

## Statistical analysis

Student's *t*-tests were used to determine the statistical significance of differences between various data sets. Results are expressed as the means  $\pm$  SEM. Statistical significance was assumed when  $p < 0.05$ .

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## References

- Metcalfe, D. D., Baram, D. and Mekori, Y. A., Mast cells. *Physiol. Rev.* 1997. 77: 1033–1079.
- Costa, J. J., Weller, P. F. and Galli, S. J., The cells of the allergic response. Mast cells, basophils, and eosinophils. *JAMA* 1997. 278: 1815–1822.
- Robbie-Ryan, M. and Brown, M. A., The role of mast cells in allergy and autoimmunity. *Curr. Opin. Immunol.* 2002. 14: 728–733.
- Kawakami, T. and Galli, S. J., Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol.* 2002. 2: 773–786.
- Siraganian, R. P., Mast cell signal transduction from the high-affinity IgE receptor. *Curr. Opin. Immunol.* 2003. 15: 639–646.
- Hsu, C. and MacGlashan, Jr. D., IgE antibody up-regulates high affinity IgE binding on murine bone marrow derived mast cells. *Immunol. Lett.* 1996. 52: 129–134.
- Yamaguchi, M., Lantz, C. S., Oettgen, H. C., Katona, I. M., Fleming, T., Yano, K., Miyajima, I., Kinet, J.-P. and Galli, S. J., IgE enhances mouse mast cell Fc $\epsilon$ RI expression *in vitro* and *in vivo*: evidence for a novel amplification mechanism in IgE-dependent reactions. *J. Exp. Med.* 1997. 185: 663–672.
- Asai, K., Kitaura, J., Kawakami, Y., Yamagata, N., Tsai, M., Carbone, D. P., Liu, F., Galli, S. J. and Kawakami, T., Regulation of mast cell survival by IgE. *Immunity* 2001. 14: 791–800.
- Kalesnikoff, J., Huber, M., Lam, V., Damen, J. E., Zang, J., Shiraganian, R. P. and Krystal, G., Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* 2001. 14: 801–811.
- Kitaura, J., Song, J., Tsai, M., Asai, K., Maeda-Yamamoto, M., Mocsai, A., Kawakami, Y., Liu, F.-T., Lowell, C. A., Barisas, B. G., Galli, S. J. and Kawakami, T., Evidence that IgE molecules mediate a spectrum of effects on mast cell survival and activation via aggregation of the Fc $\epsilon$ RI. *Proc. Natl. Acad. Sci. USA* 2003. 100: 12911–12916.
- Tanaka, S., Takasu, Y., Mikura, S., Satoh, N. and Ichikawa, A., Antigen-independent induction of histamine synthesis by immunoglobulin E in mouse bone marrow derived mast cells. *J. Exp. Med.* 2002. 196: 229–235.
- Lam, V., Kalesnikoff, J., Lee, C. W., Hernandez-Hanson, V., Wilson, B. S., Oliver, J. M. and Krystal, G., IgE alone stimulates mast cell adhesion to fibronectin via pathways similar to those used by IgE + antigen but distinct from those used by steel factor. *Blood* 2003. 102: 1405–1413.
- Gonzalez-Espinosa, C., Odom, S., Olivera, A., Hobson, J. P., Martinez, M. E. C., Oliveira-dos-Santos, A., Barra, L., Splegel, S., Penninger, J. M. and Rivera, J., Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells. *J. Exp. Med.* 2003. 197: 1453–1465.
- Huber, M., Helgason, C. D., Damen, J. E., Liu, L., Humphries, R. K. and Krystal, G., The src homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation. *Proc. Natl. Acad. Sci. USA* 1998. 95: 11330–11335.
- Pribluda, V. S. and Metzger, H., Calcium-independent phosphoinositide breakdown in rat basophilic leukemia cells. *J. Biol. Chem.* 1987. 262: 11449.
- Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallman, T. J., Jacob, R., Jaxa-Camiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E. and Rink, T. J., SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.* 1990. 271: 515–522.
- Hoth, M. and Penner, R., Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 1992. 355: 353–356.
- Venkatachalam, K., van Rossum, D. B., Patterson, R. L., Ma, H.-T. and Gill, D. L., The cellular and molecular basis of store-operated calcium entry. *Nat. Cell Biol.* 2002. 4: E263–E272.
- Prakriya, M. and Lewis, R. S., CRAC channels: activation, permeation, and the search for a molecular identity. *Cell Calcium* 2003. 33: 311–321.
- Mahmoud, S. F. and Fewtrell, C., Microdomains of high calcium are not required for exocytosis in RBL-2H3 mucosal mast cells. *J. Cell. Biol.* 2001. 153: 339–349.
- Schindl, R., Kahr, H., Graz, I., Groschner, K. and Romanin, C., Store depletion-activated CaT1 currents in rat basophilic leukemia mast cells are inhibited by 2-aminoethoxydiphenyl borate. *J. Biol. Chem.* 2002. 277: 26950–26958.
- Parekh, A. B. and Penner, R., Store depletion and calcium influx. *Physiol. Rev.* 1997. 77: 901–930.
- Strübing, C., Krapivinsky, G., Krapivinsky, L. and Clapham, D. E., TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* 2001. 29: 645–655.
- Hofmann, T., Schaefer, M., Schultz, G. and Gudermann, T., Subunit composition of mammalian transient receptor potential channels in living cells. *Proc. Natl. Acad. Sci. USA* 2002. 99: 7461–7466.
- Timmerman, L. A., Clipstone, N. A., Ho, S. N., Northrop, J. P. and Crabtree, G. R., Rapid shuttling of NF-AT in discrimination of Ca<sup>2+</sup> signals and immunosuppression. *Nature* 1996. 383: 837–840.
- Pandy, V., Mihara, S., Fensome-Green, A., Bolsover, S. and Cockcroft, S., Monomeric IgE stimulates NFAT translocation into the nucleus, a rise in cytosolic Ca<sup>2+</sup>, degranulation, and membrane ruffling in the cultured rat basophilic leukemia-2H3 mast cell line. *J. Immunol.* 2004. 172: 4048–4058.
- James, L. C., Roversi, P. and Tawfik, D. S., Antibody multispecificity mediated by conformational diversity. *Science* 2003. 299: 1362–1367.
- Coyle, A. J., Wagner, K., Bertrand, C., Tsuyuki, S., Bews, J. and Heusser, C., Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody. *J. Exp. Med.* 1996. 183: 1303–1310.
- Tanaka, S., Deai, K., Konomi, A., Takahashi, K., Yamane, H., Sugimoto, Y. and Ichikawa, A., Expression of L-histidine decarboxylase in granules of elicited mouse polymorphonuclear leukocytes. *Eur. J. Immunol.* 2004. 34: 1472–1482.
- Shiraishi, M., Hirasawa, N., Oikawa, Y., Kobayashi, Y. and Ohuchi, K., Analysis of histamine-producing cells at the late phase of allergic inflammation in rats. *Immunology* 2000. 99: 600–606.
- Bryce, P. J., Miller, M. L., Miyajima, I., Tsai, M., Galli, S. J. and Oettgen, H. C., Immune sensitization in the skin is enhanced by antigen-independent effects of IgE. *Immunity* 2004. 20: 381–392.
- Safina, F., Tanaka, S., Inagaki, M., Tsuboi, K., Sugimoto, Y. and Ichikawa, A., Expression of L-histidine decarboxylase in mouse male germ cells. *J. Biol. Chem.* 2002. 277: 14211–14215.
- Gryniewicz, G., Penie, M. and Tsien, R. Y., A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 1985. 260: 3440–3450.
- Inagaki, N., Kawasaki, H., Ueno, M., Nagai, H. and Koda, A., Potentiation of antigen-induced histamine release from rat peritoneal mast cells through a direct interaction between mast cells and non-mast cells. *Life Sci.* 1994. 54: 1403–1409.

Research

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## Sensitization of TRPV1 by EP<sub>1</sub> and IP reveals peripheral nociceptive mechanism of prostaglandins

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### Abstract

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and prostaglandin I<sub>2</sub> (PGI<sub>2</sub>) 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<sub>2</sub> or PGI<sub>2</sub> increased or sensitized TRPV1 responses through EP<sub>1</sub> 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<sub>2</sub> or PGI<sub>2</sub>, 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<sub>4</sub> and IP receptors upon exposure to PGE<sub>2</sub> and PGI<sub>2</sub>, respectively. Both PGE<sub>2</sub>-induced thermal hyperalgesia and inflammatory nociceptive responses were diminished in TRPV1-deficient mice and EP<sub>1</sub>-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<sub>1</sub> or IP activation might be one important mechanism underlying the peripheral nociceptive actions of PGE<sub>2</sub> or PGI<sub>2</sub>.

### 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  $> \sim 43^\circ C$ ), 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].

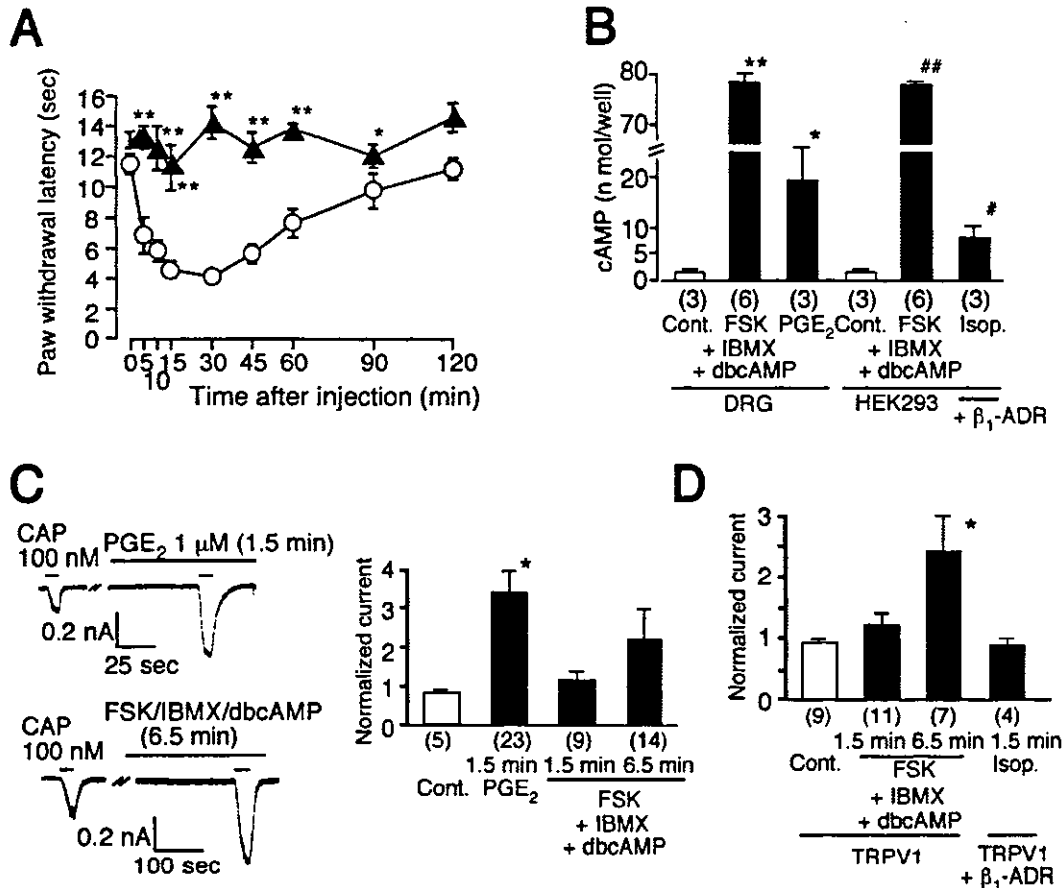
Recently, we reported that inflammatory mediators such as ATP, bradykinin and trypsin or trypsinase potentiate TRPV1 activity in a PKC-dependent manner [16-18], and identified two target serine residues in TRPV1 as substrates for PKC-dependent phosphorylation [19]. On the other hand, there are several 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<sup>-/-</sup>) mice.  $PGE_2$  (500 pmol/20  $\mu L$ ) produced a sig-

nificant 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<sup>-/-</sup> 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  $\mu M$ ) in 19 of 23 cells as previously reported [21] (Figure 1C) ( $3.36 \pm 0.55$  fold increase,  $n = 23$  for  $PGE_2$  (+);  $0.78 \pm 0.08$  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  $\mu M$ ), 3-isobutyl-1-methylxanthine (IBMX, 1 mM) and dibutyryl-cAMP (dbcAMP, 3 mM) for the same time period (1.5 min) ( $1.15 \pm 0.20$  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 ( $2.15 \pm 0.77$  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,  $1.20 \pm 0.19$  fold increase,  $n = 11$ , and treatment with isoproterenol (Isop.) in cells expressing both TRPV1 and mouse  $\beta_1$ -adrenergic receptors ( $\beta_1$ -ADR),  $0.83 \pm 0.12$  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 ( $2.39 \pm 0.60$  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<sub>2</sub> with TRPV1 in mice. (A) PGE<sub>2</sub>-induced thermal hyperalgesia in TRPV1<sup>+/+</sup> mice (○, n = 6) or TRPV1<sup>-/-</sup> mice (▲, n = 6). Reduction of paw withdrawal latency (thermal hyperalgesia) by intraplantar PGE<sub>2</sub> (500 pmol/ 20 μL) injection was significantly diminished in TRPV1<sup>-/-</sup> mice. \* p < 0.05, \*\* p < 0.01 vs. TRPV1<sup>+/+</sup> 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<sub>2</sub> (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<sub>2</sub> (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<sub>h</sub>): -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<sub>1</sub>-adrenergic receptors (β<sub>1</sub>-ADR). Numbers in parenthesis indicate cells tested. V<sub>h</sub>: -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  $\beta_1$ -ADR [24].

#### **PGE<sub>2</sub> increases TRPV1 activity through EP<sub>1</sub> receptors**

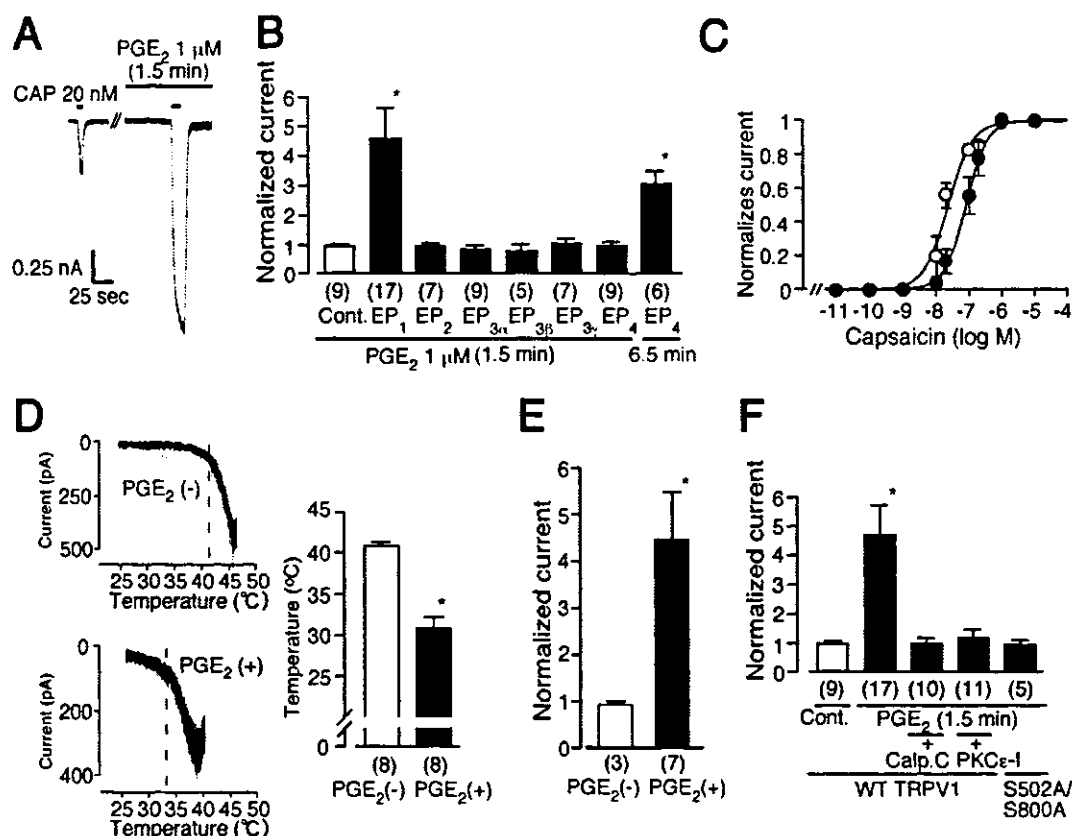
To explore the mechanism underlying the PKA-independent PGE<sub>2</sub> (1.5 min)-induced potentiation of the capsaicin-evoked responses observed in DRG neurons, we first examine the effects of PGE<sub>2</sub> on capsaicin-activated currents in HEK293 cells expressing TRPV1 and each EP receptor. PGE<sub>2</sub> (1  $\mu$ M, 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<sub>1</sub> (0.90  $\pm$  0.04 fold increase, n = 9 for control (Cont.); 4.60  $\pm$  1.03 fold, n = 17 for PGE<sub>2</sub>, 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  $\pm$  0.09 fold increase, n = 7; 0.77  $\pm$  0.13, n = 9; 0.72  $\pm$  0.24, n = 5; 0.98  $\pm$  0.18, n = 7; 0.89  $\pm$  0.15, n = 9 for EP<sub>2</sub>, EP<sub>3a</sub>, EP<sub>3b</sub>, EP<sub>3c</sub>, or EP<sub>4</sub>, respectively) (Figure 2B). Protracted (6.5 min) treatment with PGE<sub>2</sub> caused a significant increase in capsaicin-activated currents in cells expressing TRPV1 and EP<sub>4</sub>, a phenomenon like that observed following treatment with a mixture of FSK, IBMX and dbcAMP (3.03  $\pm$  0.48 fold increase, n = 6, p < 0.05 vs. Cont.) (Figure 2B), suggesting that the EP<sub>4</sub> 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<sub>2</sub> 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<sub>2</sub>. The currents were normalized to the maximal current produced by 1  $\mu$ M capsaicin in each cell. Maximal current in the presence of PGE<sub>2</sub> was almost the same as that in the absence of PGE<sub>2</sub>. The resultant dose-response curves clearly demonstrate that PGE<sub>2</sub> enhances capsaicin action on TRPV1 by lowering EC<sub>50</sub> values without altering maximal responses (EC<sub>50</sub> from 81.0 nM to 27.6 nM) (Figure 2C). We next examined the effects of PGE<sub>2</sub> on the thermal sensitivity of TRPV1. When temperature ramps were applied to HEK293 cells expressing both TRPV1 and EP<sub>1</sub> in the absence of PGE<sub>2</sub>, heat-evoked currents developed at 40.7  $\pm$  0.3 °C (n = 8) (Figure 2D). In contrast, the temperature threshold for TRPV1 activation was significantly reduced to 30.6  $\pm$  1.1 °C in the presence of PGE<sub>2</sub> (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<sub>2</sub> was observed for proton (pH 6.2)-evoked TRPV1 current responses (0.91  $\pm$  0.06 fold increase, n = 3 for control; 4.47  $\pm$  1.09 fold, n = 7 for PGE<sub>2</sub>, 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<sub>2</sub> through EP<sub>1</sub> 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  $\pm$  0.8 °C, n = 4), suggesting different actions on TRPV1 by PKA and PKC.

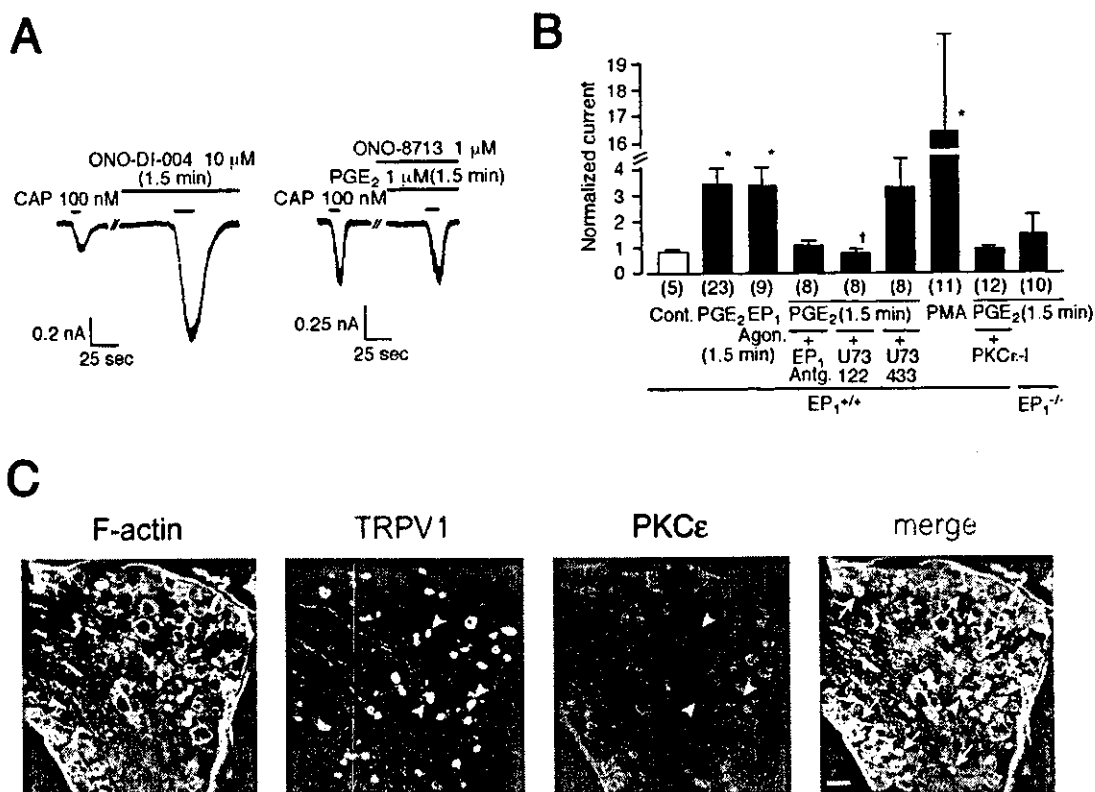
The signaling pathway downstream of EP<sub>1</sub> remains to be clarified. We have reported that G<sub>q/11</sub>-coupled metabotropic receptor activation such as ATP (P2Y), bradykinin (B2) and proteinase-activated receptor 2 (PAR2) receptors causes potentiation or sensitization of TRPV1 through the 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<sub>1</sub>. When calphostin C (Calp.C), a specific PKC inhibitor, was added to the pipette solution, the effect of PGE<sub>2</sub> was almost completely inhibited (0.92  $\pm$  0.15 fold increase, n = 10) (Figure 2F). Similarly, a PKC $\epsilon$  translocation inhibitor (PKC $\epsilon$ -I) abolished the potentiation of TRPV1 response by PGE<sub>2</sub> (1.11  $\pm$  0.25 fold increase, n = 11) (Figure 2F). These data suggest that PGE<sub>2</sub>-induced potentiation of TRPV1 responsiveness develops through activation of PKC $\epsilon$ . To further confirm the involvement of PKC-dependent phosphorylation, PGE<sub>2</sub> 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<sub>2</sub> treatment of cells expressing S502A/S800A (0.85  $\pm$  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  $\pm$  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<sub>1</sub> receptors in mouse**

To examine the involvement of EP<sub>1</sub> in PGE<sub>2</sub> (1.5 min)-induced potentiation of capsaicin-evoked response in native neurons, we used a specific EP<sub>1</sub> agonist, ONO-DI-004 [27], and a specific EP<sub>1</sub> 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<sub>2</sub> (3.36  $\pm$  0.68 fold

**Figure 2**

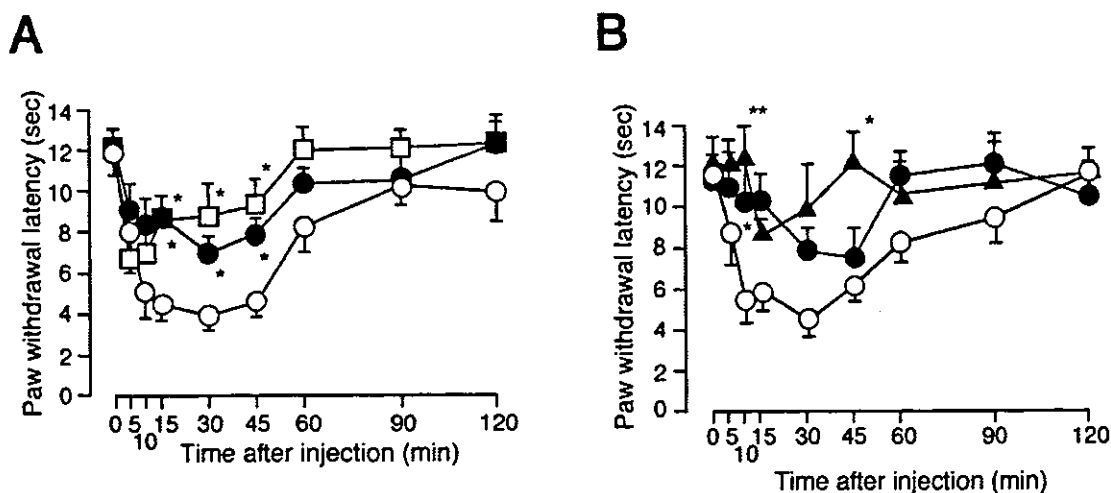
PGE<sub>2</sub> increases TRPV1 activity through EP<sub>1</sub> receptors in a PKC-dependent manner in HEK293 cells. (A) and (B) Treatment with PGE<sub>2</sub> (1.5 min) potentiates capsaicin-evoked responses in cells expressing rat TRPV1 with mouse EP<sub>1</sub> receptors, but not with other mouse EP receptors. Cells were pretreated with PGE<sub>2</sub> (1 μM) for 1.5 or 6.5 min before second capsaicin (20 nM) application. V<sub>h</sub>: -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<sub>2</sub>. Currents were normalized to the current maximally activated by 1 μM capsaicin in the absence of PGE<sub>2</sub>. Figure shows averaged data fitted with the Hill equation. EC<sub>50</sub> = 81.0 nM and Hill coefficient = 1.33 in the absence of PGE<sub>2</sub>. EC<sub>50</sub> = 27.6 nM and Hill coefficient = 1.01 in the presence of PGE<sub>2</sub>. Data were obtained from 54 different cells. (D) Temperature response profiles in the absence (upper) and presence (lower) of PGE<sub>2</sub> (left). Temperature threshold for TRPV1 activation in the presence of PGE<sub>2</sub> (30.6 ± 1.1 °C) was significantly lower than that in the absence of PGE<sub>2</sub> (40.7 ± 0.3 °C) (right). \* p < 0.05 vs. PGE<sub>2</sub> (-). Numbers in parenthesis indicate cells tested. (E) Proton-evoked TRPV1 responses were significantly potentiated by PGE<sub>2</sub> (1 μM). \* p < 0.01 vs. PGE<sub>2</sub> (-). (F) PKC-dependent pathway is involved in the PGE<sub>2</sub> (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<sub>h</sub>: -60 mV.



**Figure 3**  
**EP<sub>1</sub> receptor involvement in PGE<sub>2</sub> (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<sub>1</sub> agonist, ONO-DI-004 (10 μM, 1.5 min), and reverse of the PGE<sub>2</sub> (1.5 min)-induced potentiation by a specific EP<sub>1</sub> antagonist, ONO-8713 (1 μM). V<sub>h</sub>: -60 mV. **(B)** Effects of PGE<sub>2</sub> (1 μM), ONO-DI-004 (EP<sub>1</sub> Agon., 10 μM), PGE<sub>2</sub> plus ONO-8713 (EP<sub>1</sub> Antg., 1 μM), PGE<sub>2</sub> plus U73122 (3 μM), PGE<sub>2</sub> plus U73343 (3 μM), phorbol 12-myristate 13-acetate (PMA, 100 nM) or PGE<sub>2</sub> plus PKCε-I (200 μM) on capsaicin-activated currents in DRG neurons from wild type (EP<sub>1</sub><sup>+/+</sup>) mice, and effects of PGE<sub>2</sub> on capsaicin-activated currents in DRG neurons from EP<sub>1</sub><sup>-/-</sup> 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<sub>2</sub>, n = 23, p < 0.05 vs. control (Cont.); 3.30 ± 0.68 fold for ONO-DI-004 (EP<sub>1</sub> Agon.), n = 9, p < 0.05 vs. Cont.) (Figures 3A left and 3B). Furthermore, potentiation of the capsaicin-activated currents by PGE<sub>2</sub> was inhibited in the presence of ONO-8713 (EP<sub>1</sub> Antg., 1.00 ± 0.17 fold increase, n = 8) (Figures 3A right and 3B). These results indicate that PGE<sub>2</sub> (1.5 min)-induced potentiation of the capsaicin-activated current responses occurs

through EP<sub>1</sub> receptors in DRG neurons. To confirm the involvement of PKC-dependent events downstream of PGE<sub>2</sub> effects in DRG neurons, we first examined the effects of a specific phospholipase C (PLC) inhibitor, U73122 (3 μM). PGE<sub>2</sub>-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**

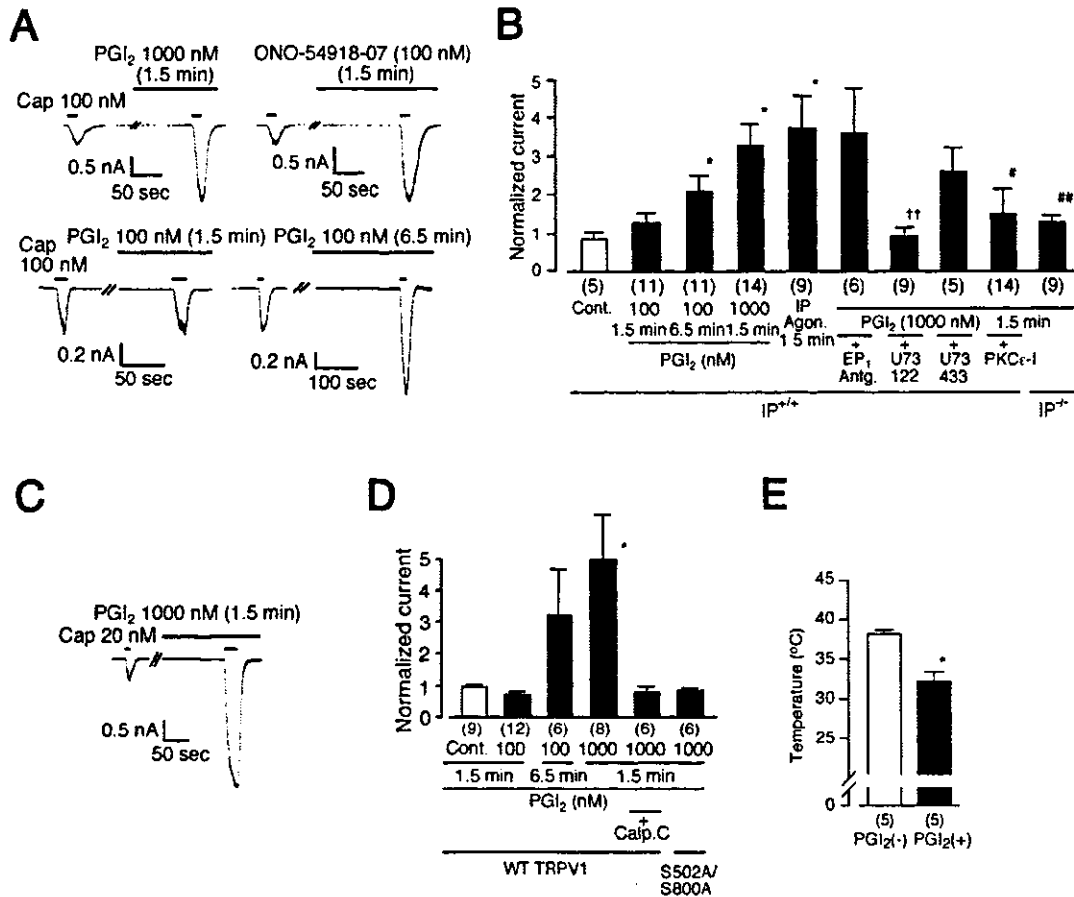
Interaction between TRPV1 and EP<sub>1</sub> receptors in a behavioral level. (A) PGE<sub>2</sub>-induced thermal hyperalgesia in wild type mice with (n = 6) or without (n = 6) pretreatment (ONO-8713, 500 pmol/ 20 µL), or in EP<sub>1</sub><sup>-/-</sup> mice (n = 6). \* p < 0.05 vs. wild type mice. (B) 10% Mustard oil-induced thermal hyperalgesia in wild type mice (n = 12), TRPV1<sup>-/-</sup> mice (n = 6) or EP<sub>1</sub><sup>-/-</sup> 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<sub>2</sub> failed to potentiate the capsaicin-activated currents when PKC $\epsilon$ -1 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<sub>1</sub> receptors, DRG neurons of EP<sub>1</sub> deficient mice (EP<sub>1</sub><sup>-/-</sup>) were subjected to patch-clamp analysis. PGE<sub>2</sub> failed to potentiate capsaicin-activated currents in the DRG neurons from EP<sub>1</sub><sup>-/-</sup> mice (1.45 ± 0.70 fold increase, n = 10) (Figure 3B). Functional interaction of PKC $\epsilon$  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-PKC $\epsilon$  antibody, supporting the TRPV1 activation pathway through PKC $\epsilon$  (Figure 3C).

We next investigated the involvement of EP<sub>1</sub> receptors in PGE<sub>2</sub>-induced thermal hyperalgesia at the behavioral level. PGE<sub>2</sub>-induced thermal hyperalgesia was significantly diminished at 15 to 45 min after injection in EP<sub>1</sub><sup>-/-</sup> mice (Figure 4A), relative to that observed in wild type

mice. The involvement of EP<sub>1</sub> receptors in the PGE<sub>2</sub>-induced hypersensitivity was supported by another behavioral analysis in which PGE<sub>2</sub> caused less reduction of paw withdrawal latency in wild type mice pretreated with a specific EP<sub>1</sub> antagonist (500 pmol/ 20 µL) than in vehicle control (Figure 4A). These results suggest that a PKC-dependent pathway downstream of EP<sub>1</sub> activation is mainly involved in PGE<sub>2</sub>-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<sub>1</sub> 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<sup>-/-</sup> mice and EP<sub>1</sub><sup>-/-</sup> mice. Thus, these data show that EP<sub>1</sub> contributes to inflammatory nociception in mice and support the hypothesis.



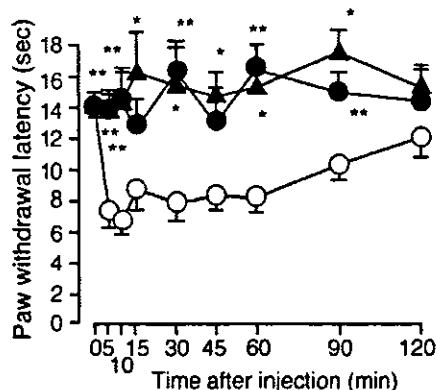


**Figure 5**

PGI<sub>2</sub> causes potentiation or sensitization of TRPV1 through mainly through PKC activation. (A) Representative traces of potentiation of capsaicin-activated currents by PGI<sub>2</sub> (1000 nM, 1.5 min), a specific IP agonist, ONO-54918-07 (100 nM, 1.5 min) or PGI<sub>2</sub> (100 nM, 6.5 min), but not by PGI<sub>2</sub> (100 nM, 1.5 min) in mouse DRG neurons. V<sub>h</sub>: -60 mV. (B) Effects of treatments (1.5 or 6.5 min) with PGI<sub>2</sub> (100 or 1000 nM), ONO-54918-07 (IP Agon., 100 nM), PGI<sub>2</sub> (1000 nM) plus ONO-8713 (EP<sub>1</sub> Antg., 1 μM), PGI<sub>2</sub> (1000 nM) plus U73122 (3 μM), PGI<sub>2</sub> (1000 nM) plus U73343 (3 μM) or PGI<sub>2</sub> (1000 nM) plus PKCε-I (200 μM) on capsaicin-activated currents in DRG neurons from wild type (IP<sup>+/+</sup>) mice, and effects of PGI<sub>2</sub> on capsaicin-activated currents in DRG neurons from IP-deficient (IP<sup>-/-</sup>) 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<sub>2</sub> (1000 nM, 1.5 min) in DRG neurons from IP<sup>+/+</sup> mice. Numbers in parenthesis indicate cells tested. (C) A representative trace of potentiation of capsaicin-activated currents by PGI<sub>2</sub> (1000 nM, 1.5 min) in HEK293 cells expressing both TRPV1 and IP. V<sub>h</sub>: -60 mV. (D) Effects of treatments (1.5 or 6.5 min) with PGI<sub>2</sub> (100 or 1000 nM) or PGI<sub>2</sub> (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<sub>2</sub> (32.2 ± 1.2°C) was significantly lower than that in the absence of PGI<sub>2</sub> (38.2 ± 0.5°C) in HEK293 cells expressing rat TRPV1 and IP. \* p < 0.01 vs. PGI<sub>2</sub> (-).

### Sensitization of TRPV1 by IP receptors

In order to determine whether the observed responses are specific to PGE<sub>2</sub>, we extended our analysis to PGI<sub>2</sub> whose receptor has been reported to be involved in nociception [8]. We first examined the effects of PGI<sub>2</sub> on capsaicin-activated currents in mouse DRG neurons. PGI<sub>2</sub> pretreatment (1000 nM, 1.5 min) potentiated capsaicin (100 nM)-activated currents ( $3.23 \pm 0.55$  fold increase,  $n = 14$  or  $0.78 \pm 0.08$  fold,  $n = 5$  with or without (Cont.) PGI<sub>2</sub>, respectively;  $p < 0.05$ ) whereas at 100 nM, PGI<sub>2</sub> (1.5 min) showed no such effects ( $1.24 \pm 0.22$  fold,  $n = 11$ ) (Figures 5A and 5B). On the other hand, long (6.5 min) treatment with PGI<sub>2</sub> (100 nM) caused significant potentiation of capsaicin-activated currents as in the treatment with a mixture of FSK, IBMX and dbcAMP ( $2.06 \pm 0.54$  fold increase,  $n = 11$ ,  $p < 0.05$  vs. Cont.) (Figures 5A and 5B). The potentiation effects of PGI<sub>2</sub> 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.,  $3.71 \pm 0.81$  fold increase,  $n = 9$ ,  $p < 0.05$  vs. Cont.) (Figures 5A and 5B) although PGI<sub>2</sub> is known to cross react with some EP receptors [2]. The fact that a specific EP<sub>1</sub> antagonist, ONO-8713 failed to prevent the PGI<sub>2</sub>-induced potentiation (+EP<sub>1</sub> Antg.,  $3.55 \pm 1.17$  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<sub>2</sub>-induced potentiation of capsaicin-activated currents was further supported by the ineffectiveness of PGI<sub>2</sub> on DRG neurons from IP-deficient mice (IP<sup>-/-</sup>) ( $1.25 \pm 0.16$  fold increase,  $n = 9$ ,  $p < 0.01$  vs. 1000 nM of PGI<sub>2</sub>) (Figure 5B). It has been reported that low concentrations of PGI<sub>2</sub> stimulate Gs protein coupled to IP receptors whereas high concentrations of PGI<sub>2</sub> stimulate not only Gs but also Gq [32]. This property might explain the dose-dependent effects of PGI<sub>2</sub> on capsaicin-activated currents: PKC-dependent sensitization of TRPV1 occurs downstream of Gq-coupled IP receptor activation at high concentrations (1000 nM) of PGI<sub>2</sub> (1.5 min) while long (6.5 min) treatment with low concentrations (100 nM) of PGI<sub>2</sub> causes potentiation of TRPV1 activity through Gs activation. To test this hypothesis, PGI<sub>2</sub> (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<sub>2</sub> (1.5 min) failed to potentiate the currents whereas U73343 exhibited no such effects, indicating the involvement of PLC activation in the potentiating process ( $0.97 \pm 0.40$  fold increase,  $n = 9$  for U73122,  $2.58 \pm 0.66$  fold,  $n = 5$  for U73343,  $p < 0.05$ ) (Figure 5B). Furthermore, PKC $\epsilon$ -I included in the pipette solution almost completely blocked the PGI<sub>2</sub> (1000 nM)-induced potentiation ( $1.49 \pm 0.60$  fold increase,  $n = 14$ ), suggesting the involvement of PKC $\epsilon$ -dependent regulation mechanism (Figure 5B).



**Figure 6**

Interaction between TRPV1 and IP receptors at a behavioral level. PGI<sub>2</sub>-induced thermal hyperalgesia in wild type mice (○,  $n = 6$ ), TRPV1<sup>-/-</sup> mice (▲,  $n = 6$ ) or IP<sup>-/-</sup> mice (□,  $n = 6$ ). Thermal hyperalgesia by intraplantar PGI<sub>2</sub> (500 pmol/ 20 μL) injection was significantly diminished in TRPV1<sup>-/-</sup> mice and IP<sup>-/-</sup> mice. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. wild type mice.

Dose-dependent PGI<sub>2</sub> (1.5 min)-induced potentiation of capsaicin-activated currents was also observed in HEK293 cells expressing TRPV1 and IP receptors ( $0.90 \pm 0.04$  fold increase,  $n = 9$  without PGI<sub>2</sub> (Cont.);  $0.68 \pm 0.08$  fold,  $n = 12$  with 100 nM of PGI<sub>2</sub>;  $0.75 \pm 0.07$  fold,  $n = 6$  with 300 nM PGI<sub>2</sub>,  $4.96 \pm 1.36$  fold,  $n = 8$  with 1000 nM of PGI<sub>2</sub>,  $p < 0.01$  vs. Cont.) (Figures 5C and 5D, and data not shown). Calp. C blocked PGI<sub>2</sub>-induced potentiation of TRPV1 currents ( $0.75 \pm 0.15$  fold increase,  $n = 6$ ) (Figure 5D). Furthermore, PGI<sub>2</sub> (1000 nM) failed to potentiate capsaicin-activated currents in HEK293 cells expressing the S502/S800 mutant ( $0.80 \pm 0.05$  fold,  $n = 6$ ) (Figure 5D). Long (6.5 min) treatment with PGI<sub>2</sub> (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 ( $3.19 \pm 1.45$  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<sub>2</sub> although both PKA-dependent and PKC-dependent pathways may contribute. The temperature threshold for TRPV1 activation was significantly reduced (from  $38.2 \pm 0.5^\circ\text{C}$ ,  $n = 5$  to  $32.2 \pm 1.2^\circ\text{C}$ ,  $n = 5$ ) in the presence of PGI<sub>2</sub>, suggesting the possibility that IP receptor activation can cause nociception at body temperature (Figure 5E). Finally, PGI<sub>2</sub>-induced thermal hyperal-

gesia observed in wild type mice disappeared almost completely in both TRPV1-deficient (TRPV1<sup>-/-</sup>) mice and IP-deficient (IP<sup>-/-</sup>) 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<sub>2</sub> and PGI<sub>2</sub>, and that TRPV1 and EP<sub>1</sub> 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<sub>2</sub> or PGI<sub>2</sub> in the context of inflammation. In the present study, 1 μM PGE<sub>2</sub> or PGI<sub>2</sub> was found to potentiate or sensitize TRPV1 activity. It is not well known how much PGE<sub>2</sub> or PGI<sub>2</sub> is released locally at the site of inflammation. However, more than micromolar-order concentrations of PGE<sub>2</sub> and PGI<sub>2</sub> have been reported to be synthesized by macrophages upon lipopolysaccharide (LPS) stimulation [33,34], suggesting that 1 μM is an attainable concentration in the context of inflammation. It has been previously reported that EP<sub>1</sub> is coupled to intracellular Ca<sup>2+</sup> mobilization in CHO cells [35]. However, the transduction events downstream of EP<sub>1</sub> signaling have been unclear. Together with a report suggesting the possible coupling of EP<sub>1</sub> with G<sub>q/11</sub>-protein [36], our data indicate that EP<sub>1</sub> receptors activate a PKC-dependent signal transduction pathway.

There has been extensive work demonstrating the activation of a PKA-dependent pathway by PGE<sub>2</sub> 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<sub>2</sub> (1.5 min)- and PGI<sub>2</sub> (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, Bhawe 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<sub>2</sub> [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<sup>2+</sup>-containing solutions, whereas we did all of our experiments under Ca<sup>2+</sup>-free conditions, to avoid Ca<sup>2+</sup>-dependent TRPV1 desensitization [43]. Potentiation of capsaicin-activated currents by PGE<sub>2</sub> 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<sup>2+</sup> 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<sub>2</sub>) 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<sub>2</sub>- and PGI<sub>2</sub>-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<sub>2</sub>- and PGI<sub>2</sub>-induced potentiation or sensitization of TRPV1 activity in mice.

The inhibition of PGE<sub>2</sub>-induced thermal hyperalgesia observed in EP<sub>1</sub><sup>-/-</sup> mice, while significant, was not very robust, compared with that in TRPV1<sup>-/-</sup> 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<sup>-/-</sup> or EP<sub>1</sub><sup>-/-</sup> 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<sup>-/-</sup> mice or EP<sub>1</sub><sup>-/-</sup> 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<sub>2</sub> and PGI<sub>2</sub> is TRPV1 as shown in our study, compounds acting on EP<sub>1</sub>, 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<sub>1</sub> or IP activation, mainly through PKC- and PKA-dependent mechanisms, might be important mechanism underlying the peripheral nociceptive actions of PGE<sub>2</sub> or PGI<sub>2</sub>.

### Methods

#### Animals

Male C57BL/6-strain mice (4 weeks, SLC, Shizuoka, Japan), EP<sub>1</sub>-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<sub>2</sub> (500 pmol/ 20 µL, Sigma) with or without ONO-8713 (500 pmol/ 20 µL), or with PGI<sub>2</sub> (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<sub>1</sub>, EP<sub>2</sub>, EP<sub>3α</sub>, EP<sub>3β</sub>, EP<sub>3γ</sub>, EP<sub>4</sub> or IP) using Lipofectamine Plus Reagent (Invitrogen). Primary cultures prepared from adult C57BL/6-strain mice, EP<sub>1</sub>-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<sub>2</sub>, 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 pre-heated 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<sup>2+</sup> [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 manufacturer'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 PKC $\epsilon$  translocation inhibitor was from Calbiochem.

#### Statistics

Values are shown as the mean  $\pm$  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.

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#### References

- 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.
- Narumiya S, Sugimoto Y, Ushikubi F: Prostanoid receptors: structures, properties, and functions. *Physiol Rev* 1999, **79**:1193-1226.
- Samad TA, Sapirstein A, Woolf CJ: Prostanoids and pain: unraveling mechanisms and revealing therapeutic targets. *Trends Mol Med* 2002, **8**:390-396.
- Breyer RM, Bagdasarian CK, Myers SA, Breyer MD: Prostanoid receptors: subtypes and signaling. *Annu Rev Pharmacol Toxicol* 2001, **41**:661-690.
- Narumiya S, FitzGerald GA: Genetic and pharmacological analysis of prostanoid receptor function. *J Clin Invest* 2001, **108**:25-30.
- 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.
- 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.
- Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, Ueno A, Ohishi S, Narumiya S: Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* 1997, **388**:678-682.
- 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.
- 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.
- Caterina MJ, Julius D: The vanilloid receptor: a molecular gateway to the pain pathway. *Annu Rev Neurosci* 2001, **24**:487-517.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Numazaki M, Tominaga T, Toyooka H, Tominaga M: Direct phosphorylation of capsaicin receptor VR1 by protein kinase C $\epsilon$  and identification of two target serine residues. *J Biol Chem* 2002, **277**:13375-13378.
- Gu Q, Kwong K, Lee LY: Ca $^{2+}$  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.
- 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.
- 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.
- Schuligoi R, Donnerer J, Amann R: Bradykinin-induced sensitization of afferent neurons in the rat paw. *Neuroscience* 1994, **59**:211-215.
- 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.
- 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, Setiadij 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. Pritchard 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. Bhawe 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, Bhawe 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. Sugiur 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. Scholtz 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 1 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.

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

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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  $20\alpha$ -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  $20\alpha$ -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  $\alpha$ -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  $\text{PGF}_{2\alpha}$  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  $\text{PGF}_{2\alpha}$  increases  $20\alpha$ -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  $\text{PGF}_{2\alpha}$  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  $\text{PGF}_{2\alpha}$  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  $\text{PGF}_{2\alpha}$  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  $\text{PGF}_{2\alpha}$  receptor knockout mice on day 19 of pregnancy. In this model, the genomic effects of physiological endogenous levels of  $\text{PGF}_{2\alpha}$  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<sub>2α</sub> receptor knockout mice with a mixed genetic background of 129/Ola and C57BL/6 strains were used [2]. Wild-type and PGF<sub>2α</sub> 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<sub>2α</sub> 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).

The arrays were hybridized for 16 hours at 45°C. After being hybridized, the arrays were washed using the fluidics station (Affymetrix, Santa Clara, CA) and scanned on a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA). Expression analysis of all experiments was performed with GeneSpring 5.1 (Silicon Genetics, Redwood City, CA) using analysis data generated by Microarray Suite software. Differential expression was defined as those transcripts that had a difference of twofold or more. Performed with GeneSpring software, the cross-gene-error model was used in combination with a one-way analysis of variance (ANOVA) (significance level set at  $p < 0.05$ ).

#### *Relative RT-PCR Analysis*

Total RNA from frozen mouse CL was isolated using Tri-Reagent following the manufacturer's instructions. One microgram of total RNA was reverse-transcribed at 42°C with the Advantage RT-for-PCR kit (Promega, Madison, WI) and later diluted to a final volume of 100  $\mu$ l. The PCR reaction mixture, containing specific oligonucleotide primers (500 pmol), deoxyribonucleotide triphosphate (150  $\mu$ M), and *Ex Taq* DNA polymerase (1 Unit) (Takara, Kyoto, Japan), was added to each tube containing 5  $\mu$ l of reverse transcription product. Each PCR reaction also included  $\beta$ -actin primers used as internal controls. Before proceeding with the semi-quantitative PCR, conditions were established such that the amplification of the products was in the exponential phase, and the assay was linear with respect to the amount of input cDNA. Band intensity was determined by using ImageJ software from NIH. Each experiment was repeated three times. A student *t* test was used to determine whether differences between groups were statistically different.

#### *Western Blotting Analysis*

Corpora lutea from wild-type mice on days 15 and 19 of pregnancy and from PGF<sub>2 $\alpha$</sub>  receptor knockout mice were homogenized in ice-cold lysis buffer (10 mM Tris-Cl, pH 8.0; 150 mM NaCl, 1% Nonidet p-40, 0.5% sodium deoxycholate, 0.1% SDS, 40  $\mu$ M PMSF, 0.3  $\mu$ M aprotinin, and 1  $\mu$ M leupeptin). This was followed by 30-min incubation on ice and centrifugation at 10,000-x g for 20 min at 4°C. The