Receptors and Signaling Mechanisms Required for Prostaglandin E\(_2\)-Mediated Regulation of Mast Cell Degranulation and IL-6 Production


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Receptors and Signaling Mechanisms Required for Prostaglandin E2-Mediated Regulation of Mast Cell Degranulation and IL-6 Production

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Mast cells are implicated in the pathogenesis of a broad spectrum of immunological disorders. These cells release inflammatory mediators in response to a number of stimuli, including IgE-Ag complexes. The degranulation of mast cells is modified by PGs. To begin to delineate the pathway(s) used by PGs to regulate mast cell function, we examined bone marrow-derived mast cells (BMMC) cultured from mice deficient in the EP1, EP2, EP3, and EP4 receptors for PGE2. Although BMMCs express all four of these PGE2 receptors, potentiation of Ag-stimulated degranulation and IL-6 cytokine production by PGE2 is dependent on the EP3 receptor. Consistent with the coupling of this receptor to Gαi, PGE2 activation of the EP3 receptor leads to both inhibition of adenylate cyclase and increased intracellular Ca2+. The magnitude of increase in intracellular Ca2+ induced by EP3 activation is similar to that observed after activation of cells with IgE and Ag. Although PGE alone is not sufficient to initiate BMMC degranulation, stimulation of cells with PGE along with PMA induces degranulation. These actions are mediated by the EP3 receptor through signals involving Ca2+ mobilization and/or decreased cAMP levels. Accordingly, these studies identify PGE2/EP3 as a proinflammatory signaling pathway that promotes mast cell activation. The Journal of Immunology, 2002, 169: 4586–4593.
The existence of multiple EP receptors coupled to different intracellular signals provides a molecular basis for the diverse physiological actions of PGE₂. However, the roles of the individual EP receptor subtypes in mediating specific actions of PGE₂ in various cell types and tissues, including mast cells, have not been well characterized. This is due in part to the lack of subtype-specific EP agonists and antagonists. Accordingly, using mouse lines deficient in the EP₁, EP₂, EP₃, or EP₄ receptors, we examined the modulation of mast cell functions and signaling by PGE₂. We find that the actions of PGE₂ in mast cells are mediated primarily through the EP₃ receptor.

Materials and Methods

Mice

The generation of mice deficient in EP₁, EP₂, EP₃, and EP₄ receptors has been previously reported (11–14). All mice used were at least 8 wk old and were bred at the University of Texas Health Science Center, Galveston, TX, and at the University of North Carolina (Chapel Hill, NC), in accordance with the Institutional Animal Care and Use Committee guidelines.

Preparation of bone marrow-derived mast cells (BMMCs)

Bone marrow was isolated from the femurs of 2- to 3-mo-old mice and placed in culture. Cells were grown for at least 4 wk in the presence of IL-3-supplemented culture medium to select for pure populations of mast cells as described previously (15). Briefly, bone marrow-derived cells were grown in RPMI medium supplemented with 10% FCS, 8% mouse IL-3 culture supplement (Collaborative Biomedical Products, Bedford, MA), 20 mM HEPES, 4 mM t-glutamine, 0.08 U/ml penicillin, 0.08 mg/ml streptomycin, 800 μM nonessential amino acids, 800 μM sodium pyruvate, 0.04 mg/ml gentamicin, and 92 μM 2-ME (BMMCs culture medium). Cell cultures were maintained in a constant environment (humidified 37°C, 5% CO₂). To deplete adherent cells such as macrophages and monocytes from culture, cells in suspension were transferred to fresh dishes a few days after harvesting and then weekly at a concentration of 10⁵-10⁶ cells/ml. Cells cultured in this manner were examined visually after fixation with Carnoy’s fixative and toluidine blue staining. Although the percentage of mast cells of all cultures was not tested before each experiment, we have since examined populations of cells cultured from two different EP₂-deficient mice, two wild-type mice, and one culture derived from each of the following: an EP₁⁻/-, an EP₃⁻/-, and an EP₄⁻/- mouse. Cultured cells were comprised of 98% toluidine blue positive staining granulocytes, regardless of the genotype.

RNA analysis of EP receptor expression

Total RNA from cultured BMMCs was obtained using RNAzol B (Teltest, Friendswood, TX) according to the manufacturer’s instructions. Total RNA (20 μg) was fractionated by gel electrophoresis under denaturing conditions (1.2% agarose/1.1% formaldehyde). The contents of the gel were then transferred for Northern blot analysis. Hybridization to [3²P]RNA probes, synthesized from BMMC RNA by reverse transcription according to the manufacture’s protocol, and PCR amplification was conducted according to the manufacturer’s protocol, and PCR amplification was conducted using the following primers: EP₁-1F (5’-GTGGCCCCTGCTCCCGAAATG-3’) and EP₁-2R (5’-GGCAAGAGGACATATGCGGAAGTT-3’). The presence of EP₁ receptor isoforms was analyzed by RT-PCR analysis using isoform-specific primers as previously described (16).

Measurement of hexosaminidase release

BMMCs from wild-type and EP₁⁻/-, EP₂⁻/-, EP₃⁻/-, and EP₄⁻/- deficient mice were loaded with murine DNP-specific IgE mAb (clone SPE-7 from Sigma-Aldrich, St. Louis, MO) overnight at a concentration of 100 ng/ml per million cells. They were washed twice with Siraganian buffer (119 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6 mM glucose, 1 mM CaCl₂, 0.4 mM MgCl₂, and 0.1% BSA, pH 7.2) to eliminate any excess Ab. BMMCs were then resuspended (5 × 10⁶ cells per 75 μl) in Siraganian buffer, transferred to 96-well microtiter plates, and preincubated for 15 min at 37°C. Cells were then exposed to 25 μl of prewarmed PGE₁ or PGE₂ (Cayman Chemicals, Ann Arbor, MI), PGE₁ or PGE₂, and PMA solutions for 20 min, followed by addition of 100 μl of Ag (PMA and DNP-human serum albumin (HSA); Sigma-Aldrich). In some experiments, Ag was added immediately after PGE₁ or PGE₂ treatment, as noted in Fig. 2B. Plates were then incubated for 30 min (humidified 37°C, 5% CO₂) followed by centrifugation at 500 × g for 5 min (4°C). A 100-μl aliquot of the supernatant was then taken and the mast cell pellets with the remaining supernatants were lysed with 100 μl of 0.2% Triton X-100. The supernatant and an aliquot of the cell lysate were then transferred to a well containing 100 μl of 1 mM p-nitrophenyl-N-acetyl-B-D-glucosaminide (Sigma-Aldrich) in citrate buffer (0.1 M citric acid, 0.1 M sodium citrate, pH 4.5). The reactions were then incubated for 1 h (humidified 37°C, 5% CO₂), then halted with the addition of 100 μl of 0.1 M Na₂CO₃/0.1 M NaHCO₃. The absorbance was read at 405 nm to measure hexosaminidase activity. Data are expressed as the percentage of released hexosaminidase relative to the total cellular hexosaminidase content.

Measurement of IL-6 production

As described above, BMMCs were loaded with IgE overnight. After a 30-min preincubation with 10 μM indomethacin to inhibit endogenous prostaglandin formation, cells were washed twice in BMMC culture medium. Cells were then resuspended at 5 × 10⁶ cells in 100 μl of fresh medium, plated in microtiter plates, and treated with Ag (50 ng/ml), PGE₂ (10 μM), or Ag and PGE₂ in a final volume of 200 μl. BMMCs were then incubated for 7 h (humidified 37°C, 5% CO₂). At the end of the incubation period, mast cells were centrifuged (1500 × g for 4 min), washed twice in calcium buffer and transferred to new wells and aliquots were stored at −80°C until assayed. Samples were diluted 10-fold in BMMC culture medium to fit within the standard curve. IL-6 content was determined by enzyme immunoassay (ELISA) (PerSeptive Biosystems, Framingham, MA) according to the manufacturer’s protocol.

Measurement of LTβ production

As described above, BMMCs were loaded with IgE overnight. BMMCs were washed twice and resuspended in fresh culture medium, and 5 × 10⁶ cells in 100 μl of medium were transferred to 96-well tissue culture plates, treated with Ag (50 ng/ml), PGE₂ (10 μM), or Ag and PGE₂, and incubated at 37°C for 20 min. After the incubation period, cells were centrifuged (800 × g for 5 min), washed in calcium buffer and transferred to new wells and stored at −80°C until assayed by ELISA according to the manufacturer’s protocol (Cayman Chemicals).

Measurement of intracellular calcium concentrations ([Ca²⁺]ᵢ)

BMMCs were loaded with murine anti-DNP IgE by overnight incubation with the mAb as described above. Cells were incubated in the presence of 10 μM indomethacin (Sigma-Aldrich) for 30 min and then transferred to calcium buffer (135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, 0.1% BSA, and 10 μM indomethacin, pH 7.4). After washing, cells were resuspended in calcium buffer (10² cells/ml) and loaded with 10 μM fura 2-aceatoxyethyl ester (fura 2-AM; Molecular Probes, Eugene, OR) for 45 min (humidified 37°C, 5% CO₂). Cells were then washed in calcium buffer and transferred to a cuvette. Fluoresce in intracellular free [Ca²⁺]ᵢ in response to the indicated treatments of Ag and PGE₂ was recorded with a luminescence spectrometer (LS50B, PerkinElmer, Wellesley, MA) at excitation wavelengths of 340/380 nm and an emission wavelength of 510 nm. The fluorescence ratio signal was calibrated by adding 1 μM ionomycin (Sigma-Aldrich) and the addition of 5 mM EGTA to determine the maximal and minimal fluorescence, respectively. After incubation with fura 2-AM, cells were then stimulated by adding 10 μM fura 2 Ca²⁺ of 224 mM according to the method previously reported (17). Stock solutions of PGE₂ and indomethacin were prepared in 100% ethanol. Fura 2-AM and ionomycin were dissolved in DMSO.

Measurement of intracellular cAMP levels ([cAMP]ᵢ)

BMMCs were washed twice with Tyrode’s solution (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.2 mM Na₂HPO₄, 12 mM NaHCO₃, 5.5 mM glucose) and resuspended at 2 × 10⁶ cells per 200 μl of solution. Cells were then transferred to a sample vial and incubated at 37°C with stirring for 1 min and then treated with PGE₂, PGE₂-prostacyclin (PGL₃), or vehicle control for 1 min. Each treatment was conducted on three different cell samples. Purification and EIA analysis of [cAMP], were then conducted according to the manufacturer’s protocol (Amersham Pharma cia, Piscataway, NJ). The triplicate data points were then used to calculate baseline levels of [cAMP], and the change in [cAMP], with results expressed as the percent of the control baseline level.
Results

EP receptors expressed by BMMCs

BMMCs were cultured from EP<sub>1</sub>-deficient (EP<sub>1</sub><sup>R<sup>−/−</sup></sup>), EP<sub>2</sub>-deficient (EP<sub>2</sub><sup>R<sup>−/−</sup></sup>), EP<sub>3</sub>-deficient (EP<sub>3</sub><sup>R<sup>−/−</sup></sup>), and EP<sub>4</sub>-deficient (EP<sub>4</sub><sup>R<sup>−/−</sup></sup>) mice. Total RNA was prepared from these cells and the level of expression of the PG receptors was examined by Northern analysis. The EP<sub>2</sub> receptor was easily detected in control BMMCs (Fig. 1, left). EP<sub>2</sub> mRNA could not be detected in either the normal or the mutant cell lines by Northern analysis of total RNA; however, RT-PCR analysis of the RNA indicated low levels of the EP<sub>2</sub> receptor transcript in BMMCs (data not shown). Expression of the EP<sub>3</sub> receptor was easily detected in wild-type BMMCs but not in the cells derived from the EP<sub>3</sub>-deficient animals (Fig. 1, center). To determine which EP<sub>3</sub> receptor splice variants are expressed by BMMCs, RT-PCR analysis was conducted using primer sets specific for each of the isoforms. After gel electrophoresis and ethidium bromide staining, PCR products corresponding to EP<sub>3</sub>α and EP<sub>3</sub>β isoforms were easily detected (data not shown). However, expression of the EP<sub>3</sub>γ isoform was not observed. In contrast, all three EP<sub>3</sub> isoforms were easily detected on similar analysis of RNA prepared from mouse kidney. By Northern analysis, high levels of EP<sub>4</sub> expression were easily detected in the BMMCs (Fig. 1, right).

Effect of PGs on mast cell degranulation

Degranulation of BMMCs was monitored by measuring the release of hexosaminidase into the culture supernatant. PGE<sub>2</sub> and PGE<sub>1</sub> bind with equal affinity to the four known EP receptors. However, PGE<sub>1</sub> has a higher affinity than PGE<sub>2</sub> for the IP prostacyclin receptor (18). Incubation of mast cells with either PGE<sub>1</sub> or PGE<sub>2</sub> alone had no effect on hexosaminidase release by wild-type mast cells (Fig. 2). By contrast, in the presence of IgE-Ag receptor complexes, PGE<sub>1</sub> and PGE<sub>2</sub> significantly augmented release of hexosaminidase in a dose-dependent manner, even at very low doses of 1 × 10<sup>−8</sup> M and across the range of Ag concentrations that were tested (Fig. 2A). No measurable difference in the relative effectiveness of PGE<sub>1</sub> and PGE<sub>2</sub> was observed. To reconcile the differing results of previous studies, which have found that PGs can inhibit or potentiate mast cell mediator release, it has been suggested that the effect might depend on the timing of the addition of this lipid mediator, relative to exposure of the cells to Ag (19). Therefore, we examined the ability of PGE<sub>1</sub> or PGE<sub>2</sub> to modulate degranulation of BMMCs when added either immediately before Ag or 20 min before stimulation with Ag. As can be seen in Fig. 2, under both experimental conditions, exposure to either PGE<sub>1</sub> or PGE<sub>2</sub> enhanced mast cell degranulation. To identify the EP receptor subtype that mediates the enhanced degranulation, we compared the effects of PGE<sub>1</sub> or PGE<sub>2</sub> in mast cell cultures prepared from each of the EP-deficient mouse lines. No difference in the response of the four different EP-deficient mast cell cultures to IgE and Ag alone was observed, suggesting that these PGE<sub>2</sub> receptors are not required for the development of these cells or for the ability of these cells to be activated via the FcεR1 receptor (Fig. 3). We next examined the ability of PGE<sub>1</sub> or PGE<sub>2</sub> to augment IgE-mediated degranulation. The enhancement of mast cell degranulation by PGE<sub>2</sub> continued to be observed on examination of mast cells prepared from EP<sub>1</sub><sup>R<sup>−/−</sup></sup> mice (Fig. 3A). Similarly, PGE<sub>2</sub> continued to augment degranulation of BMMCs that lacked the EP<sub>2</sub> receptor (Fig. 3B). Examination of BMMCs prepared from

![FIGURE 1.](image1)

Isolated total cellular RNA (20 µg) was analyzed for the presence of the EP<sub>1</sub>-receptor, EP<sub>3</sub>-receptor, and EP<sub>4</sub>-receptor mRNA transcripts by Northern blot analysis using cDNA probes specific for the EP<sub>1</sub>, EP<sub>3</sub>, and EP<sub>4</sub> receptors. The integrity and amount of the RNA samples was monitored by subsequent analysis using radiolabeled β-actin mouse probe. Expression of EP<sub>1</sub>, EP<sub>3</sub>, and EP<sub>4</sub> receptors is easily detected in wild-type BMMCs. EP<sub>1</sub>, EP<sub>3</sub>, and EP<sub>4</sub> expression cannot be detected in RNA prepared from the EP<sub>1</sub><sup>R<sup>−/−</sup></sup>, EP<sub>3</sub><sup>R<sup>−/−</sup></sup>, and EP<sub>4</sub><sup>R<sup>−/−</sup></sup> BMMCs, respectively.

![FIGURE 2.](image2)

PGE<sub>1</sub> and PGE<sub>2</sub> potentiate Ag-induced mast cell degranulation. BMMCs were treated overnight with anti-DNP IgE. In the experiment shown in A, after removal of excess Ab, degranulation was initiated by addition of the indicated amount of DNP-HSA Ag immediately after the addition of the vehicle used for preparation of PGE<sub>1</sub> and PGE<sub>2</sub> (M). In parallel, samples received Ag together with various amounts of PGE<sub>1</sub> or PGE<sub>2</sub> (ائد<sub>1</sub>) × 10<sup>−8</sup> M; ⬤, 1 × 10<sup>−7</sup> M; ⬤, 1 × 10<sup>−6</sup> M; and □, 1 × 10<sup>−5</sup> M). The experiments shown in B differ only in that the PGE<sub>2</sub> was added 20 min before the addition of Ag. Thirty minutes after the addition of Ag, degranulation was assessed by measurement of the fraction of hexosaminidase released into the supernatant. Addition of PGE<sub>1</sub> or PGE<sub>2</sub> enhanced hexosaminidase release by BMMCs in response to Ag in a dose-dependent manner.
mice also responded in a similar manner to control populations when exposed to both Ag and PGE\textsubscript{1} (Fig. 3D). In stark contrast, the enhanced response to IgE-Ag after PGE\textsubscript{1} treatment was completely abrogated in EP\textsubscript{3}-deficient BMMCs (Fig. 3C). These results demonstrate that the enhancement of Fc\textsubscript{e}RI-mediated degranulation by PGs is mediated by EP\textsubscript{3} receptors.

Effects of PGs on cytokine and leukotriene release by mast cells

Activation of mast cells also stimulates cytokine release. Thus, we next determined whether PGs could modify release of cytokines and whether this effect was also mediated by the EP\textsubscript{3} receptor. As described above, mast cells were incubated with IgE and then treated with Ag alone or Ag plus PGE\textsubscript{1}. Seven hours later, the supernatant was harvested and IL-6 levels were determined. After Ag stimulation, high levels of IL-6 were released from mast cells (Fig. 4) and this IL-6 release was significantly enhanced in the wild-type mast cells by the treatment with PGE\textsubscript{1}. In contrast, PGE\textsubscript{1} failed to enhance the release of IL-6 in the EP\textsubscript{3}-deficient mast cells. Similar to wild-type control BMMCs, IL-6 release by EP\textsubscript{2}- and EP\textsubscript{4}-deficient BMMCs was enhanced upon exposure to PGE\textsubscript{1} after IgE-Ag/Fc\textsubscript{e}RI receptor complex formation (data not shown).

Along with IL-6, leukotriene (LT) production is another important event associated with mast cell activation. Incubation of wild-type mast cells with PGE\textsubscript{1} alone did not increase LT release. In contrast, LT release was significantly stimulated after treatment of mast cells with IgE-Ag. In contrast to its effects on IL-6, PGE\textsubscript{1} did not further augment LT release in BMMCs exposed to IgE-Ag.

**FIGURE 3.** Prostaglandin E potentiates Ag-induced mast cell degranulation via the EP\textsubscript{3} receptor. BMMCs were cultured from mouse lines lacking one of the four known EP receptors and matched controls. BMMCs were treated overnight with anti-DNP IgE. After removal of excess Ab, cells were treated with various concentrations of either PGE\textsubscript{1} or PGE\textsubscript{2} for 20 min before the initiation of degranulation by addition of the indicated amount of DNP-HSA Ag. After 30 min, degranulation was assessed by measurement of the fraction of hexosaminidase released into the supernatant. A single experiment compared the response of EP\textsubscript{1} cells and wild-type BMMCs to the various concentrations of PGE\textsubscript{2} (A). The response of the EP\textsubscript{1} cells (right) did not differ from that of the control wild-type mast cells (left). Two independent experiments were conducted using EP\textsubscript{1} cells and wild-type cells. As shown in B, the EP\textsubscript{2} receptor does not play a role in the PGE\textsubscript{2}-mediated enhancement of degranulation. One of five experiments conducted with EP\textsubscript{2} cells is shown in C. All experiments demonstrate that EP\textsubscript{3} cells display no enhancement of mast cell degranulation in response to PGE\textsubscript{1}, at all concentrations of PGE\textsubscript{1} examined. Three independent experiments were conducted using EP\textsubscript{3} cells and one is shown in D. EP\textsubscript{3} cells did not lose their ability to respond to PGE\textsubscript{1}. The concentration of PGE\textsubscript{1} used is as follows: ■, vehicle only; ▲, 1 × 10\textsuperscript{-9} M; ▼, 1 × 10\textsuperscript{-8} M; ●, 1 × 10\textsuperscript{-7} M; ○, 1 × 10\textsuperscript{-6} M; □, 1 × 10\textsuperscript{-5} M.

**FIGURE 4.** PGE\textsubscript{1}-dependent release of IL-6 from Ag-activated BMMCs is mediated through the EP\textsubscript{3} receptor. BMMCs were loaded with anti-DNP IgE and then exposed to the indicated concentrations of DNP-HSA Ag, PGE\textsubscript{1}, or Ag plus PGE\textsubscript{1}. Control populations were loaded with IgE and treated only with vehicle. Cells were then incubated for either 7 h or 30 min to assess the production and release of IL-6 (A) and LT\textsubscript{B} (B), respectively. At completion of the incubation, the supernatant was harvested and the IL-6 and LT\textsubscript{B} were present measured by ELISA. Values are shown as mean ± SEM. *, p = 0.0004 for EP\textsubscript{3} vs wild-type cells.

**EP\textsubscript{3}** mice also responded in a similar manner to control populations when exposed to both Ag and PGE\textsubscript{1} (Fig. 3D). In stark contrast, the enhanced response to IgE-Ag after PGE\textsubscript{1} treatment was completely abrogated in EP\textsubscript{3}-deficient BMMCs (Fig. 3C). These results demonstrate that the enhancement of Fc\textsubscript{e}RI-mediated degranulation by PGs is mediated by EP\textsubscript{3} receptors.
EP₃ receptor signaling in BMMCs

Based on our finding that mast cells express multiple EP₃ receptor isoforms and the diverse signaling pathways used by these isoforms, we explored EP₃ receptor signaling in BMMCs by comparing [cAMP], of wild-type and EP₃-deficient cells after incubation with PGE₁ and PGE₂. Treatment of wild-type BMMCs with PGE₁ significantly increased [cAMP]. In contrast, exposure of the cells to PGE₂ did not significantly alter levels of cAMP in wild-type cells (Fig. 5A). Because PGE₁, but not PGE₂, may also activate the Gₛ₅-coupled IP receptor, we examined the effect of PGI₂ on BMMCs. IP receptor activation in mast cells causes a marked stimulation of cAMP levels (Fig. 5A). To obtain maximum stimulation of adenylate cyclase we used PGE₁ in subsequent experiments to determine the impact of loss of the EP₃ receptor on [cAMP]. Stimulation of cAMP levels by PGE₁ was markedly enhanced in EP₃−/− compared with wild-type BMMCs, suggesting that EP₃ receptors couple to Gₛ₅ proteins in BMMCs (Fig. 5B).

We next examined the effect of PGE₁ and PGE₂ on calcium signaling in mast cells. As shown, the addition of PGE₂ alone to BMMCs causes a brisk increase in [Ca²⁺], (Fig. 6A, left). Moreover, the magnitude of the change in [Ca²⁺], induced by PGE₂ is similar to that elicited by Ag treatment (Fig. 6B). This increase in [Ca²⁺], in response to PGE₂ is completely dependent on expression of the EP₃ receptor because PGE₂ had no effect on [Ca²⁺], in EP₃−/− BMMCs (Fig. 6A, right). We next examined whether PGE₂ could enhance IgE-Ag-induced Ca²⁺ mobilization. In wild-type cells, this increase in [Ca²⁺], is substantially enhanced after Ag activation, IgE-loaded wild-type cells were treated with Ag alone followed 1 min later by addition of PGE₂. Treatment with PGE₂ resulted in a further increase in [Ca²⁺], over that observed upon treatment with Ag alone. Data shown are representative of three independent experiments.

FIGURE 5. Regulation of [cAMP], by prostaglandins. BMMCs were washed and resuspended (2 × 10⁶ cells) in Tyrode’s solution. Cells were then treated with 1 × 10⁻⁷ M PGE₁, E₂, or I₂ or vehicle for 1 min, after which [cAMP], levels were assayed by ELISA. The amount of cAMP measured in the vehicle-treated cells was designated as the control amount of cAMP for the cell preparation. The percent change in the [cAMP], from this baseline after treatment with the indicated prostaglandin is shown. Treatment of the cells with either PGE₁ or PGI₂ resulted in a significant increase in [cAMP], (A; *p < 0.01; **p < 0.05). In contrast, PGE₂ treatment failed to increase [cAMP], in these cells. B. The increase in [cAMP], observed in the EP₃−/− BMMCs in response to PGE₁ was significantly higher than that measured in cells obtained from wild-type littermates (B; ***p < 0.05), demonstrating that the EP₃ receptor limits the increase in [cAMP]. Baseline cAMP levels: for control mast cells, 0.74 ± 0.2 pmols/well; and for EP₃−/− mast cells, 0.57 ± 0.1 pmols/well.

FIGURE 6. Activation of the EP₃ receptor results in the mobilization of [Ca²⁺]., and potentiates FcεRI-dependent Ca²⁺ mobilization. A. Wild-type and EP₃−/− BMMCs were loaded with fura 2-AM and changes in [Ca²⁺], were monitored after treatment with Ag, 10 μM PGE₂, and 1 μM ionomycin. As expected, no change in [Ca²⁺], was observed upon treatment of cells with Ag alone. Treatment of the wild-type cells with PGE₂ resulted in an easily measurable increase in [Ca²⁺],. No change in [Ca²⁺], was observed in the EP₃−/− cells. The loading of these cells with fura-2 was confirmed by observation of an increase in [Ca²⁺], upon exposure to ionomycin. B. Changes in [Ca²⁺], in IgE-loaded EP₃−/− and wild-type BMMCs were monitored after addition of Ag or Ag and PGE₂. As expected, treatment of IgE-loaded BMMCs with Ag resulted in a rapid increase in [Ca²⁺],. This change was observed in both the EP₃−/− and wild-type BMMCs. Addition of PGE₂ together with Ag resulted in an enhancement of this response in wild-type BMMCs. In contrast, no enhanced response was observed in the EP₃−/− cells. C. To further demonstrate the ability of PGE₂ to enhance Ca²⁺ mobilization observed after Ag activation, IgE-loaded wild-type cells were treated with Ag alone followed 1 min later by addition of PGE₂. Treatment with PGE₂ resulted in a further increase in [Ca²⁺],, over that observed upon treatment with Ag alone. Data shown are representative of three independent experiments.
was dependent on expression of the EP3 receptor, because no increase in dase was increased with increasing concentrations of PMA. This response PMA and PGE 1.

Several PGE2 receptors and the G

were not observed in the EP3-deficient mast cells (data not shown). Both PGE1 and PGE2 had similar effects on [Ca2+]i and inhibition of adenylate cyclase activity. Expression analysis and pharmacological studies have indicated that mast cells express a number of prostanoid receptors, including several PGE2 receptors and the Gαs-coupled prostacyclin receptor. Pharmacological studies are consistent with the expression of EP1 and EP4 on IL-3-dependent mast cell lines and on rat peritoneal mast cells (20–22). Expression analysis of MC/9 mouse mast cell line revealed the expression of EP1, EP3, and EP4 (19). We show here that BMMCs express high levels of the Gαs-coupled EP4 receptor. Although we could not detect expression of the EP2 receptor by Northern analysis, RT-PCR studies indicate that mast cells do express this receptor. This finding is consistent with the cloning of the mouse EP2 receptor from a mouse mastocytoma cell line. The relatively low expression of the EP2 receptor in comparison to EP1 in virtually all tissues that coexpress these two receptors has been noted previously (22). In addition to these Gαs-coupled receptors, we show that BMMCs express high levels of the EP1 receptor, including both the EP1α and EP1β isoforms. Consistent with previous reports, we detected high levels of EP1 receptor expression. Activation of this receptor has been shown to result in an increase in [Ca2+]i.

We show that treatment of BMMCs with PGE1 or PGE2 alone does not induce degranulation of BMMCs. However, addition of PGE to BMMCs stimulated with IgE and Ag potentiates degranulation of these cells. This is consistent with the findings reported by Leal-Berumen et al. (23), using rat peritoneal mast cells, and with results obtained using BMMCs and IL-3-dependent mast cell lines (19, 20). Our results contrast sharply, however, with other studies that showed that PGE2 could inhibit the release of mediators from both rat peritoneal mast cells (24, 25) and human lung mast cells (26, 27). It is likely that at least some of these differences reflect the use of different mast cell populations in these studies. As discussed below, these observations suggest that the impact of PGE2 on mast cell function may be dependent on the maturity, tissue type, and perhaps activation state of the cell.

The studies reported here also show that PGE2 can enhance the production of IL-6 by IgE-Ag-stimulated mast cells. Unlike previous reports, we failed to observe an increase in production of IL-6 by cells stimulated by PGE alone. Stimulation of rat peritoneal mast cells with PGE1 or PGE2 was reported to induce a significant increase in the release of IL-6 from 3 to 18 h after treatment (23). In similar studies using BMMCs, no significant increase in IL-6 release was observed at early time points on comparison of medium-treated and PGE2-treated BMMCs (19). However, in these same studies IL-6 production in response to PGE1 alone was noted at later time points. We did not observe substantial release of IL-6 in the response to PGE1 or PGE2 alone, even at these later time points (data not shown).

The enhancement of Ag-stimulated degranulation of BMMCs by PGE1 and PGE2, is perhaps surprising in light of our demonstration that these cells express both the EP2 and the EP4 receptors and of the established coupling of these receptors to stimulatory G proteins in all cell types examined to date. Agents that stimulate an increase in [cAMP], have been shown to inhibit mast cell degranulation (28), and an increase in [cAMP], due to PGE1 addition is believed to precede the inhibition of histamine release from rat peritoneal mast cells in response to stimulation of IgE receptors (27). There are a number of possible explanations for the observed lack of inhibition of BMMC degranulation after treatment with PGE1 and the similarity of the response of EP2- or EP4-deficient mast cells compared with wild-type cells. It is possible, for instance, that although these cells express very high levels of EP2 receptor mRNA, this does not correspond to high numbers of EP2 receptors at the cell surface of BMMCs. Alternatively, the coupling of the EP4 receptors to adenylate cyclase might be inefficient in these immature mast cells, or rapid desensitization of this receptor in BMMCs might reduce their impact on cell physiology. Alteration of the EP4/IP3 inhibitory pathways as mast cells mature after migration to various tissues might alter the response of mast

Discussion

BMMCs have provided an important means for examining the receptors and intracellular pathways that regulate mast cell function. These cell populations, in combination with the increasing availability of mice deficient in receptors implicated in mast cell biology, provide an important method for dissecting the requirements for mast cell degranulation and production of inflammatory mediators. Using this approach, we show that PGs can potentiate mast cell degranulation and cytokine production and that these actions are mediated primarily by the EP3 receptor. Furthermore, we show that EP3 receptor activation is associated with both increases in [Ca2+]i and inhibition of adenylate cyclase activity. Expression analysis and pharmacological studies have indicated that mast cells express a number of prostanoid receptors, including several PGE2 receptors and the Gαs-coupled prostacyclin receptor. Pharmacological studies are consistent with the expression of EP1 and EP4 on IL-3-dependent mast cell lines and on rat peritoneal mast cells (20–22). Expression analysis of MC/9 mouse mast cell line revealed the expression of EP1, EP3, and EP4 (19). We show here that BMMCs express high levels of the Gαs-coupled EP4 receptor. Although we could not detect expression of the EP2 receptor by Northern analysis, RT-PCR studies indicate that mast cells do express this receptor. This finding is consistent with the cloning of the mouse EP2 receptor from a mouse mastocytoma cell line. The relatively low expression of the EP2 receptor in comparison to EP1 in virtually all tissues that coexpress these two receptors has been noted previously (22). In addition to these Gαs-coupled receptors, we show that BMMCs express high levels of the EP1 receptor, including both the EP1α and EP1β isoforms. Consistent with previous reports, we detected high levels of EP1 receptor expression. Activation of this receptor has been shown to result in an increase in [Ca2+]i.

We show that treatment of BMMCs with PGE1 or PGE2 alone does not induce degranulation of BMMCs. However, addition of PGE to BMMCs stimulated with IgE and Ag potentiates degranulation of these cells. This is consistent with the findings reported by Leal-Berumen et al. (23), using rat peritoneal mast cells, and with results obtained using BMMCs and IL-3-dependent mast cell lines (19, 20). Our results contrast sharply, however, with other studies that showed that PGE2 could inhibit the release of mediators from both rat peritoneal mast cells (24, 25) and human lung mast cells (26, 27). It is likely that at least some of these differences reflect the use of different mast cell populations in these studies. As discussed below, these observations suggest that the impact of PGE2 on mast cell function may be dependent on the maturity, tissue type, and perhaps activation state of the cell.

The studies reported here also show that PGE2 can enhance the production of IL-6 by IgE-Ag-stimulated mast cells. Unlike previous reports, we failed to observe an increase in production of IL-6 by cells stimulated by PGE alone. Stimulation of rat peritoneal mast cells with PGE1 or PGE2 was reported to induce a significant increase in the release of IL-6 from 3 to 18 h after treatment (23). In similar studies using BMMCs, no significant increase in IL-6 release was observed at early time points on comparison of medium-treated and PGE2-treated BMMCs (19). However, in these same studies IL-6 production in response to PGE1 alone was noted at later time points. We did not observe substantial release of IL-6 in the response to PGE1 or PGE2 alone, even at these later time points (data not shown).

The enhancement of Ag-stimulated degranulation of BMMCs by PGE1 and PGE2, is perhaps surprising in light of our demonstration that these cells express both the EP2 and the EP4 receptors and of the established coupling of these receptors to stimulatory G proteins in all cell types examined to date. Agents that stimulate an increase in [cAMP], have been shown to inhibit mast cell degranulation (28), and an increase in [cAMP], due to PGE1 addition is believed to precede the inhibition of histamine release from rat peritoneal mast cells in response to stimulation of IgE receptors (27). There are a number of possible explanations for the observed lack of inhibition of BMMC degranulation after treatment with PGE1 and the similarity of the response of EP2- or EP4-deficient mast cells compared with wild-type cells. It is possible, for instance, that although these cells express very high levels of EP2 receptor mRNA, this does not correspond to high numbers of EP2 receptors at the cell surface of BMMCs. Alternatively, the coupling of the EP4 receptors to adenylate cyclase might be inefficient in these immature mast cells, or rapid desensitization of this receptor in BMMCs might reduce their impact on cell physiology. Alteration of the EP4/IP3 inhibitory pathways as mast cells mature after migration to various tissues might alter the response of mast

![Figure 7](http://www.jimmunol.org/)

**Figure 7.** PGE1 with PMA activates BMMCs. BMMCs were stimulated for 30 min at 37°C with various concentrations of PMA in the presence or absence of 10 μM PGE1. Degranulation of the BMMCs was assessed by measuring the release of hexosaminidase into the supernatant. As expected, treatment of mast cells with PMA up to 100 ng/ml did not result in a significant increase in hexosaminidase release in either wild-type or EP3R−/− cells. However, in the presence of PGE1, release of hexosaminidase was increased with increasing concentrations of PMA. This response was dependent on expression of the EP3 receptor, because no increase in hexosaminidase release was observed in the EP3R−/− BMMCs treated with both PGE1 and PMA. Data shown are representative of three independent experiments.
cells to PGE2, resulting in a primarily inhibitory function for this lipid mediator. This later interpretation provides an explanation for the inhibitory response to PGE2 observed in a number of studies that used mature tissue mast cells.

The observed enhancement of Ag-mediated BMMC degranulation upon treatment with PGE1 or PGE2 is consistent with earlier pharmacological studies that implicated the inhibitory response to PGE2 observed in a number of studies. This later interpretation provides an explanation for the failure of mast cells to degranulate in response to PGE2 alone. Similarly, the ability of PGE to enhance IL-6 production is abolished in EP2-deficient mast cells. These results are consistent with earlier pharmacological studies that implicated either the EP3 or the EP1 receptor in the activation of mast cells and/or mast cell lines (19, 20). When EP2 receptor-deficient mice were tested directly, they exhibited a degranulation response that was sensitive to PG potentiation. Together with the complete absence of a PGE response in the EP3-deficient mast cells, this suggests that the EP1 receptor is not likely involved in this response in BMMCs.

To further examine the mechanism by which activation of the EP3 receptor can modify mast cell function, we have examined the impact of loss of EP3 receptor expression on cAMP accumulation and on alterations in [Ca2+]i. Transfection of both the EPα and EPβ receptors into Chinese hamster ovary (CHO) cells has been reported to inhibit forskolin-induced cAMP accumulation (29). We show here that treatment of BMMCs with PGE1 leads to an increase in [cAMP]i and that, consistent with the activation of Gαs, this accumulation of [cAMP]i is enhanced in the EP3 receptor-deficient cells.

In addition to their ability to inhibit cAMP accumulation, both the EPα and the EPβ isoforms have been shown to stimulate an increase in [Ca2+]i upon transfection into CHO cells. This increase in [Ca2+]i was pertussis toxin-sensitive, consistent with the coupling of both of these isoforms to Gαs proteins (30). We report that treatment of BMMCs with PGE1 or PGE2 resulted in a rapid increase in [Ca2+]i. Furthermore, we show that this response is completely dependent on the expression of the EP3 receptor. Therefore, it is likely that in BMMCs, similar to the CHO cells, the EPα and the EPβ are coupled to Gαs proteins, whose Gαs subunits can mediate increases in [Ca2+]i, by activation of phospholipase C. It is likely that this in turn is responsible for the potentiation of mast cell degranulation observed on treatment with PGE2. However, we cannot rule out the possibility that PGE/EP3 contribute to this response, at least in part, by inhibition of adenylate cyclase activity and [cAMP]i accumulation. This seems less likely given the observation that the loss of EP4 and EP2 receptors had no effect on mast cell degranulation.

There are a number of possible explanations for the inability of PGE to initiate mast cell degranulation, despite the substantial increase in [Ca2+]i. First, it is possible that subtle differences in the magnitude, duration, and/or the stores of Ca2+ released lead to the differential effect on mast cell activation. Alternatively, it is possible that although activation of the EP3 receptor leads to changes in Ca2+ sufficient for mast cell degranulation, activation of additional pathways is necessary to bring about these physiological changes. It has been suggested that activation of PKC plays an important role in mast cell degranulation. To determine whether the failure of mast cells to degranulate in response to PGE2 alone is related to its inability to activate the PKC pathway, we examined mast cell degranulation in EP3-deficient and wild-type BMMCs treated with PMA alone and PMA together with PGE1. PMA treatment of BMMCs did not result in any changes in [Ca2+]i. The ability of EP3 receptor activation to act synergistically with PMA to bring about degranulation of mast cells suggests that PKC activity can contribute to mast cell activation and that the failure of PGE2 to activate this pathway prevents PGE2 alone from initiating mast cell degranulation in these cells.

We cannot formally rule out the possibility that other cell types might contribute to the observed differences between the responses of the BMMCs derived from the various EP-deficient mouse lines. Because the number of mast cells present was not determined for every preparation of cells (only representative cultures of each genotype) used in the reported studies, we cannot rule out the possibility that differences in the number of mast cells or presence of contaminating populations contribute to the observed differences in the response of wild-type and EP3/−/− populations to PGE2. However, the fact that the baseline response to IgE and Ag was similar in all of the EP-deficient cells (only the ability of PGE to potentiate this response was altered in the EP3-deficient cells) suggests that loss of these receptors does not alter the development and/or growth of BMMCs under these culture conditions.

The studies reported here, together with those of other investigators, begin to develop a model that unifies the often “opposing” actions of prostanoids on mast cell physiology. The effect of PGE2 is determined by both the expression profile of the various prostanoid receptors and by the activity of the intracellular pathways to which they are coupled. It is easy to envision a model in which this contributes to the ability of mast cells to modulate their responses in both a temporal and spatial manner. For example, early during an inflammatory process, PGE2 produced upon local tissue damage leads to heightened production of cytokines necessary for the recruitment of inflammatory cells. This could lead to altered expression or activity of the various EP receptors on the mast cells, with a shift from the Gαi- to the Gαs-linked receptors and restoration of homeostasis as the inflammation is resolved. It is also possible that different EP receptor pathways dominate in the various mast cell populations. This would contribute to the adaptation of mast cells to the particular organ in which it is located. The generation of mouse lines with deficiencies in a single or combination of receptors and the study of these animals in model systems in which a role for mast cells has been defined should help us answer these questions in the future.

References


