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EP_4 Receptor Mediation of Prostaglandin E_2-Stimulated Mucus Secretion by Rabbit Gastric Epithelial Cells

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ABSTRACT. Prostaglandin (PG) E receptors are divided into four subtypes (EP_1-EP_4). We investigated the EP receptor subtype involved in PGE_2-stimulated mucus secretion by rabbit gastric epithelial cells. Northern blot analysis revealed that epithelial cells express EP_3 and EP_4 receptor mRNAs, but neither EP_1 nor EP_2 receptor mRNAs were detected. PGE_2, 11-deoxy-PGE_1 (an EP_3/EP_4/EP_2 agonist) and 16,16-dimethyl-PGE_2 (an EP_3/EP_2/EP_4 agonist) concentration-dependently promoted mucus secretion. In contrast, 17-phenyl-PGE_2 (an EP_3/EP_1 agonist), sulprostone (an EP_3/EP_1 agonist), and butaprost (an EP_2 agonist) failed to stimulate secretion. The effective concentrations of PGE_2, 11-deoxy-PGE_1, and 16,16-dimethyl-PGE_2 were associated with their affinities for the EP_4 receptor. In addition, PGE_2, 11-deoxy-PGE_1, and 16,16-dimethyl-PGE_2 increased cyclic AMP (cAMP) production, but the other prostanoids had no effect. SQ22536 [9-(tetrahydro-2′-furyl)adenine; an adenylate cyclase inhibitor] inhibited both the increased cAMP production and mucus secretion induced by PGE_2, 11-deoxy-PGE_1, and 16,16-dimethyl-PGE_2. H-89 (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinoline sulfonamide; a protein kinase A inhibitor) also abolished the stimulatory effects of the prostanoids on mucus secretion, but calphostin C (a protein kinase C inhibitor) did not. These results indicate that PGE_2 promotes mucus secretion by rabbit gastric epithelial cells, mediated through EP_4 receptor stimulation and the subsequent activation of protein kinase A. BIOCHEM PHARMACOL **58**;12:1997–2002, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. prostaglandin; EP receptor; mucus secretion; gastric epithelial cells

PGs§, especially PGE_2, play crucial roles in regulating a variety of gastric functions, such as blood flow and acid, mucus, and bicarbonate secretion [1, 2]. PGE receptors are divided pharmacologically into at least four subtypes (EP_1, EP_2, EP_3, and EP_4), and their cDNAs have been cloned [3, 4]. Morimoto *et al.* [5] reported that mRNA expression of EP_1, EP_3, and EP_4 receptors is observed in the gastric mucosa of mice by *in situ* hybridization analysis. However, there have been only a few studies on the relationship between EP receptor subtypes and gastric functions, i.e. EP_2/EP_3 receptors in acid secretion [6, 7] and EP_1 receptor in bicarbonate secretion [8].

Gastric mucus is well known to play an important role in mucosal protection by forming a viscoelastic layer [9]. The promotion of mucus secretion induced by PGE_2 is considered to lead to mucosal protection [1, 2, 9], and several studies have demonstrated that PGE_2 and PGE analogues stimulate mucus secretion by cultured gastric epithelial cells [10, 11]. However, the EP receptor subtype mediating mucus secretion in gastric epithelial cells has not been characterized.

Therefore, we investigated the EP receptor subtype mediating the PGE_2-stimulated mucus secretion in rabbit gastric epithelial cells, using EP receptor-selective agonists. Furthermore, we examined the intracellular signal transduction of EP receptor-mediated mucus secretion in epithelial cells.

MATERIALS AND METHODS

Materials

PGE_2, 17-phenyl-PGE_2, and 11-deoxy-PGE_1 were purchased from Cayman Chemicals. Sulprostone, butaprost, and 16,16-dimethyl-PGE_2 were provided by the Ono Pharmaceutical Co. Indomethacin, forskolin, 3-isobutyl-1-methylxanthine, dibutyryl cAMP (Sigma), SQ22536 [9-(tetrahydro-2′-furyl)adenine], calphostin C (Research Biochemicals International), and H-89 (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinoline sulfonamide; Seikagaku Kogyo) were used. SQ22536 was dissolved in distilled water. The other agents were dissolved in ethanol or DMSO, followed by dilution with medium to the desired concentration. The final concentrations of ethanol and DMSO were less than 0.25%, at which concentration cell viability and mucus secretion were unaffected. Indometha-

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§ *Abbreviations*: PG, prostaglandin; cAMP, cyclic AMP; cDNA, complementary DNA; and 17-phenyl-PGE_2, 17-phenyl-ω-trinor-PGE_2.

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cin, 3-isobutyl-1-methylxanthine, SQ22536, and the protein kinase inhibitors were added 30 min before stimulation with PGE_2 or PGE analogues. All other chemicals used were of reagent grade.

Preparation of Gastric Epithelial Cells

Gastric epithelial cells were prepared from rabbit stomachs as described previously [12, 13]. In brief, male Japanese White rabbits (Nihon SLC), weighing 2.5 to 3.5 kg, were anesthetized with Nembutal (50 mg/kg, i.v.; Abbott). After the stomach had been excised, the surface of the oxyntic mucosa was removed with a razor blade and minced immediately. The minced tissue was incubated in Hanks' balanced salt solution containing 0.07% collagenase (Wako Pure Chemicals) and 1 mg/mL of BSA for 15 min at 37°, and then washed with Ca^{2+}- and Mg^{2+}-free Hanks' solution containing 1 mg/mL of BSA and 1 mM EDTA. These procedures were repeated twice. The epithelial cells were obtained by filtration through metal meshes (pore sizes, 300 and 100 μm). The viability of the isolated cells was more than 85%, as determined by the dye exclusion test [14].

Cell Culture

Coon's modified Ham's F12 medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL), 100 U/mL of penicillin, 100 U/mL of streptomycin, and 0.25 μg/mL of amphotericin B, and culture plates and dishes coated with collagen type I (Sigma) were used. Gastric epithelial cells (2×10^5/2 mL) were inoculated onto 12-well plates. For measurement of cAMP content, cells (2×10^6/5 mL) were inoculated onto 35-mm dishes. The cultures were maintained at 37° under 5% CO_2 in air, the medium being changed daily. The cells were allowed to grow to confluence for 2 or 3 days. Most of the cultured cells were morphologically epithelial-like, and 80–90% of them were confirmed to be mucus-producing cells by periodic acid-Schiff staining [12].

Northern Blot Analysis

Total RNAs were extracted from gastric epithelial cells and from rabbit kidney and thymus by means of the acid guanidinium thiocyanate-phenol-chloroform method [15], using TRIZOL Reagent (GIBCO BRL). Subsequently, poly $(A)^+$ RNAs were purified with Oligotex dT30 (TaKaRa). Poly $(A)^+$ RNAs (0.5 μg) were separated by electrophoresis on 1.2% agarose gels and then were transferred to nylon membranes (Gene Screen Plus; New England Nuclear). cDNA probes for mouse EP_1 [16], EP_2 [17], EP_3 [18], and EP_4 [19, 20] receptors (supplied by Drs. Y. Sugimoto and A. Ichikawa, Kyoto University) were ^{32}P-labeled by the random primer method (Ready-To-Go; Pharmacia LKB Biotech). Hybridization was carried out overnight at 58° in 6x SSC (900 mM NaCl and 90 mM sodium citrate) containing 5x Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% BSA) and 0.5% SDS, and the membranes were washed at 60° in 2x SSC containing 1% SDS. The detection of hybridized mRNAs was performed with an imaging analyzer (BAS-5000MAC; Fuji Film).

Determination of Mucus Secretion

Mucus secretion was assessed as secretion of high M_r prelabeled glycoproteins, as described previously [11]. Gastric epithelial cells grown to confluence were incubated with 0.5 mL of medium containing [^{3}H]glucosamine (37 kBq, 2.0 TBq/mmol; New England Nuclear) at 37° for 24 hr. Medium alone, without cells, was used as a blank. After being washed with PBS, epithelial cells were incubated in 0.5 mL of medium containing PGE_2, PGE analogues, or dibutyryl cAMP for 4 hr. To exclude the influence of endogenous PGs, epithelial cells were treated with 10 μM indomethacin, which potently inhibited PGE_2 production to below 10% of the normal value. Thereafter, 0.1 mL of 5 mM dithiothreitol solution was added to each well, and the cells were held on ice for 10 min to detach mucus glycoproteins from the cell surface. The medium was recovered, followed by centrifugation at 500 *g* for 2 min. The resulting supernatant was mixed with 0.1 mL of 5% Triton X-100. An aliquot (0.5 mL) of the mixture was applied onto a Sepharose CL-4B column (6 mL), which had been equilibrated with 10 mM sodium phosphate buffer (pH 7.2) containing 150 mM NaCl, 0.1 mM dithiothreitol, and 1% Triton X-100. The radioactivity in the void fractions was measured with a liquid scintillation counter (Beckman Instruments). About 70% of the total radioactivity loaded on the column was eluted in the void fractions.

Determination of cAMP Production

Gastric epithelial cells grown to confluence were incubated at 37° in 2 mL of medium containing PGE_2, PGE analogues, or forskolin in the presence of 2 mM 3-isobutyl-1-methylxanthine for 1 hr. To exclude the influence of endogenous PGs, epithelial cells were treated with 10 μM indomethacin. Thereafter, the medium was discarded, and 1 mL of ice-cold 5% trichloroacetic acid was added. Ten minutes later, the cells were scraped and sonicated, followed by centrifugation at 1000 *g* for 15 min. cAMP was extracted twice from the supernatant with 8 mL of water-saturated diethyl ether. After the diethyl ether was evaporated, cAMP content in the residue was measured using a cAMP ELISA kit (Cayman Chemicals).

Statistical Analysis

The data are presented as means ± SEM for 8 cultures. Statistical differences in the concentration-response studies were evaluated by Dunnett's multiple comparison test.

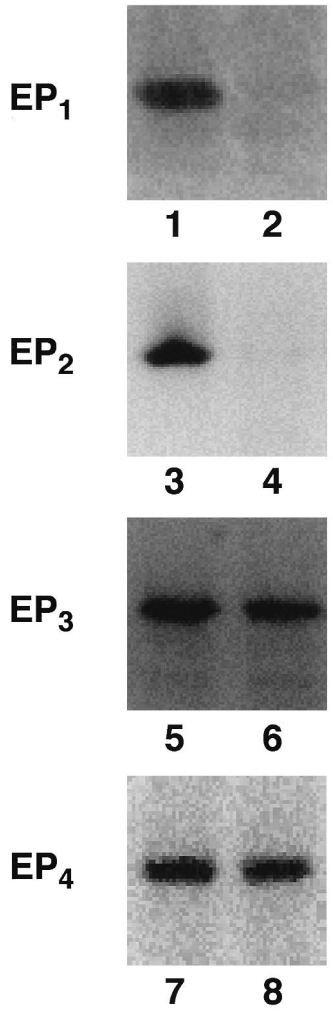

FIG. 1. EP receptor mRNA expression in rabbit gastric epithelial cells. Poly $(A)^+$ RNAs (0.5 μg) were subjected to northern blot analysis. Lanes 1 and 5, kidney; lanes 2, 4, 6, and 8, gastric epithelial cells; lanes 3 and 7, thymus.

Student's *t*-test was applied for the comparison between two groups. Values of $P < 0.05$ were regarded as significant.

RESULTS

We examined the mRNA expression of EP_1-EP_4 receptors in rabbit gastric epithelial cells (Fig. 1). Kidney and thymus poly $(A)^+$ RNAs were used as positive controls. Northern blot analysis revealed that both EP_3 and EP_4 receptor mRNAs are expressed in gastric epithelial cells. In contrast, neither EP_1 nor EP_2 receptor mRNAs were detected. This indicates that gastric epithelial cells possess two subtypes (EP_3 and EP_4) of PGE receptors.

To clarify the EP receptor subtype involved in stimulation of mucus secretion, we examined the effects of PGE analogues on mucus secretion by gastric epithelial cells (Fig. 2A). Gastric epithelial cells secreted mucus glycoproteins without any external stimulus. When epithelial cells were incubated with PGE_2 for 4 hr, mucus secretion was stimulated in a concentration-dependent manner. 11-Deoxy-PGE_1 (an $EP_3/EP_4/EP_2$ agonist) and 16,16-dimethyl-PGE_2 (an $EP_3/EP_2/EP_4$ agonist) also concentration-dependently increased the secretion. In contrast, mucus secretion was not affected by 17-phenyl-PGE_2 (an EP_3/EP_1 agonist), sulprostone (an EP_3/EP_1 agonist), or butaprost (an EP_2 agonist). The mucus secretion stimulated by these agents at the highest concentration is summarized in Fig. 2B. PGE_2 at 0.3 μM, 11-deoxy-PGE_1 at 3 μM, and 16,16-dimethyl-PGE_2 at 3 μM significantly stimulated secretion to about 190, 170, and 180%, respectively, of that in the control. 11-Deoxy-PGE_1 and 16,16-dimethyl-PGE_2 had nearly the same potency in stimulating mucus secretion, although their effects were slightly weaker than that of PGE_2.

It is known that PGE_2 and PGE analogues increase cAMP production in gastric epithelial cells [21, 22]. Therefore, the effects of PGE analogues on cAMP production

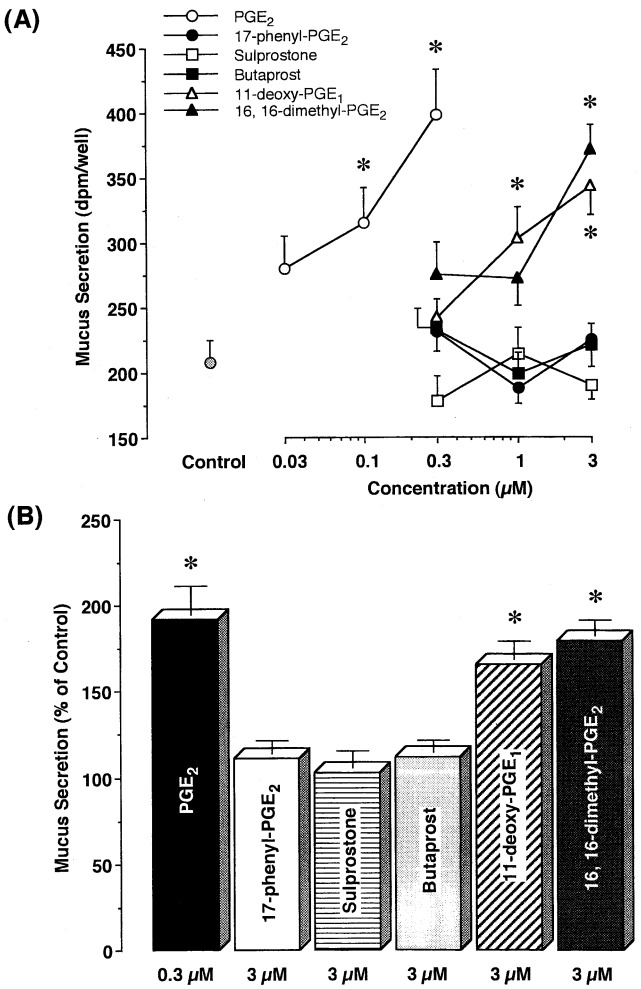

FIG. 2. (A) Concentration-dependent effects of various EP agonists on mucus secretion by rabbit gastric epithelial cells. After the cells had been cultured in the presence of $[^3H]$glucosamine, they were incubated further with the indicated prostanoids for 4 hr. The radioactivity of high M_r glycoproteins released into the medium was determined. (B) Effects of various EP agonists at the highest concentration on mucus secretion. Data are presented as means ± SEM for 8 cultures. Key: (*) statistically significant difference from the control, $P < 0.05$.

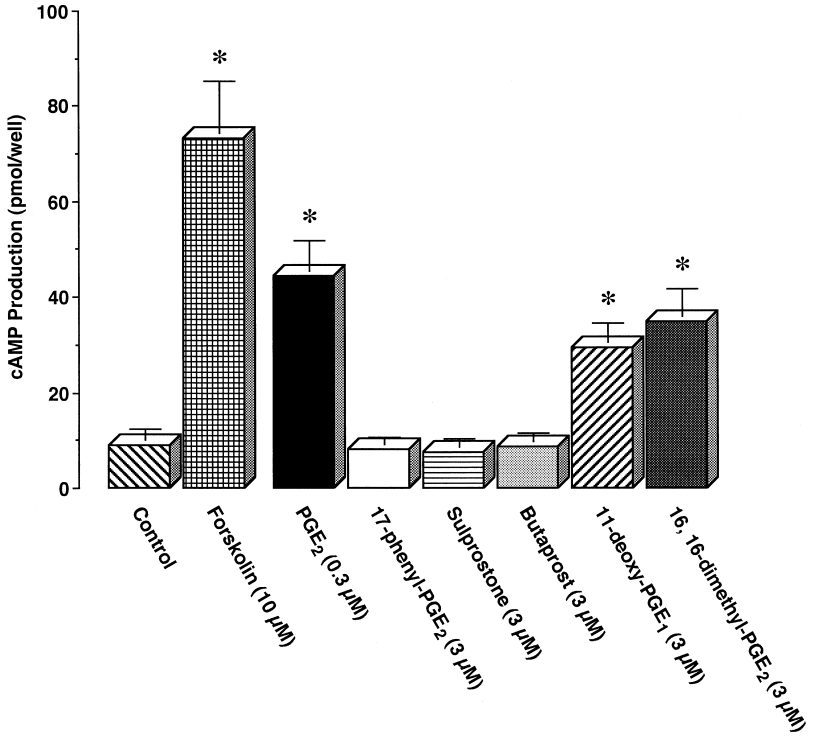

FIG. 3. Effects of various EP agonists on cAMP production in rabbit gastric epithelial cells. The cells were incubated with the indicated prostanoids for 1 hr. cAMP content in the cells was determined by ELISA. Data are presented as means ± SEM for 8 cultures. Key: (*) statistically significant difference from the control, $P < 0.05$.

were examined (Fig. 3). Forskolin (an adenylate cyclase activator) at 10 μM and PGE_2 at 0.3 μM stimulated cAMP production significantly. 11-Deoxy-PGE_1 at 3 μM and 16,16-dimethyl-PGE_2 at 3 μM significantly increased cAMP production by 3.4- and 3.8-fold, respectively. However, 17-phenyl-PGE_2, sulprostone, and butaprost at 3 μM failed to elevate cAMP production.

Exposure of gastric epithelial cells to 1 mM dibutyryl cAMP caused a significant increase in mucus secretion, the increase rate being 65.1%. When the cells were treated with SQ22536 (an adenylate cyclase inhibitor), the increased cAMP production induced by 0.3 μM PGE_2, 3 μM 11-deoxy-PGE_1, and 3 μM 16,16-dimethyl-PGE_2 was inhibited (Fig. 4). SQ22536 concentration-dependently reduced the increase in cAMP production caused by the prostanoids. The effects of the prostanoids were abolished nearly completely by 1 mM SQ22536. The basal cAMP level also was reduced by 1 mM SQ22536 to about 30% of the normal value.

In addition, we examined the effects of SQ22536, H-89 (a protein kinase A inhibitor), and calphostin C (a protein kinase C inhibitor) on mucus secretion stimulated by 0.3 μM PGE_2, 3 μM 11-deoxy-PGE_1, and 3 μM 16,16-dimethyl-PGE_2 (Fig. 5). Similar to inhibition of cAMP production, 1 mM SQ22536 potently suppressed the mucus secretion in response to PGE_2, 11-deoxy-PGE_1, and 16,16-dimethyl-PGE_2. H-89 also exerted a potent inhibitory effect on the stimulated mucus secretion. The secretion stimulated by dibutyryl cAMP (1 mM) also was abolished completely by 1 mM H-89. SQ22536 and H-89 slightly reduced basal secretion to 86.3 and 80.2% of the normal value, respectively, but their effects were not significant. In contrast, calphostin C had no effect on stimulated or basal mucus secretion.

The effects of the prostanoids and dibutyryl cAMP on cell viability also were examined. Treatment with the

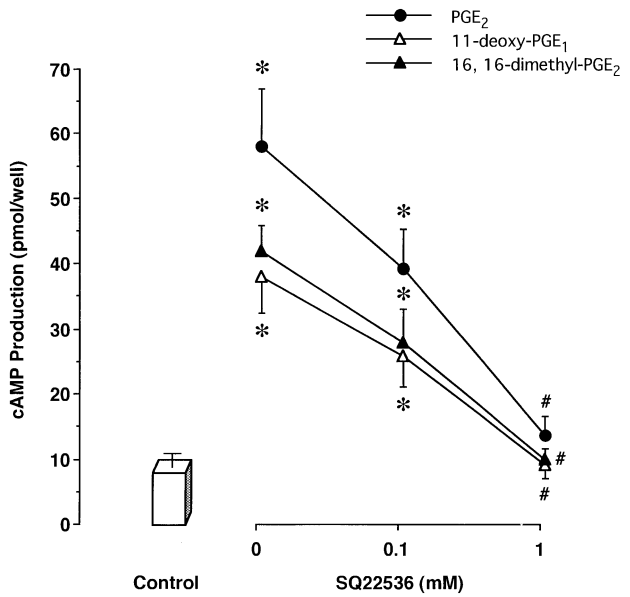

FIG. 4. Effect of SQ22536 on the increased cAMP production induced by PGE_2, 11-deoxy-PGE_1, and 16,16-dimethyl-PGE_2. Gastric epithelial cells were incubated with the indicated prostanoids and/or SQ22536 for 1 hr. cAMP content in the cells was determined by ELISA. Data are presented as means ± SEM for 8 cultures. Key: (*, #) statistically significant difference from the control (basal) and the corresponding prostanoid alone (without SQ22536), respectively, $P < 0.05$.

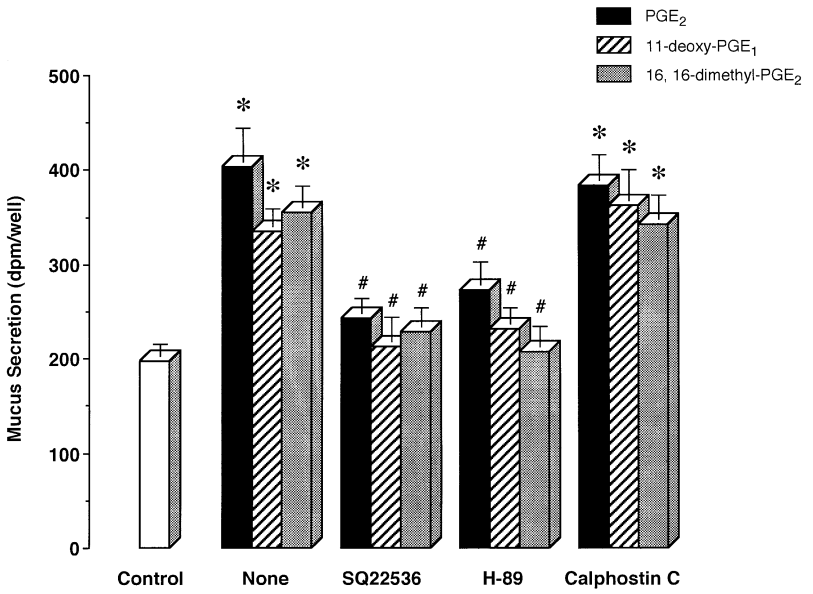

FIG. 5. Effects of SQ22536, H-89, and calphostin C on the mucus secretion stimulated by PGE_2, 11-deoxy-PGE_1, and 16,16-dimethyl-PGE_2. After the cells had been cultured in the presence of [^{3}H]glucosamine, they were incubated further with the indicated prostanoids and/or inhibitors for 4 hr. The radioactivity of high M_r glycoproteins released into the medium was determined. Data are presented as means ± SEM for 8 cultures. Key: (*, #) statistically significant difference from the control and the corresponding prostanoid (without SQ22536), respectively, $P < 0.05$.

agents did not cause any loss of cell viability, as determined by the dye exclusion test (data not shown).

DISCUSSION

Hassan *et al*. [23] claimed that PGE_2 stimulates mucus synthesis by rat gastric epithelial RGM1 cells, probably through the EP_4 receptor. However, they did not exclude the possibility that the effect of PGE_2 may be mediated through the EP_2 receptor, since there were no data on EP_2 receptor expression or the effects of EP agonists on the synthesis. In addition, their measurement of total hexosamine content in the cells is not suitable for determination of the amount of mucus glycoproteins, because not all hexosamines are incorporated into high M_r mucus glycoproteins. In contrast, we examined the mRNA expression of all EP receptors and assessed the secretion of high M_r glycoproteins as mucus secretion. In our study, release of the radioactivity of high M_r glycoproteins into the medium reflected secretion of mucus glycoproteins by gastric epithelial cells. The possibility that the stimulation of mucus secretion was due to disruption of membrane integrity of the epithelial cells was ruled out, because we confirmed that the viability of the epithelial cells was not affected by treatment with the agents used here.

In the present study, we clarified the EP receptor subtype mediating PGE_2-stimulated mucus secretion by rabbit gastric epithelial cells. Northern blot analysis revealed that only EP_3 and EP_4 receptor mRNAs are expressed in gastric epithelial cells. Therefore, to determine which EP receptor subtype mediates the effect of PGE_2, a pharmacological study was also performed. The ligands stimulating mucus secretion have high affinity for the EP_4 receptor. The K_i values of PGE_2, 11-deoxy-PGE_1, and 16,16-dimethyl-PGE_2 for the EP_4 receptor are reported to be 1.9, 23, and 43 nM, respectively [24]. 11-Deoxy-PGE_1 and 16,16-dimethyl-PGE_2 also can bind to EP_2 and EP_3 receptors with affinities similar to those of PGE_2. However, neither 17-phenyl-PGE_2 nor sulprostone affected mucus secretion, although their affinities for EP_3 and EP_1 receptors are nearly the same as those of PGE_2 [24]. An EP_2-specific agonist, butaprost, also had no effect on the secretion. The stimulatory effects of PGE_2, 11-deoxy-PGE_1, and 16,16-dimethyl-PGE_2 on mucus secretion were associated with their affinities for the EP_4 receptor. These results indicate that the EP_4 receptor is essential for PGE_2-stimulated mucus secretion by rabbit gastric epithelial cells.

We previously reported that rabbit gastric epithelial cells constitutively produce PGE_2, and the concentration of PGE_2 in the medium is around 0.05 μM [14]. Based on the present results, endogenous PGE_2 is likely to regulate normal basal mucus secretion in an autocrine manner.

Furthermore, we examined the intracellular mechanism by which PGE_2 stimulates mucus secretion by gastric epithelial cells. It has been reported that exposure of gastric epithelial cells to PGE_2 and PGE analogues induces an increase in cAMP production [21, 22], but there has been no evidence that cAMP plays a key role in the PGE_2-stimulated secretion of gastric mucus. As we confirmed here that dibutyryl cAMP enhances mucus secretion, cAMP is considered to be one of the intracellular mediators stimulating mucus secretion. Among PGE receptor subtypes, the EP_2 and EP_4 receptors are linked to the stimulatory GTP-binding protein/adenylate cyclase system [3, 4]. Expression of EP_4 receptor mRNA was observed, but EP_2 receptor mRNA was undetectable in gastric epithelial cells. 11-Deoxy-PGE_1 and 16,16-dimethyl-PGE_2 promoted cAMP production, but the other PGE analogues including butaprost had no effect. These results indicate that only the EP_4 receptor is involved in the increased cAMP production in response to PGE_2. In addition, the inhibition by SQ22536 of EP_4 receptor-linked cAMP production resulted in a decrease in stimulated mucus secretion. H-89, but not calphostin C, also potently reduced the EP_4 receptor-

dependent increase in mucus secretion. These results strongly suggest that PGE_2 activates the cAMP/protein kinase A pathway via the EP_4 receptor, thereby leading to the promotion of mucus secretion in gastric epithelial cells.

Both SQ22536 and H-89 failed to suppress basal mucus secretion significantly. In this study, experiments were carried out in the presence of indomethacin to exclude the influence of endogenous PGs. Under this condition, the cAMP level and mucus secretion were significantly lower than in normal cells without indomethacin. Consequently, it seems that the effects of SQ22536 and H-89 on basal secretion of mucus did not appear clearly.

Morimoto *et al.* [5] observed mRNA expression of EP_3 and EP_4 receptors in epithelial cells in the mouse stomach. Our results are consistent with their finding. Hassan *et al.* [23] also confirmed the presence of EP_4 mRNA in rat gastric epithelial RGM1 cells. However, the role of the EP_3 receptor in gastric epithelial cells remains unknown. Further study is needed for elucidation of the physiological significance of the EP_3 receptor in gastric epithelial cells.

We conclude that PGE_2 promotes mucus secretion by rabbit gastric epithelial cells, mediated through EP_4 receptor stimulation and the subsequent activation of protein kinase A.

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