-activated currents in mouse DRG neurons. PGI_2 pretreatment (1000 nM, 1.5 min) potentiated capsaicin (100 nM)-activated currents (3.23 ± 0.55 fold increase, n = 14 or 0.78 ± 0.08 fold, n = 5 with or without (Cont.) PGI_2, respectively; $p < 0.05$) whereas at 100 nM, PGI_2 (1.5 min) showed no such effects (1.24 ± 0.22 fold, n = 11) (Figures. 5A and 5B). On the other hand, long (6.5 min) treatment with PGI_2 (100 nM) caused significant potentiation of capsaicin-activated currents as in the treatment with a mixture of FSK, IBMX and dbcAMP (2.06 ± 0.54 fold increase, n = 11, $p < 0.05$ vs. Cont.) (Figures 5A and 5B). The potentiation effects of PGI_2 appear to occur through IP receptors because a specific IP agonist, ONO-54918-07 (100 nM) [31] caused similar potentiation of capsaicin-activated currents (Agon., 3.71 ± 0.81 fold increase, n = 9, $p < 0.05$ vs. Cont.) (Figures 5A and 5B) although PGI_2 is known to cross react with some EP receptors [2]. The fact that a specific EP_1 antagonist, ONO-8713 failed to prevent the PGI_2-induced potentiation (+EP_1 Antg., 3.55 ± 1.17 fold increase, n = 6) (Figure 5B) further suggests the involvement of IP receptors in the potentiation process. The involvement of IP receptors in the PGI_2-induced potentiation of capsaicin-activated currents was further supported by the ineffectiveness of PGI_2 on DRG neurons from IP-deficient mice ($IP^{-/-}$) (1.25 ± 0.16 fold increase, n = 9, $p < 0.01$ vs. 1000 nM of PGI_2) (Figure 5B). It has been reported that low concentrations of PGI_2 stimulate Gs protein coupled to IP receptors whereas high concentrations of PGI_2 stimulate not only Gs but also Gq [32]. This property might explain the dose-dependent effects of PGI_2 on capsaicin-activated currents: PKC-dependent sensitization of TRPV1 occurs downstream of Gq-coupled IP receptor activation at high concentrations (1000 nM) of PGI_2 (1.5 min) while long (6.5 min) treatment with low concentrations (100 nM) of PGI_2 causes potentiation of TRPV1 activity through Gs activation. To test this hypothesis, PGI_2 (1.5 min)-induced potentiation of capsaicin-activated currents was examined in the presence of U73122. When U73122 was included in the pipette solution, PGI_2 (1.5 min) failed to potentiate the currents whereas U73343 exhibited no such effects, indicating the involvement of PLC activation in the potentiating process (0.97 ± 0.40 fold increase, n = 9 for U73122, 2.58 ± 0.66 fold, n = 5 for U73343, $p < 0.05$) (Figure 5B). Furthermore, PKCε-I included in the pipette solution almost completely blocked the PGI_2 (1000 nM)-induced potentiation (1.49 ± 0.60 fold increase, n = 14), suggesting the involvement of PKCε-dependent regulation mechanism (Figure 5B).

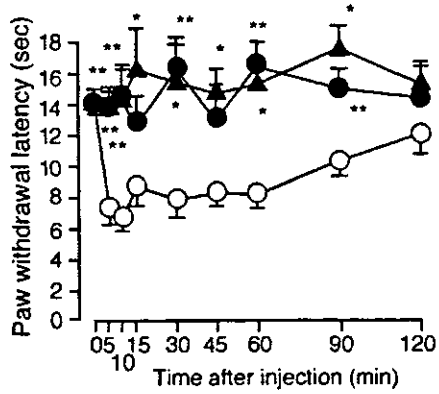

Figure 6

Interaction between TRPV1 and IP receptors at a behavioral level. PGI_2-induced thermal hyperalgesia in wild type mice (○, n = 6), $TRPV1^{-/-}$ mice (▲, n = 6) or $IP^{-/-}$ mice (•, n = 6). Thermal hyperalgesia by intraplantar PGI_2 (500 pmol/ 20 μL) injection was significantly diminished in $TRPV1^{-/-}$ mice and $IP^{-/-}$ mice. * $p < 0.05$, ** $p < 0.01$ vs. wild type mice.

Dose-dependent PGI_2 (1.5 min)-induced potentiation of capsaicin-activated currents was also observed in HEK293 cells expressing TRPV1 and IP receptors (0.90 ± 0.04 fold increase, n = 9 without PGI_2 (Cont.); 0.68 ± 0.08 fold, n = 12 with 100 nM of PGI_2; 0.75 ± 0.07 fold, n = 6 with 300 nM PGI_2, 4.96 ± 1.36 fold, n = 8 with 1000 nM of PGI_2, $p < 0.01$ vs. Cont.) (Figures 5C and 5D, and data not shown). Calp. C blocked PGI_2-induced potentiation of TRPV1 currents (0.75 ± 0.15 fold increase, n = 6) (Figure 5D). Furthermore, PGI_2 (1000 nM) failed to potentiate capsaicin-activated currents in HEK293 cells expressing the S502/S800 mutant (0.80 ± 0.05 fold, n = 6) (Figure 5D). Long (6.5 min) treatment with PGI_2 (100 nM) caused an increase in capsaicin-activated currents in 4 out of 6 cells, as did long treatment with a mixture of FSK, IBMX and dbcAMP in HEK293 cells expressing TRPV1 (3.19 ± 1.45 fold increase, n = 6, p = 0.16). These results suggest that a mechanism involving PKC is predominantly involved in the regulation of TRPV1 activity during short treatment with PGI_2 although both PKA-dependent and PKC-dependent pathways may contribute. The temperature threshold for TRPV1 activation was significantly reduced (from 38.2 ± 0.5°C, n = 5 to 32.2 ± 1.2°C, n = 5) in the presence of PGI_2, suggesting the possibility that IP receptor activation can cause nociception at body temperature (Figure 5E). Finally, PGI_2-induced thermal hyperal-

gesia observed in wild type mice disappeared almost completely in both TRPV1-deficient ($TRPV1^{-/-}$) mice and IP-deficient ($IP^{-/-}$) mice, suggesting that the functional interaction of TRPV1 with IP causes thermal hyperalgesia at the behavioral level (Figure 6).

Discussion

The data presented herein demonstrate that TRPV1 is essential for the development of thermal hyperalgesia *in vivo* induced by two major inflammation-associated prostaglandins, PGE_2 and PGI_2, and that TRPV1 and EP_1 or IP receptors can functionally interact, mainly through a PKC-dependent pathway. The temperature threshold for TRPV1 activation is reduced below 35°C in the presence of prostaglandins, so that TRPV1 can be activated at normal body temperature, possibly leading to spontaneous pain sensation. This interaction might be one important underlying mechanism for the well-recognized peripheral nociceptive actions of PGE_2 or PGI_2 in the context of inflammation. In the present study, 1 μM PGE_2 or PGI_2 was found to potentiate or sensitize TRPV1 activity. It is not well known how much PGE_2 or PGI_2 is released locally at the site of inflammation. However, more than micromolar-order concentrations of PGE_2 and PGI_2 have been reported to be synthesized by macrophages upon lipopolysacharide (LPS) stimulation [33,34], suggesting that 1 μM is an attainable concentration in the context of inflammation. It has been previously reported that EP_1 is coupled to intracellular Ca^{2+} mobilization in CHO cells [35]. However, the transduction events downstream of EP_1 signaling have been unclear. Together with a report suggesting the possible coupling of EP_1 with $G_{q/11}$-protein [36], our data indicate that EP_1 receptors activate a PKC-dependent signal transduction pathway.

There has been extensive work demonstrating the activation of a PKA-dependent pathway by PGE_2 that influences capsaicin- or heat-mediated actions in rat sensory neurons [20-22,37,38] as well as interactions between cloned TRPV1 and PKA [26,39-42]. These results suggest that PKA plays a pivotal role in the development of hyperalgesia and inflammation by prostaglandins. In our experiments using mouse DRG neurons and HEK293 cells expressing TRPV1, a PKC-dependent pathway was found to be predominantly involved in both PGE_2 (1.5 min)- and PGI_2 (1.5 min)-induced responses. The reason that there has been no study describing the involvement of a PKC-dependent pathway in the regulation of TRPV1 following prostaglandin receptor activation is not clear. In the present study, it was found that both PKA- and PKC-dependent pathways are involved downstream of prostaglandin actions on TRPV1 although the PKC-dependent one appears to predominate. A PKA-dependent pathway took a relatively long time to exert its potentiating effects on TRPV1 activity, suggesting some difference between PKA- and PKC-dependent phosphorylation of TRPV1. Indeed, Bhave et al. treated cells with 8-Br-cAMP for 30 min to inhibit TRPV1 desensitization through phosphorylation [39], and significant potentiation of capsaicin-activated currents in rat DRG neurons was observed upon prolonged (greater than 10 min) exposure to PGE_2 [21]. Furthermore, there is a report describing the ineffectiveness of PKA stimulation on TRPV1 currents in *Xenopus* oocytes treated with 8-Br-cAMP and IBMX for relatively short periods [24]. Both PKA-dependent and PKC-dependent pathways might work in concert in native cells. Patch-clamp recordings in the previous studies were performed in the Ca^{2+}-containing solutions, whereas we did all of our experiments under Ca^{2+}-free conditions, to avoid Ca^{2+}-dependent TRPV1 desensitization [43]. Potentiation of capsaicin-activated currents by PGE_2 was observed in embryonic rat DRG neurons [21] while we used adult mouse DRG neurons. Furthermore, potentiation of heat-activated currents [26], inhibition of desensitization of capsaicin-activated currents [39,41,44] or anandamide-induced cytosolic Ca^{2+} increase [40] but not potentiation of capsaicin-activated current response were examined in the previous studies investigating the involvement of PKA-dependent pathway in TRPV1 activity. Thus, difference in experimental conditions or readout might also account for the different outcomes. The physiological relevance of the two different pathways downstream of prostaglandin exposure remains to be elucidated. The fact that only PKC activation leads to the reduction of temperature threshold for TRPV1 activation might be pertinent to this issue. Disruption of interaction between phosphatidylinositol-4, 5-bisphosphate (PIP_2) and TRPV1 has also been reported to be involved in the sensitization of TRPV1 downstream of PLC activation [45,46]. In our study, however, both PGE_2- and PGI_2-induced potentiation of TRPV1 activity was completely inhibited by treatments with two kinds of PKC inhibitors. Thus, we believe that a PKC-dependent pathway is predominantly involved in the PGE_2- and PGI_2-induced potentiation or sensitization of TRPV1 activity in mice.

The inhibition of PGE_2-induced thermal hyperalgesia observed in $EP_1^{-/-}$ mice, while significant, was not very robust, compared with that in $TRPV1^{-/-}$ mice (Figure 4). Other pathways, most likely including one involving PKA, might account for the residual component. Further, inhibition of mustard oil-induced thermal hyperalgesia observed in $TRPV1^{-/-}$ or $EP_1^{-/-}$ mice might seem not to be robust or dramatic (Figure 4). Since many inflammatory factors activating PLC-coupled receptors are involved in the inflammatory response [47,48]. In such a complicated environment, thermal hyperalgesia was significantly diminished in $TRPV1^{-/-}$ mice or $EP_1^{-/-}$ mice albeit at a few time points, suggesting the importance of the two molecules in the context of inflammatory pain sensation.

Given the fact that one of the final targets of both PGE_2 and PGI_2 is TRPV1 as shown in our study, compounds acting on EP_1, IP or TRPV1, or interfering with their interaction could prove useful in the treatment of pain and inflammation.

Conclusions

Potentiation or sensitization of TRPV1 activity through EP_1 or IP activation, mainly through PKC- and PKA-dependent mechanisms, might be important mechanism underlying the peripheral nociceptive actions of PGE_2 or PGI_2.

Methods

Animals

Male C57BL/6-strain mice (4 weeks, SLC, Shizuoka, Japan), EP_1-deficient mice (4 weeks, from Dr. Narumiya), IP-deficient mice (4 weeks, from Dr. Narumiya) or TRPV1-deficient mice (4 weeks, from Dr. Julius, UCSF) were used. They were housed in a controlled environment (12 h light/dark cycle, room temperature 22–24°C, 50–60% relative humidity) with free access to food and water. All procedures involving the care and use of mice were carried out in accordance with institutional (Mie University) guidelines and the National Institute of Health guide for the care and use of laboratory animals.

Behavioral study

Thermal nociceptive threshold was assessed using the paw withdrawal test. Mice were placed in a transparent Perspex box on a thin glass platform (Plantar test, Ugo Basile, Italy). They were injected intraplantarly with PGE_2 (500 pmol/ 20 μL, Sigma) with or without ONO-8713 (500 pmol/ 20 μL), or with PGI_2 (500 pmol/ 20 μL, Sigma), or applied topically to the plantar surface of right hind paw with 10% mustard oil (Sigma) (diluted with mineral oil), and the paw withdrawal latency to radiant heat applied to the plantar surface of hind paw was measured as the time from onset of the radiant heat to the withdrawal of the mouse hind paw.

Cell culture

Human embryonic kidney-derived (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen; supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine) and transfected with 0.5 μg of rat TRPV1 cDNA and 0.5 μg of mouse EP or IP receptor cDNAs (EP_1, EP_2, $EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$, EP_4 or IP) using Lipofectamine Plus Reagent (Invitrogen). Primary cultures prepared from adult C57BL/6-strain mice, EP_1-deficient mice or IP-deficient mice dorsal root ganglion (DRG) neurons were incubated in medium containing nerve growth factor (Sigma, 100 ng/ml).

Electrophysiology

Whole-cell patch-clamp recordings were performed 1 day after transfection to HEK293 cells or dissociation of the DRG neurons. Standard bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 10 mM glucose, pH7.4 (adjusted with NaOH). Pipette solution contained 140 mM KCl, 5 mM EGTA, 10 mM HEPES, pH7.4 (adjusted with KOH). All patch-clamp experiments were performed at room temperature (22°C). Thermal stimulation was applied by increasing the bath temperature at a rate of 1.0°C/sec with a preheated solution. When the heat-activated currents started to inactivate, the preheated solution was changed to a 22°C one. Chamber temperature was monitored with a thermocouple placed within 100 μm of the patch-clamped cell. For this analysis, heat-evoked current responses were compared between different cells, rather than within the same cell, because repetitive heat-evoked currents show significant desensitization even in the absence of extracellular Ca^{2+} [13] and because the thermal sensitivity of TRPV1 increases with repeated heat application [49]. Threshold temperature for activation was defined as the intersection where two lines approximating the stable baseline current and the clearly increasing temperature-dependent current cross in the temperature-response profile. The sensitivity of DRG neurons to capsaicin is slightly lower than that of TRPV1-transfected HEK293 cells as previously reported [18,50]. Therefore, we applied capsaicin at 100 nM to DRG neurons and at 20 nM to HEK293 cells.

cAMP measurement

Intracellular cAMP level was examined using 'cAMP Biotrak Enzymeimmunoassay System' according to the manufacture's direction (Amersham Biosciences). In brief, intracellular cAMP released upon membrane hydrolysis of treated cells (10,000 cells/ well) after stimulation (90 sec) was measured based on competition between unlabelled cAMP and a fix quantity of Peroxidase-labeled cAMP for a limited number of the binding sites on a cAMP specific antibody.

Immunostaining

DRG was removed from male C57BL/6-strain mice and frozen in liquid nitrogen, and the frozen tissue was cut on a cryostat at a 10 μm thickness. The sections were incubated with the rabbit anti-rat TRPV1 polyclonal antibody (1: 500; Oncogene) and anti-rat PKCε monoclonal antibody (1: 250; Transduction lab) at 4°C for 2 days. Slides with the section were washed with PBS, followed by incubation with Alexa 488-conjugated goat anti-rabbit IgG (1: 700, Molecular Probes), Alexa 350-conjugated anti-mouse IgG (1: 500, Molecular Probes) and Texas Red-phalloidin (1: 500, Molecular Probes). Images were obtained using an Olympus fluorescent microscope with

a cooled-CCD camera (ORCA-ER, Hamamatsu Photonics) and IP-Lab Image software (Scanalytics Inc.).

Chemicals

ONO-DI-004, ONO-8713 and ONO-54918-07 were obtained from Ono Pharmaceutical Co., Ltd (Osaka, Japan). Calphostin C, phorbol 12-myristate 13-acetate, forskolin, 3-isobutyl-1-methylxanthine, dibutyryl-cAMP, isoproterenol, U73122 and U73343 were from Sigma, and PKCε translocation inhibitor was from Calbiochem.

Statistics

Values are shown as the mean ± S.E. and data are analyzed using an unpaired *t* test. P values of < 0.05 were considered significant.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

TM and TH carried out most of the experiments in this study. KT carried out the immunostaining experiments. TI carried out some electrophysiological experiments. ES made and maintained EP_1- and IP-deficient mice, and participated in the interpretation of data. YS and SN participated in experimental design and discussion. TT carried out some biochemical experiments. MT contributed to all aspects of the study and wrote the manuscript.

Acknowledgements

We thank D. Julius (University of California, San Francisco) for giving us TRPV1-deficient mice, and M.J. Caterina (Johns Hopkins University), N. Saito (Kobe University) and M. Numazaki (University of Tsukuba) for their critical reading of the manuscript, and N. Suzuki and H. Tsumura (Mie University) for their support for maintaining mice. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan, Japan Brain Foundation, Yamanouchi Foundation for Research on Metabolic Disorders, Uehara Memorial Foundation, AstraZeneca Research Foundation and ONO Medical Research Foundation to M.T.

References

1. Tilley SL, Coffman TM, Koller BH: **Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes.** *J Clin Invest* 2001, **108**:15-23.
2. Narumiya S, Sugimoto Y, Ushikubi F: **Prostanoid receptors: structures, properties, and functions.** *Physiol Rev* 1999, **79**:1193-1226.
3. Samad TA, Sapirstein A, Woolf CJ: **Prostanoids and pain: unraveling mechanisms and revealing therapeutic targets.** *Trends Mol Med* 2002, **8**:390-396.
4. Breyer RM, Bagdassarian CK, Myers SA, Breyer MD: **Prostanoid receptors: subtypes and signaling.** *Annu Rev Pharmacol Toxicol* 2001, **41**:661-690.
5. Narumiya S, FitzGerald GA: **Genetic and pharmacological analysis of prostanoid receptor function.** *J Clin Invest* 2001, **108**:25-30.
6. Oida H, Namba T, Sugimoto Y, Ushikubi F, Ohishi H, Ichikawa A, Narumiya S: **In situ hybridization studies of prostacyclin receptor mRNA expression in various mouse organs.** *Br J Pharmacol* 1995, **116**:2828-2837.
7. Sugimoto Y, Shigemoto R, Namba T, Negishi M, Mizuno N, Narumiya S, Ichikawa A: **Distribution of the messenger RNA for the prostaglandin E receptor subtype EP3 in the mouse nervous system.** *Neuroscience* 1994, **62**:919-928.
8. Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, Ueno A, Oh-ishi S, Narumiya S: **Altered pain perception and inflammatory response in mice lacking prostacyclin receptor.** *Nature* 1997, **388**:678-682.
9. Minami T, Nakano H, Kobayashi T, Sugimoto Y, Ushikubi F, Ichikawa A, Narumiya S, Ito S: **Characterization of EP receptor subtypes responsible for prostaglandin E2-induced pain responses by use of EP1 and EP3 receptor knockout mice.** *Br J Pharmacol* 2001, **133**:438-444.
10. Stock JL, Shinjo K, Burkhardt J, Roach M, Taniguchi K, Ishikawa T, Kim HS, Flannery PJ, Coffman TM, McNeish JD, Audoly LP: **The prostaglandin E2 EP1 receptor mediates pain perception and regulates blood pressure.** *J Clin Invest* 2001, **107**:325-331.
11. Caterina MJ, Julius D: **The vanilloid receptor: a molecular gateway to the pain pathway.** *Annu Rev Neurosci* 2001, **24**:487-517.
12. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D: **The capsaicin receptor: a heat-activated ion channel in the pain pathway.** *Nature* 1997, **389**:816-824.
13. Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D: **The cloned capsaicin receptor integrates multiple pain-producing stimuli.** *Neuron* 1998, **21**:531-543.
14. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D: **Impaired nociception and pain sensation in mice lacking the capsaicin receptor.** *Science* 2000, **288**:306-313.
15. Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA: **Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia.** *Nature* 2000, **405**:183-187.
16. Dai Y, Moriyama T, Higashi T, Togashi K, Kobayashi K, Yamanaka H, Tominaga M, Noguchi K: **Proteinase-activated receptor 2-mediated potentiation of transient receptor potential vanilloid subfamily 1 activity reveals a mechanism for proteinase-induced inflammatory pain.** *J Neurosci* 2004, **24**:4293-4299.
17. Sugiura T, Tominaga M, Katsuya H, Mizumura K: **Bradykinin lowers the threshold temperature for heat activation of vanilloid receptor 1.** *J Neurophysiol* 2002, **88**:544-548.
18. Tominaga M, Wada M, Masu M: **Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia.** *Proc Natl Acad Sci U S A* 2001, **98**:6951-6956.
19. Numazaki M, Tominaga T, Toyooka H, Tominaga M: **Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues.** *J Biol Chem* 2002, **277**:13375-13378.
20. Gu Q, Kwong K, Lee LY: **Ca2+ transient evoked by chemical stimulation is enhanced by PGE2 in vagal sensory neurons: role of cAMP/PKA signaling pathway.** *J Neurophysiol* 2003, **89**:1985-1993.
21. Lopshire JC, Nicol GD: **The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: whole-cell and single-channel studies.** *J Neurosci* 1998, **18**:6081-6092.
22. Smith JA, Davis CL, Burgess GM: **Prostaglandin E2-induced sensitization of bradykinin-evoked responses in rat dorsal root ganglion neurons is mediated by cAMP-dependent protein kinase A.** *Eur J Neurosci* 2000, **12**:3250-3258.
23. Schuligoi R, Donnerer J, Amann R: **Bradykinin-induced sensitization of afferent neurons in the rat paw.** *Neuroscience* 1994, **59**:211-215.
24. Lee YS, Lee JA, Jung J, Oh U, Kaang BK: **The cAMP-dependent kinase pathway does not sensitize the cloned vanilloid receptor type 1 expressed in xenopus oocytes or Aplysia neurons.** *Neurosci Lett* 2000, **288**:57-60.
25. Moriyama T, Iida T, Kobayashi K, Higashi T, Fukuoka T, Tsumura H, Leon C, Suzuki N, Inoue K, Gachet C, Noguchi K, Tominaga M: **Possible involvement of P2Y2 metabotropic receptors in ATP-induced transient receptor potential vanilloid receptor 1-**

mediated thermal hypersensitivity. *J Neurosci* 2003, **23**:6058-6062.
26. Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C, Kress M: **PKA/AKAP/VR-1 module: A common link of Gs-mediated signaling to thermal hyperalgesia.** *J Neurosci* 2002, **22**:4740-4745.
27. Shibuya I, Setiadji SV, Ibrahim N, Harayama N, Maruyama T, Ueta Y, Yamashita H: **Involvement of postsynaptic EP4 and presynaptic EP3 receptors in actions of prostaglandin E2 in rat supraoptic neurones.** *J Neuroendocrinol* 2002, **14**:64-72.
28. Watanabe K, Kawamori T, Nakatsugi S, Ohta T, Ohuchida S, Yamamoto H, Maruyama T, Kondo K, Narumiya S, Sugimura T, Wakabayashi K: **Inhibitory effect of a prostaglandin E receptor subtype EP(1) selective antagonist, ONO-8713, on development of azoxymethane-induced aberrant crypt foci in mice.** *Cancer Lett* 2000, **156**:57-61.
29. Woolf CJ, Wall PD: **Relative effectiveness of C primary afferent fibers of different origins in evoking a prolonged facilitation of the flexor reflex in the rat.** *J Neurosci* 1986, **6**:1433-1442.
30. Reeh PW, Kocher L, Jung S: **Does neurogenic inflammation alter the sensitivity of unmyelinated nociceptors in the rat?** *Brain Res* 1986, **384**:42-50.
31. Inoue M, Rashid MH, Kawashima T, Matsumoto M, Maeda T, Kishioka S, Ueda H: **The algogenic-induced nociceptive flexion test in mice: studies on sensitivity of the test and stress on animals.** *Brain Res Bull* 2003, **60**:275-281.
32. Namba T, Oida H, Sugimoto Y, Kakizuka A, Negishi M, Ichikawa A, Narumiya S: **cDNA cloning of a mouse prostacyclin receptor. Multiple signaling pathways and expression in thymic medulla.** *J Biol Chem* 1994, **269**:9986-9992.
33. Tang Y, Di Pietro L, Feng Y, Wang X: **Increased TNF-alpha and PGI(2), but not NO release from macrophages in 18-month-old rats.** *Mech Ageing Dev* 2000, **114**:79-88.
34. Ikegami R, Sugimoto Y, Segi E, Katsuyama M, Karahashi H, Amano F, Maruyama T, Yamane H, Tsuchiya S, Ichikawa A: **The expression of prostaglandin E receptors EP2 and EP4 and their different regulation by lipopolysaccharide in C3H/HeN peritoneal macrophages.** *J Immunol* 2001, **166**:4689-4696.
35. Watabe A, Sugimoto Y, Honda A, Irie A, Namba T, Negishi M, Ito S, Narumiya S, Ichikawa A: **Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor.** *J Biol Chem* 1993, **268**:20175-20178.
36. Tabata H, Tanaka S, Sugimoto Y, Kanki H, Kaneko S, Ichikawa A: **Possible coupling of prostaglandin E receptor EP(1) to TRP5 expressed in Xenopus laevis oocytes.** *Biochem Biophys Res Commun* 2002, **298**:398-402.
37. Pitchford S, Levine JD: **Prostaglandins sensitize nociceptors in cell culture.** *Neurosci Lett* 1991, **132**:105-108.
38. Distler C, Rathee PK, Lips KS, Obreja O, Neuhuber W, Kress M: **Fast Ca2+-induced potentiation of heat-activated ionic currents requires cAMP/PKA signaling and functional AKAP anchoring.** *J Neurophysiol* 2003, **89**:2499-2505.
39. Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW: **cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation.** *Neuron* 2002, **35**:721-731.
40. De Petrocellis L, Harrison S, Bisogno T, Tognetto M, Brandi I, Smith GD, Creminon C, Davis JB, Geppetti P, Di Marzo V: **The vanilloid receptor (VR1)-mediated effects of anandamide are potently enhanced by the cAMP-dependent protein kinase.** *J Neurochem* 2001, **77**:1660-1663.
41. Hu HJ, Bhave G, Gereau RW: **Prostaglandin and protein kinase A-dependent modulation of vanilloid receptor function by metabotropic glutamate receptor 5: potential mechanism for thermal hyperalgesia.** *J Neurosci* 2002, **22**:7444-7452.
42. Sugiuar T, Bielefeldt K, Gebhart GF: **TRPV1 function in mouse colon sensory neurons is enhanced by metabotropic 5-hydroxytryptamine receptor activation.** *J Neurosci* 2004, **24**:9521-9530.
43. Numazaki M, Tominaga T, Takeuchi K, Murayama N, Toyooka H, Tominaga M: **Structural determinant of TRPV1 desensitization interacts with calmodulin.** *Proc Natl Acad Sci U S A* 2003, **100**:8002-8006.
44. Mohapatra DP, Nau C: **Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway.** *J Biol Chem* 2003, **278**:50080-50090.
45. Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D: **Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition.** *Nature* 2001, **411**:957-962.
46. Prescott ED, Julius D: **A modular PIP2 binding site as a determinant of capsaicin receptor sensitivity.** *Science* 2003, **300**:1284-1288.
47. Julius D, Basbaum AI: **Molecular mechanisms of nociception.** *Nature* 2001, **413**:203-210.
48. Scholz J, Woolf CJ: **Can we conquer pain?** *Nat Neurosci* 2002, **5 Suppl**:1062-1067.
49. Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D: **A capsaicin-receptor homologue with a high threshold for noxious heat.** *Nature* 1999, **398**:436-441.
50. Shin JS, Wang MH, Hwang SW, Cho H, Cho SY, Kwon MJ, Lee SY, Oh U: **Differences in sensitivity of vanilloid receptor I transfected to human embryonic kidney cells and capsaicin-activated channels in cultured rat dorsal root ganglion neurons to capsaicin receptor agonists.** *Neurosci Lett* 2001, **299**:135-139.

BOR Papers in Press. Published on January 12, 2005 as DOI:10.1095/biolreprod.104.037598

Changes in the Expression of Steroidogenic and Antioxidant Genes in the Mouse Corpus Luteum during Luteolysis

Nastaran Foyouzi*, Zailong Cai*, Yukihiko Sugimoto†, Carlos Stocco*

*Department of Obstetrics, Gynecology & Reproductive Science, Yale University School of Medicine. 333 Cedar St., New Haven, CT 06520

†Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan.

Corresponding author: Carlos Stocco. Yale University School of Medicine. 333 Cedar St., P.O. Box 208063 New Haven, CT 06520. Phone: (203) 785-2878. Fax: (203) 785-7134. E-mail: carlos.stocco@yale.edu

Running Title: Luteolysis and Steroidogenic/Anti-oxidant Genes

Abstract

Luteal cell death plays a key role in the regulation of the reproductive process in all mammals. It is also known that prostaglandin (PG) $F_{2\alpha}$ is one of the main factors that cause luteal demise; still, the effects of $PGF_{2\alpha}$ on luteal gene transcription have not been fully explored. Using microarray and RT-PCR, we have profiled gene expression in the corpus luteum (CL) of wild-type (WT) and $PGF_{2\alpha}$ receptor knockout mice on day 19 of pregnancy. Western blot analysis of selected genes was also performed. Since luteolysis has been shown to be associated with increased oxygen radical production and decreased progesterone synthesis, we report here changes observed in the expression of antioxidant and steroidogenic genes. We found that luteal cells express all genes necessary for progesterone synthesis, whether they had undergone luteolysis or not; however, an increase in mRNA levels of enzymes involved in androgen production, along with a decrease in the expression of enzymes implicated in estrogen synthesis, was observed. We also identified six genes committed to the elimination of free radical species that are dramatically down-regulated in the CL of WT animals with respect to $PGF_{2\alpha}$ receptor knockout mice. Similar changes in the expression of steroidogenic and antioxidant genes were found in the CL of WT animals between days 15 and 19 of pregnancy. It is proposed that an increase in the androgen/estrogen biosynthesis ratio, along with a significantly reduced expression of free radical scavenger proteins, may play an important role in the luteolytic process.

Introduction

Luteal cell death plays a key role in the regulation of the reproductive process in all mammals. Because of the negative effect of luteal progesterone on the secretion of gonadotrophin hormones, the demise of the corpus luteum (CL) is essential for the initiation of a new reproductive cycle. In species such as rats, mice, and goats, where the CL is the only source of progesterone throughout pregnancy, luteolysis is also necessary for parturition to occur. Prostaglandin (PG) $F_{2\alpha}$ plays a key role in the initiation of labor and parturition in rodents, and it is vital for the induction of the luteolytic process. The luteolytic effect of $PGF_{2\alpha}$ was first reported in 1969 by Pharriss and Wyngarden [1]. Later in the last century, it was demonstrated that mice rendered deficient for the $PGF_{2\alpha}$ receptor gene do not show the normal pre-partum drop in progesterone and, as a consequence, do not give birth [2]. Despite this dramatic phenotype, $PGF_{2\alpha}$ receptor knockout mice have a normal gestation, and normal fetuses can be rescued by cesarean or ovariectomy, indicating that the primary role of $PGF_{2\alpha}$ is limited to the days before parturition and its principal target is the ovary [2]. Subsequently, researchers demonstrated that the CL of $PGF_{2\alpha}$ receptor knockout mice fails to express the enzyme 20α-hydroxysteroid dehydrogenase (E.C. 1.1.1.149) at the end of pregnancy, resulting in sustained levels of progesterone in circulation [3]. This evidence indicates that, in rodents, the CL is the main ovarian structure affected by $PGF_{2\alpha}$ at the end of pregnancy. Whether the expression of other steroidogenic enzymes, besides 20α-hydroxysteroid dehydrogenase, is affected by the lack of $PGF_{2\alpha}$ receptor expression is not known.

It is now firmly established that the CL produces significant amounts of reactive oxygen species (ROS) at regression. It has been shown that hydrogen peroxide and lipid peroxides are generated during luteolysis associated with ascorbic acid and α-tocopherol depletion [4-6]. There is an increase in superoxide anion production in luteal membranes after challenge *in vivo* with $PGF_{2\alpha}$ [7], or in isolated luteal cells treated with $PGF_{2\alpha}$ [8-10]. However, whether reactive oxygen species mediate the action of $PGF_{2\alpha}$ or are a consequence of luteal regression is an unresolved question. Similarly, it is not known

whether changes in the expression of genes involved in the metabolism of ROS take place during the luteolytic process.

The mechanisms by which $PGF_{2\alpha}$ affects luteal function have been extensively studied and reviewed [11]; however, the effects of this hormone on luteal gene transcription at the end of pregnancy in rodents have only lately begun to be studied. For instance, although early experiments clearly established that $PGF_{2\alpha}$ increases 20α-hydroxysteroid dehydrogenase activity [12], the discovery that this effect is due to the stimulation of the *Akr1c18* gene was only recently published [3, 13]. A gene expression profile of the rat corpus luteum has been previously reported [13]. In that report, rats on day 19 of pregnancy were treated with $PGF_{2\alpha}$ or vehicle and the expression of several genes was accessed 24 hours afterward. Although this is a valid model in which to study the effect of $PGF_{2\alpha}$ on luteal gene expression, it does not represent a physiological situation, and questions are raised regarding dose and time of treatment. In addition, only a limited number of genes directly involved in luteal function were included. As mentioned before, gestation proceeds without any alteration in $PGF_{2\alpha}$ receptor knockout mice, suggesting normal luteal function. Strikingly, the corpus luteum of these animals fails to undergo luteolysis, and luteal function persists beyond the normal period of pregnancy. The aim of this investigation was to profile gene expression in the CL of wild type and $PGF_{2\alpha}$ receptor knockout mice on day 19 of pregnancy. In this model, the genomic effects of physiological endogenous levels of $PGF_{2\alpha}$ were studied at the time when the normal luteolytic process takes place. We report here changes observed in the expression of genes involved in steroidogenesis and ROS metabolism.

Materials and Methods

Animals

$PGF_{2\alpha}$ receptor knockout mice with a mixed genetic background of 129/Ola and C57BL/6 strains were used [2]. Wild-type and $PGF_{2\alpha}$ receptor knockout mice were maintained at 23°C under a 12-hour light cycle. Virgin females (9 to 12 weeks of age) housed overnight with males were checked the following morning for vaginal plugs. The day the plug was found was counted as day 1 of pregnancy. Wild-type animals were killed in the morning of days 15 or 19 of pregnancy, whereas CL of $PGF_{2\alpha}$ receptor knockout mice were obtained only in the morning of day 19 of pregnancy. Animal care and handling conformed to the National Institutes of Health (NIH) guidelines for animal research. The experimental protocol was approved by the Yale University Animal Resources Center.

Affymetrix Gene Chip Analysis

We utilized the mouse 430 2.0 chip array from Affymetrix. Total RNA CL was isolated using TRIzol reagent and further purified using the RNeasy kit (Qiagen, Valencia, CA). The quality of total RNA was evaluated by A260/A280 ratio and by electrophoresis. Preparation of labeled cRNA for hybridization onto Affymetrix GeneChips was performed following the recommended Affymetrix protocol. Double-stranded cDNA was synthesized from 6 μg of total RNA by using The Superscript Choice System (Invitrogen, Carlsbad, CA), with an HPLC-purified oligomer (dT) 24 primer containing a T7 RNA polymerase promoter sequence (Genset, La Jolla, CA). The second cDNA strand was synthesized through the use of *E. coli* DNA polymerase I, RNase H, and DNA ligase. The cDNA was precipitated with ethanol and resuspended in RNase-free water. Labeled cRNA was generated from cDNA by *in vitro* transcription, using a Bioarray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY), following the manufacturer's instructions and incorporating Cy5-UTP. Labeled cRNA was purified using an RNeasy column prior to fragmenting to a size of 35-200 bases by incubating at 94 °C for 35 minutes in fragmentation buffer (40 μM Tris-acetate, pH 8.1, 100 μM potassium acetate, 30 μM magnesium acetate).