Cells by the Cooperation of Two Prostaglandin E\textsubscript{2} Receptor Subtypes, EP3 and EP4* 

In this study, we investigated the role of PGE\textsubscript{2} in mouse mastocytoma P-815 cell adhesion to extracellular matrix proteins (ECMs) \textit{in vitro}. We report that PGE\textsubscript{2} accelerated ProNectin F\textsuperscript{TM} (a proteolytic fragment of fibronectin)-mediated adhesion, which was abolished by the addition of the GRGDS peptide, an inhibitor of the RDG binding site of ProNectin F\textsuperscript{TM}. We show that the cAMP level and cAMP-regulated protein kinase (PKA) activity were critical mediators of this PGE\textsubscript{2} effect, because the cell-permeable cAMP analogue 8-Br-cAMP accelerated P-815 cell adhesion to ProNectin F\textsuperscript{TM} and the pharmacological inhibitor of PKA, H-89, blocked PGE\textsubscript{2}-mediated adhesion. Consistent with mRNA expression of the G\textsubscript{s}-coupled EP4- and G\textsubscript{i}-coupled EP3-PGE\textsubscript{2} receptor subtypes, P-815 cell adhesion was accelerated by treatment with a selective EP4 agonist, ONO-AE1–329, but not a selective EP1/EP3 agonist, sulprostone. However, simultaneous treatment with ONO-AE1–329 and sulprostone resulted in augmentation of both the cAMP level and cell adhesion. The augmentation of EP3-mediated cAMP synthesis was dose-dependent, without affecting the half-maximal concentration for EP4-mediated G\textsubscript{s}-activity, which was inhibited by a G\textsubscript{i} inhibitor, pertussis toxin. In conclusion, these findings suggest that PGE\textsubscript{2} accelerates RGD-dependent adhesion via cooperative activation between EP3 and EP4 and contributes to the recruitment of mast cells to the ECM during inflammation.

Differentiated mast cells (MCs),\textsuperscript{1} which originate from bone marrow stem cells, traffic throughout the circulation and adhere to the extracellular matrix (ECM) in various tissues. MCs are widely distributed in tissues throughout the body, especially in connective tissues, serosal cavities, and mucosal surfaces under normal physiological conditions. This characteristic distinguishes MCs from other bone marrow-derived hematopoietic cells, such as basophils, neutrophils, and eosinophils. MCs congregate around nerves, blood vessels, and lymphatic vessels. MCs therefore interact with not only the ECM but with other cells as well. As well known for rodent connective tissue-typed MCs and mucosal-typed MCs, the biological activity of MCs vary with their interactions with the ECM and other cells.

MCs are widely distributed along basement membranes, indicating that MCs might adhere to laminin. Supporting this fact, mouse bone marrow-derived mast cells (BMMC) have been reported to adhere to laminin, when the cells were activated by phorbol 12-myristate 13-acetate (PMA) (1–3) or agent-stimulated aggregation of FceRI (4). In addition to laminin, MCs can adhere to other matrix components such as fibronectin (5) and vitronectin (6, 7). As with laminin, the adherence of BMMC to fibronectin has been reported to occur through activation with PMA or after aggregation of FceRI. These adherence activities required calcium (3). In contrast to BMMC, the mouse PT18 cell line spontaneously adhered to laminin (1), and human skin mast cells also spontaneously adhered to laminin and fibronectin (8). These previous findings indicate that the interactions between MCs and matrix components may depend on the cells involved and the kinds of stimuli.

PGE\textsubscript{2}, which is involved in inflammation section (9), affects both differentiation and growth of MCs \textit{in vitro}. PGE\textsubscript{2} enhances mast cell differentiation from cord blood mononuclear cells (10) and in the fibroblast co-culture system (11). Very recently, Dormond et al. (12) reported that a COX-2 inhibitor suppressed α\textsubscript{v}β\textsubscript{3}-dependent HUVEC spreading, migration, and angiogenesis through Rac activation (12), and PGE\textsubscript{2} accelerated α\textsubscript{v}β\textsubscript{3}-mediated HUVEC responses in a cAMP-dependent manner (13). However, no reports have published the effects of PGE\textsubscript{2} on MC adhesion to the ECM.

The PGE\textsubscript{2} receptors (EP) are comprised of four subtypes, EP1, EP2, EP3, and EP4, which are coupled to different G proteins and signal pathways (14, 15). Among these subtypes, EP2 and EP4 couple to G\textsubscript{i}, resulting in increases of intracellular cAMP concentrations (16, 17), while EP3 couples to G\textsubscript{s}, causing a decrease in cAMP levels (18–21). Very recently, we and other investigators have reported that activation of the G\textsubscript{s}-coupled EP3 receptor was able to augment adenylyl cyclase activity via stimulation of a G\textsubscript{i}-coupled receptor (22, 23). We previously reported that the mouse EP3β receptor was able to augment EP2-induced adenylyl cyclase activity in both EP2- and EP3-transfected COS-7 cells (22). Southall and Vasko (23) showed that the simultaneous depletion of rat EP3C and EP4 was essential to abolish PGE\textsubscript{2}-stimulated cAMP production and neuropeptide release in rat sensory neurons (23). These permissive actions between the two subtypes may be involved in the events of a number of physiological actions of PGE\textsubscript{2} (15).
Here we examined the effects of the PGE2 on adhesion of mouse mastocytoma P-815 cells to the fibronectin component in a PKA-dependent manner, and we examined the correlation between CAMP levels and adhesion in P-815 cells to see whether EP3 could augment EP4-mediated CAMP synthesis.

**EXPERIMENTAL PROCEDURES**

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**Materials**—Sputrose was a generous gift from Dr. M. P. L. Caton of Rhone-Poulenc Lto, ONO-AEI-259 and ONO-AEI-329 were generous gifts from ONO Pharmaceuticals (Osaka, Japan). ProNectin FTM (Proment-F) and ProNectin LTM (Proment-L) were generous gifts from Sanyo Chemical Industries, Ltd. (Osaka, Japan). Collagen I-coated plates were from BD Pharmingen Labware, the 125I-deoxycholate, and 0.1% SDS, and incubated for 2 h at 4 °C. For protection against proteolytic degradation, a mixture of protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 100 μg benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml E-64, and 1 mg/ml pepstatin A) was added. The mixture was then centrifuged at 10,000 × g for 15 min at 4 °C. The resulting supernatant was dissolved in Laemmli buffer and heated to 30 °C, and the precipitated proteins were separated by SDSPAGE on 10% polyacrylamide gels, and transferred electrophoretically onto a polyvinylidene difluoride membrane and then incubated with peroxidase-conjugated antibodies. The membranes were developed with an ECL Western blot detection reagent. The relative intensities of the bands were quantitated by a computerized densitometer.

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**Expression of the Adenyl Cyclase Subtypes**—Expression of adenyl cyclase II, IV, and VII were determined as reported previously (25). PGE2-induced cAMP synthesis was assayed by incubation with 4 nM [3H] PGE2 at 30 °C for 10 min, and the mixture was then calculated with the following formula shown in Equation 1.

% cell adhesion = adherent cell number × 100/(adherent cell number + non-adherent cell number) (Eq. 1)

**RESULTS**

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**P-815 Cell Adhesion to Pronectin-F-coated Plates**—When P-815 cells were cultured in Fischer’s medium containing 10% fetal bovine serum together with 10 μM indomethacin, almost no attachment to non-coated plates was observed. However, after incubation with 1 μM PGE2 for 8 h, ~11% of P-815 cells were retained on the non-coated plates as adhered cells, after being washed three times with PBS and then preincubated overnight in TTBS containing 5% nonfat milk at 4 °C. The membrane fraction was then incubated with anti-A cyclase antibodies (A cyclase II, IV, 1206; A cyclase VII, 1:100) in TTBS containing 5% nonfat milk at 1 h at 37 °C. After washing three times with TTBS (containing 0.05% Tween 20) at room temperature, the membrane fraction was incubated with peroxidase-conjugated anti-rabbit or -goat IgG in TTBS for 1 h at room temperature, and then detected with the ECL Western blot detection reagent.

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**Measurement of CAMP Formation**—Cyclic AMP levels in P-815 cells were determined as reported previously (25). P-815 cells (2 × 10⁶ cells/assay) were suspended with 10 μM indomethacin in HEPES-buffered saline containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 11 mM glucose, and 15 mM HEPES (pH 7.4), and preincubated for 10 min at 37 °C. Reactions were started by addition of test agents along with 500 μM 3-isobutyl-1-methyl-xanthine (Sigma). After incubation for 10 min at 37 °C, reactions were terminated by the addition of 10% trichloroacetic acid. The level of cAMP was measured by radioimmunoassay with an Amersham Biosciences cAMP assay system.
Expression of the EP3 and EP4 Subtypes in P-815 Cells

**Fig. 1.** Adhesion of PGE2-stimulated P-815 cells to Pronectin-F-coated plates. A, P-815 cells were incubated at 37 °C for 8 h with or without 1 μM PGE2. B, P-815 cells were incubated at 37 °C for 8 h in wells coated with or without Pronectin-F (10 μg/ml) in the presence (solid bars) or absence (open bars) of 1 μM PGE2, and the percentages of P-815 cell adhesion were determined as described under “Experimental Procedures.” Values are shown as the means ± S.E. of triplicate experiments. *, p < 0.01 versus non-coat. C, P-815 cells were incubated at 37 °C for 8 h in wells coated with Pronectin-F (10 μg/ml) in the presence (closed circles) or absence (open circles) of 1 μM PGE2, together with the indicated concentrations of soluble GRGDS peptide, and the percentages of P-815 cell adhesion were determined as described under “Experimental Procedures.” Values are means ± S.E. for triplicate experiments. *, p < 0.01 versus without the GRGDS peptide.

almost completely disappeared with addition of the GRGDS peptide (Fig. 1C), which is a soluble inhibitor for the RDG site of fibronectin as well as Pronectin-F (28). Furthermore, this PGE2-treated cell adhesion was not affected by further incubation with 0.02% EDTA for 30 min, but the cells could be detached by 0.25% trypsin treatment.

Expression of the EP3 and EP4 Subtypes in P-815 Cells—The RT-PCR experiment revealed that P-815 cells express mRNAs for EP3, EP4, and IP (Fig. 2A). We confirmed the expression of the EP3 and EP4 receptor proteins by the replacement of the binding of 4 nM [3H]PGE2 to membrane fractions with 10 μM PGE2, sulprostone (EP1 and EP3 agonist), or ONO-AE1-329 (EP4 agonist). The order of replacement potency was found to be PGE2 > sulprostone > ONO-AE1-329 (Fig. 2B). These results indicate that PGE2 can bind to EP3 and EP4 present in the membrane fractions of P-815 cells.

PGE2-induced cAMP and Protein Kinase A-dependent P-815 Cell Adhesion—Along with PGE2, 8-Br-cAMP, and IBMX accelerated the adhesion of P-815 cells to Pronectin-F-coated plates (Fig. 3A). PGE2-mediated cell adhesion was greatly reduced by pretreatment with 10 μM H-89, a PKA inhibitor (Fig. 3B). On the other hand, phorbol 12-myristate 13-acetate (PMA) had no effect on cell adhesion in the absence of PGE2, and in addition, PGE2 did not change the intracellular Ca2+ level (data not shown). These results suggest that the PGE2-induced adhesion of P-815 cells to Pronectin-F is via a cAMP-dependent and cAMP-protein kinase A-dependent pathway.

Sulprostone-induced Augmentation of ONO-AE1-329-induced cAMP Formation in P-815 Cells—Since P-815 cells express the Gs-coupled EP3 as well as the Gi-coupled EP4, it is possible that PGE2 may bind to both subtype receptors at the same time, and hence the cAMP level may reflect the difference between Gs and Gi/α protein activation. We therefore examined the cAMP levels in P-815 cells when treated with the EP1/EP3 agonist sulprostone and/or the EP4 agonist ONO AE1-329 (Fig. 4A). The cAMP level was increased by treatment with ONO-AE1-329 and reached a plateau level at 0.1 μM. However, cAMP accumulation induced by various concentrations of the EP4 agonist were all augmented by the simultaneous addition of sulprostone (1 μM), without affecting the half-maximal concentration, although sulprostone alone did not stimulate cAMP accumulation (Fig. 4A). The half-maximal concentration of sulprostone for EP3-mediated augmentation in PGE2-induced cAMP synthesis was calculated to be ~5 × 10⁻⁸ M (data not shown). Furthermore, sulprostone-induced augmentation of ONO-AE1-329-activated cAMP formation was absolutely inhibited by treatment with PT, and the cAMP level was almost equal to that observed in the cells stimulated by ONO-AE1-329 alone (Fig. 4B). These findings suggest that EP3-mediated augmentation of Gi activity is achieved through Giβγ subunits resulting from G1α protein activation (29), although EP4-mediated Gi activity is not.

Sulprostone-induced Augmentation of ONO-AE1-329-stimulated P-815 Cell Adhesion to Pronectin-F—We next examined whether EP3 stimulation could augment EP4-mediated adhe-
triplicate experiments. *, p < 0.01 versus untreated. B, P-815 cells were preloaded with or without 10 μM H-89 for 30 min, then the cells were incubated for 8 h in the presence or absence of 1 μM PGE₂ with or without 10 μM H-89. The percentages of P-815 cell adhesion were determined as described under "Experimental Procedures." Values are shown as the means ± S.E. for triplicate experiments. *, p < 0.01 versus without H-89 treatment.

Fig. 3. Adhesion of PGE₂-stimulated P-815 cells to Pronectin-F-coated wells mediated by PKA activation. A, P-815 cells were incubated at 37 °C for 8 h in wells coated with Pronectin-F (10 μg/ml) in the presence of 1 μM PGE₂, 1 mM 8Br-cAMP, or 0.5 mM IBMX, and the percentages of P-815 cell adhesion were determined as described under "Experimental Procedures." Values are shown as the means ± S.E. for triplicate experiments. *, p < 0.01 versus untreated. B, P-815 cells were preloaded with or without 10 μM H-89 for 30 min, then the cells were incubated for 8 h in the presence or absence of 1 μM PGE₂ with or without 10 μM H-89. The percentages of P-815 cell adhesion were determined as described under "Experimental Procedures." Values are shown as the means ± S.E. for triplicate experiments. *, p < 0.01 versus without H-89 treatment.

Fig. 4. Sulprostone-induced augmentation of ONO-AE1–329-stimulated cAMP formation in P-815 cells. A, P-815 cells were incubated at 37 °C for 10 min with 1 μM sulprostone in the presence of indicated concentrations of ONO-AE1–329. cAMP formation was determined as described under "Experimental Procedures." Values are the means ± S.E. of triplicate experiments. *, p < 0.01 versus stimulation with the indicated concentrations of ONO-AE1–329 alone. B, P-815 cells were pretreated with 25 ng/ml PT for 7 h, and the treated cells were then incubated at 37 °C for 10 min with 1 μM PGE₂, 1 μM sulprostone, 1 μM ONO-AE1–329, or both 1 μM sulprostone and 1 μM ONO-AE1–329, and cAMP formation was determined as described under "Experimental Procedures." Values are shown as the means ± S.E. for triplicate experiments. *, p < 0.01 versus without PT treatment.

Fig. 5. Effect of PGE₂ on adhesion of P-815 cells to Pronectin-F-coated wells. P-815 cells were incubated at 37 °C for 8 h in wells coated with Pronectin-F (10 μg/ml) in the presence or absence of 1 μM PGE₂, 1 μM sulprostone, 1 μM ONO-AE1–329, or both 1 μM sulprostone and 1 μM ONO-AE1–329. The percentages of P-815 cell adhesion were determined as described under "Experimental Procedures." Values are shown as the means ± S.E. for triplicate experiments. *, p < 0.01 versus stimulation with ONO-AE1–329 alone.

Discussion

PGE₂ was previously reported to affect both differentiation and growth of mast cells in vitro. For example, PGE₂ is essential for the differentiation of IL-3-dependent mouse mast cells from spleen cells (30), PGE₂ enhanced human mast cell differentiation from cord blood mononuclear cells by inhibiting the production of macrophage-derived GM-CSF (10), and PGE₂ enhanced the growth of differentiated mast cells in a fibroblast co-culture system (11). It is therefore possible that PGE₂ plays a role in the interaction of mast cells with fibroblastic cells and extracellular matrix components. The interaction between mast cells and extracellular matrix components have profound influences on the targeting of mast cell progenitors to specific locations, the distribution of mast cell subsets, and the biological responsiveness of mast cells in tissues. The ability of mast cells to adhere to fibronectin, which involves the RGD sequence located within the cell-attachment domain of the fibronectin molecule, may play a role in the migration of mast cells in various tissues (5). In the present report, we found that PGE₂ is able to stimulate the adhesion of P-815 cells to Pronectin-F through the RGD cell attachment domain of fibronectin, and that this adherent activity is mediated via a cAMP-dependent pathway induced by the activation of the EP4 and/or EP3 receptors. It is possible that the cAMP-protein kinase A pathway may be involved in the induction of these cell attachment molecules, as PGE₂-induced cell attachment was inhibited by treatment with H-89 (Fig. 3B) and cycloheximide (data not shown). However, neither PGE₂ nor 8Br-cAMP augmented the expression of VLA-5, one of the fibronectin receptors (data not shown). Therefore, cycloheximide may affect the signaling pathway of the fibronectin receptors. Further experiments are required to clarify these points.

Prostaglandin E₂ acts through binding to its specific receptors, which are comprised of four subtypes, EP1, EP2, EP3, and EP4 (14, 15). Among them, EP3 and EP4 couple to Gₛ and Gᵢ, and result in inhibition and stimulation of adenylyl cyclase activity, respectively (17–21). The mouse EP3 receptor is comprised of three isoforms, EP₃α, EP₃β, and EP₃γ, which differ in their C terminus. Among them, EP₃β, which was used in this experiment is known to be coupled to the Gᵢ protein (19). Very recently, activation of a Gᵢ-coupled receptor has been reported to augment the adenylyl cyclase activity induced by the stimulation of a Gₛ-coupled receptor in COS-7 cells. For example, activation of Gₛ-coupled receptors such as α₂ adrenoreceptor (29) and bradykinin B2 receptor (32) lead to the augmentation of Gₛ-stimulated adenylyl cyclase in COS-7 cells. This synergistic effect has not been clearly shown in mammalian cells. Southall and Vasko (23) showed that the simultaneous depl-
tion of both rat EP3C and EP4 was essential for abolishing PGE2-stimulated cAMP production and neuroepitope release in rat sensory neurons (23). In the current experiment we showed that the Gt-coupled EP3 agonist sulprostone augmented cAMP formation stimulated in P-815 cells. Similarly, we found that sulprostone was able to augment cAMP formation in P-815 cells activated by an IP agonist, carbacycin, suggesting that the augmentative effects of EP3 can be observed irrespective of Gt activation. The mechanism underlying these phenomena was thought to be via Gβγ-mediated activation of type IV adenylyl cyclase (29), because pretreatment with PT inhibited the augmentation by the activation of the Gt-coupled EP3 receptor, and P-815 cells express type IV adenylyl cyclase (data not shown). Therefore, Gβγ-mediated activation of type IV adenylyl cyclase may be involved in EP3-mediated signaling to augment EP4-stimulated adenylyl cyclase activity in P-815 cells.

Although the involvement of phosphatidylinositol 3-kinase (PI3K) in cell adhesion to matrix proteins is shown in a variety of cell types (33), the role of PI3K in mast cell adhesion is still unknown. Kinashi et al. (31) reported that the adhesion of platelet-derived growth factor receptor-expressed bone marrow-derived mast cells was induced by the addition of the PI3K inhibitor wortmannin. To understand whether PI3K is activated by PGE2 during P-815 cell adhesion, we examined the effect of the PI3K inhibitors, wortmannin (100 nM) and LY294002 (10 μM) on EP3/EP4-agonist induced cell adhesion to fibronectin. As a result, these inhibitors showed an additive effect on the augmentation of PGE2-induced cell adhesion (wortmannin: 81.7 ± 0.1% and LY294002: 82.4 ± 0.2%, compared with PGE2 stimulation alone: 24.8 ± 0.6%). Furthermore, each inhibitor alone had an effect on cell adhesion (wortmannin: 20.4 ± 0.1% and LY294002: 24.1 ± 0.1%, compared with the absence of these inhibitors (3.3 ± 0.1%). Therefore, these results indicate that P-815 cell attachment to fibronectin is regulated by two independent signaling pathways involving PKA and PI3K. Further experiments are necessary to clarify the fundamental differences in the signaling involving PKA and PI3K in P-815 cell attachment to fibronectin. In summary, this study clearly demonstrates that two subtypes of the PGE2 receptor, EP3 and EP4, are cooperatively involved in PGE2-evoked and cAMP-mediated functions of P-815 cell adhesion.

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