Inhibition of ACh-stimulated exocytosis by NSAIDs in guinea pig antral mucous cells: autocrine regulation of mucin secretion by PGE$_2$

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Shimamoto, Chikao, Shoko Fujiwara, Masumi Kato, Shigenori Ito, Ken-ichi Katsu, Hiroshi Mori, and Takashi Nakahari. Inhibition of ACh-stimulated exocytosis by NSAIDs in guinea pig antral mucous cells: autocrine regulation of mucin secretion by PGE$_2$. Am J Physiol Gastrointest Liver Physiol 288: G39–G47, 2005. First published September 2, 2004; doi:10.1152/ajpgi.00060.2004.—The effects of indomethacin (IDM) and aspirin (ASA) on ACh (10 μM)-stimulated exocytotic events were studied in guinea pig antral mucous cells by using video optical microscopy. IDM or ASA, which inhibits cyclooxygenase (COX), decreased the frequency of ACh-stimulated exocytotic events by 30% or 60%, respectively. The extent of inhibition induced by ASA (60%) decreased by 30% when IDM or arachidonic acid (AA, the substrate of COX) was added. IDM, unlike ASA, appears to induce the accumulation of AA, which enhances the frequency of ACh-stimulated exocytotic events in ASA-treated cells. ONO-8713 (100 μM; an inhibitor of the EP1–EP4 prostaglandin receptors) and N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide, HCl (H-89, 20 μM; an inhibitor of PKA) also decreased the frequency of ACh-stimulated exocytotic events by 60%. However, the supplementation of PGE$_2$ (1 μM) prevented the IDM-induced decrease in the frequency of ACh-stimulated exocytotic events. SC-560 (an inhibitor of COX-1) decreased the frequency of ACh-stimulated exocytotic events by 30%, but NS-398 (an inhibitor of COX-2) did not. Moreover, IDM decreased the frequency of exocytotic events stimulated by ionomycin, suggesting that COX-1 activity is stimulated by an increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). ACh and ionomycin increased PGE$_2$ release in antral mucosal cells. In conclusion, in ACh-stimulated antral mucous cells, an increase in [Ca$^{2+}$] activates Ca$^{2+}$-regulated exocytotic events and PGE$_2$ release mediated by COX-1. The released PGE$_2$ induces the accumulation of cAMP, which enhances the Ca$^{2+}$-regulated exocytosis. The autocrine mechanism mediated by PGE$_2$ maintains the high-level mucin release from antral mucous cells during ACh stimulation.

gastric mucin secretion; cyclic adenosine 5’-monophosphate; cyclooxygenase-1; indomethacin; aspirin

MUCIN EXOCYTOSIS in guinea pig antral mucous cells is regulated by two mechanisms: Ca$^{2+}$-regulated exocytosis and cAMP-regulated exocytosis (8, 13, 15–17). The Ca$^{2+}$-regulated exocytosis is the major mechanism operating in antral mucous cells, because the frequency of exocytosis induced by cAMP accumulation was <10% of that induced by the increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) (8, 13, 16, 17). The Ca$^{2+}$-regulated exocytosis in pancreatic acinar cells consists of two biochemically distinct steps: ATP-dependent priming and Ca$^{2+}$-dependent fusion (19, 32). In our previous study (13), cAMP accumulation was shown to enhance the Ca$^{2+}$-regulated exocytosis by increasing the number of primed granules and Ca$^{2+}$ sensitivity of fusion in antral mucous cells. This cAMP modulation of Ca$^{2+}$-regulated exocytosis may play an important role in mucin release from antral mucous cells.

Many secretagogues, such as PGE$_2$, β-adrenergic agonists, gastrin, secretin, and histamine, induce the accumulation of cAMP in the gastric mucosa (7). PGE$_2$, which induces the accumulation of cAMP in antral mucous cells via the EP4 prostaglandin receptor (16, 17), protects the gastric mucosa from lesions. In contrast, the inhibition of PGE$_2$ production by NSAIDs, such as indomethacin (IDM) or aspirin [acetylsalicylic acid (ASA)], induces gastric lesions. The pathogenic mechanisms inducing these lesions are considered to involve multiple factors, such as a decrease in mucin amount, inhibition of glycoprotein synthesis, and gastric hypermotility (20–22, 26, 27, 34); the decrease in mucin release may also be an important factor inducing gastric lesions, especially during a meal.

On the other hand, cholinergic stimulations were reported to enhance PGE$_2$ release from the gastric mucosa (5, 7, 21), and P2Y$_2$ receptor stimulation was reported to induce PGE$_2$ release from the rat inner medullary collecting duct (31). Both cholinergic and purinergic stimulations increase [Ca$^{2+}$]. PGE$_2$ is generated from arachidonic acid (AA) mediation via cyclooxygenase (COX), which is a Ca$^{2+}$-dependent enzyme (9). In ACh-stimulated antral mucous cells, an increase in [Ca$^{2+}$], may also stimulate PGE$_2$ release. The released PGE$_2$, which induces cAMP accumulation, may enhance Ca$^{2+}$-regulated exocytosis in antral mucous cells (15). Therefore, the inhibition of PGE$_2$ synthesis may decrease the frequency of ACh-stimulated exocytotic events. NSAIDs, which inhibit COX, are a tool for inhibiting PGE$_2$ synthesis. The goal of the present study is to confirm whether released PGE$_2$ enhances Ca$^{2+}$-regulated exocytotic events in ACh-stimulated guinea pig antral mucous cells.

MATERIALS AND METHODS

Solutions and chemicals. Solution I contained (in mM): 121 NaCl, 4.5 KCl, 25 NaHCO$_3$, 1 MgCl$_2$, 1.5 CaCl$_2$, 5 Na-HEPES, 5 H-HEPES, and 5 glucose. To prepare a NaCl-free solution, CaCl$_2$ was excluded from solution I and 1 mM EGTA was added. The pHs of the solutions were all adjusted to 7.4 by adding HCl (1 M). The solutions were gassed with 95% O$_2$-5% CO$_2$ at 37°C. N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide, HCl (H-89, 20 μM; an inhibitor of PKA) was added. IDM or ASA, which inhibits cyclooxygenase (COX), which is a Ca$^{2+}$ buffering agent (13). PGE$_2$ (1 μM) was added to the solution. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
namyl) amino) ethyl]-5-isoquinolinesulfonamide, HCl (H-89), ionomycin, SC-560, and NS-398 were purchased from Calbiochem-Novabiochem (La Jolla, CA). ACh was from Daiichi Pharmaceuticals (Osaka, Japan), and PGE2, IBMX, IDM, ASA, collagenase (for cell dispersion; 180–220 U/mg), and BSA were from Wako Pure Chemical Industries (Osaka, Japan). ONO-8713 was a generous gift from ONO Pharmaceutical (Osaka, Japan). All reagents were dissolved in DMSO and were prepared to their final concentrations immediately before the experiments. The concentration of DMSO never exceeded 0.1%, and, at this concentration, DMSO does not have any effect on the exocytotic events in antral mucous cells.

Cell preparations. Hartley-strain male guinea pigs weighing ~250 g were purchased from Shimizu Experimental Animals (Kyoto, Japan) and were maintained on standard pellet food and water. The guinea pigs were anesthetized by inhalation of ether, after which they were killed by cervical dislocation. The experiments were approved by the Animal Research Committee of Osaka Medical College, and the animals were cared for according to the guidelines of this committee. The procedures for cell preparation have been previously described in detail (8, 15, 16, 25). Briefly, the antrum was excised and the mucosal layer was stripped from the muscle layer in cooled saline (4°C). The suspension was stored at 4°C and used in the experiments within 3 h.

Observation of exocytosis. Isolated antral mucous cells were mounted on a coverslip precoated with neutralized Cell-Tak (Becton Dickinson Labware, Bedford, MA) for the firm attachment of cells. The coverslip with cells was set in a perfusion chamber that was mounted on the stage of a differential interference contrast microscope (BX50Wi; Olympus, Tokyo, Japan) connected to a video-enhanced contrast system (model ARGUS-10; Hamamatsu Photonics, Hamamatsu, Japan) (1, 8, 10, 13–17). Images were recorded continuously with a video recorder. The experiments were performed at 37°C. The volume of the perfusion chamber was ~20 μl, and the rate of perfusion was 200 μl/min. Exocytotic events, which were detected as rapid changes in the light intensity of granules (1, 3, 10, 11, 13, 14, 16, 17), were counted in five to six cells every 30 s and were normalized to the number of events (events per cell per 30 s). The frequencies of exocytotic events in three to seven experiments were expressed as means ± SE. Our previous report showed that ACh (10 μM) increased the frequency of exocytotic events transiently (8, 13); therefore, the initial peak frequency that was observed within 2 min from the start of stimulation (events per cell per 30 s) was used for comparison.

[Ca2+]i measurements. The isolated antral mucous cells were incubated in solution I containing 2% BSA and 2.5 μM fura-2 AM (Dojindo, Kumamoto, Japan) for 25 min at room temperature (22–24°C). They were then washed with solution I containing 2% BSA three times. Fura-2-loaded cells were resuspended and stored in the solution I-containing 2% BSA at 4°C and then mounted on a coverslip precoated with neutralized Cell-Tak. The coverslip with cells was set in a perfusion chamber, which was then mounted on the stage of an inverted microscope (IX70; Olympus, Tokyo, Japan) connected to an image-analysis system (ARGUS/HiSCA; Hamamatsu Photonics, Hamamatsu, Japan) (8, 13, 14, 16, 33). All experiments were performed at 37°C. The volume of the perfusion chamber was ~80 μl, and the rate of perfusion was 500 μl/min. Fura-2 was excited at 340 nm and 380 nm, and emission was measured at 510 nm. Fluorescence ratio (F340/F380) was calculated and stored in the image-analysis system. The calibration curve was obtained from F340/F380 of the cell-free Ca2+ calibration solutions containing 10 μM fura-2. Solution II contained (in mM) 130 KCl, 20 NaCl, 2 EGTA, and 10 HEPES. To prepare the cell-free Ca2+-calibration solutions, an appropriate amount of CaCl2 (0.2–2 mM), calculated by using a computer program, was added to solution II. The pH was adjusted to 7.05 by adding KOH (1 M). The dissociation constant of Ca2+ and EGTA used was 214 nM (37°C, pH 7.05) (12). One experiment was performed using five to six coverslips, and F340/F380s of three cells on two to three coverslips were expressed as means ± SE.

Measurement of cAMP. The stripped antral mucosa was incubated for 5 min at 37°C in the control solution (10 ml) aerated with a gas mixture (95% O2-5% CO2), to which ACh (10 μM), ACh (10 μM) + IDM (10 μM), ionomycin (1 μM), ionomycin (1 μM) + IDM (10 μM), PGE2 (1 μM), or IBMX (1 mM) was added. In the control experiment, DMSO (10 μl) was added to the control solution. After a 5-min incubation, the antral mucosa was immediately frozen in liquid nitrogen and lyophilized at ~80°C for 24–48 h. After the lyophilized, antral mucosa was weighed, its cAMP content was measured by using a cAMP EIA kit (Amersham International, Little Chalfont, England). The cAMP contents were expressed as moles per milligram of dry weight (14, 16).

Measurement of PGE2. The stripped antral mucosa was weighed and then stored in the control solution at 4°C for 30 min before the start of experiments. After the 2-min warmup period, the stripped antral mucosa was incubated in the control solution (10 ml) and aerated with a gas mixture (95% O2-5% CO2) for 10 min before the addition of ACh (10 μM), ionomycin (10 μM), ASA (10 μM), or IDM (10 μM) and was then incubated for a further 10 min. In the control experiments, DMSO (10 μl) was added instead of ACh or ionomycin. When the inhibitors (ASA and IDM) were used with ACh or ionomycin, the antral mucosa, after the 2-min warmup period, was incubated with the inhibitors for 10 min before the stimulation with ACh or ionomycin. The incubation solution of 500 μl was transferred to a microtube immediately before and 10 min after the stimulation. The microtube with sample was immediately cooled on ice and stored at −30°C until the PGE2 contents assay. PGE2 contents were measured by using a PGE2 EIA kit (Cayman Chemical, Ann Arbor, MI) and were expressed as micrograms per gram of wet weight tissue. The released PGE2 for 10 min was calculated from the difference between values before and 10 min after the stimulation.

The statistical significance of the difference between mean values was assessed by using ANOVA as appropriate. Differences were considered significant at P < 0.05.

RESULTS

In the present study, the concentration of ACh used was 10 μM. ACh (10 μM) induced submaximal responses in exocytotic events and increased the frequency of exocytotic events transiently in antral mucous cells (8, 13). In the control experiments, the frequencies of ACh-stimulated exocytotic events 1 min and 4 min after ACh stimulation were 48.6 ± 1.2 events·cell−1·30 s−1 (n = 5) and 5.0 ± 1.2 events·cell−1·30 s−1, respectively (Fig. 1A).

Effects of NSAIDs. The dose effects of IDM on the frequency of ACh-stimulated exocytotic events were examined. The cells were treated with IDM for 10 min before ACh stimulation. In unstimulated antral mucous cells, low concentrations (0.1–1 μM) of IDM did not induce any exocytotic events. However, high concentrations (10 or 100 μM) of IDM slightly increased the frequency of exocytotic events (1–3 events·cell−1·30 s−1) in some cells 5–8 min after IDM addition (10/25 experiments). The subsequent stimulation with ACh increased the frequency of exocytotic events. A low concentration of IDM (1 μM) had no effect on the frequency.
of ACh-stimulated exocytotic events (Fig. 1A). However, IDM (10 μM) decreased the initial peak frequency of ACh-stimulated exocytotic events by ~30% (initial peak frequency = 31.6 ± 4.2 events·cell⁻¹·30 s⁻¹; n = 5; Fig. 1B). Similar results were also obtained by using 100 μM IDM (Fig. 1C). The dose effects of IDM on the initial peak frequencies are summarized in Fig. 1D. The initial peak frequencies were significantly smaller in the presence of 10–100 μM IDM than in the control experiments (P < 0.05).

The effects of ASA were also examined (Fig. 2). ASA (0.01–100 μM), unlike IDM (10–100 μM), did not induce any exocytotic events in unstimulated antral mucous cells. Pretreatment with ASA for 10 min suppressed ACh-stimulated exocytotic events in a dose-dependent manner. ASA (10 μM) decreased the initial peak frequency by ~60%. The initial peak frequency of ACh-stimulated exocytotic events was 19.9 ± 2.9 events·cell⁻¹·30 s⁻¹ (n = 5; Fig. 2A). The dose effects of ASA on ACh-stimulated exocytotic events are summarized in Fig. 2B. The initial peak frequencies were significantly smaller in the presence of 1–100 μM ASA than those in the control experiments (P < 0.05).

ASA (10 μM) decreased the frequencies of ACh-stimulated exocytotic events to a greater extent than IDM (10 μM) (P < 0.05). ASA and IDM differ in their pharmacological actions, such as binding site and binding reversibility, although they both inhibit PGH synthesis. COX-1 and -2 are capable of synthesizing (15R)-15-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid (15R-HETE) in addition to PGH. ASA, which induces acetylation of COX-1 and -2, inhibits PGH synthesis but not 15R-HETE synthesis (4, 18, 24). However, IDM inhibits the synthesis of both 15R-HETE and PGH (4). These reports suggest that IDM may induce the accumulation of AA, which is a substrate of COX. The accumulation of AA may modulate Ca²⁺-regulated exocytotic events, because AA is already known to mobilize Ca²⁺ from the intracellular stores (29). We examined the effects of IDM and AA on ASA-treated antral mucous cells. In Fig. 3A, cells were pretreated with both IDM (100 μM) and ASA (10 μM) for 10 min and then stimulated with ACh. The addition of IDM enhanced the initial peak frequency of ACh-stimulated exocytotic events in ASA-treated cells (34.6 ± 2.5 events·cell⁻¹·30 s⁻¹; n = 4). In Fig. 3B, cells were pretreated with both AA (20 μM) and ASA (10 μM) for 10 min and then stimulated with ACh. The addition of AA enhanced the initial peak frequency of ACh-stimulated exocytotic events in ASA-treated cells (34.3 ± 3.0 events·cell⁻¹·30 s⁻¹; n = 4). Thus IDM is suggested to induce accumulation of AA, which appears to enhance the frequency of ACh-stimulated exocytotic events.

**Effects of PKA inhibitor.** IDM or ASA inhibits PGE₂ synthesis in many cell types (7, 18, 31). Our previous reports (16, 17) showed that PGE₂ induces accumulation of cAMP mediated by the EP4 receptor in antral mucous cells. We then examined the effects of a PKA inhibitor (H-89; 20 μM). Cells were pretreated with H-89 (20 μM) for 5 min. H-89 (20 μM) did not induce any increase in the frequency of exocytotic events in unstimulated cells. The subsequent ACh stimulation increased the frequency of exocytotic events; however, the initial peak frequency of ACh-evoked exocytotic events de-
increased by ~60% compared with that of the control experiments (Fig. 4A). The initial peak frequency of ACh-stimulated exocytotic events was 20.7 ± 1.9 events·cell⁻¹·30 s⁻¹ (n = 4). When cells were pretreated with both ASA (10 μM) and H-89 (20 μM) for 5 min, ACh-stimulated exocytotic responses were inhibited similarly (the initial peak frequency = 19.1 ± 1.3 events·cell⁻¹·30 s⁻¹; n = 6). Cells were also pretreated with both IDM (10 μM) and H-89 (20 μM) for 5 min and subsequently stimulated with ACh. IDM decreased the initial peak frequency of ACh-evoked exocytotic events by ~30% in H-89-treated cells (Fig. 4B). The initial peak frequency of ACh-stimulated exocytotic events was 34.9 ± 3.7 (n = 4), which was similar to that observed in cells treated with IDM alone. Thus IDM, unlike ASA, still enhanced the initial peak frequency of ACh-stimulated exocytotic events in H-89-treated cells.

Effects of PGE2. The effects of PGE2 on ACh-stimulated exocytotic events were examined in IDM-treated antral mucous cells. The antral mucous cells were pretreated with 10 μM IDM for 5 min and then stimulated with PGE2 (1 μM) and ACh. Stimulation with PGE2 increased the initial peak frequency of ACh-stimulated exocytotic events by 15% even in the presence of 10 μM IDM (Fig. 5). The initial peak frequency was 56.3 ± 2.5 events·cell⁻¹·30 s⁻¹ (n = 5). However, in the presence of 20 μM H-89, PGE2 did not potentiate the frequencies of ACh-stimulated exocytotic events in IDM-treated cells, and the initial peak frequency was 35.0 ± 2.8 events·cell⁻¹·30 s⁻¹ (n = 5).

To confirm PGE2 release during ACh stimulation, an inhibitor of the EP receptor (100 μM ONO-8713) was used. A low concentration of ONO-8713 inhibits the EP1 receptor selectively; however, it inhibits other EP receptor subtypes at a high concentration, such as 100 μM [The binding affinity to the EP1 receptor is 0.13 nM and those to EP2, EP3, and EP4 are 2.8 μM, 3.2 μM, and 3.3 μM, respectively (Refs. 16 and 28 and personal communication from ONO Pharmaceutical)].

Cells were treated with ONO-8713 (100 μM) for 5 min and then stimulated with ACh. ONO-8713 (100 μM) alone did not increase the frequency of exocytotic events. The subsequent stimulation with ACh increased the frequency of exocytotic events. The initial peak frequency was 21.3 ± 1.0 events·cell⁻¹·30 s⁻¹ (n = 4). Thus ONO-8713 (100 μM) decreased the frequency of ACh-stimulated exocytotic events by ~60% (Fig. 6A).

The effects of ONO-8713 (100 μM) on PGE2-stimulated exocytotic events were examined. Cells were pretreated with ONO-8713 (100 μM) for 5 min. The subsequent addition of PGE2 increased the frequency of exocytotic events. The initial peak frequency was 21.3 ± 1.0 events·cell⁻¹·30 s⁻¹ (n = 4). Thus ONO-8713 (100 μM) decreased the frequency of ACh-stimulated exocytotic events by ~60% (Fig. 6A).

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The effects of ONO-8713 (100 μM) on PGE2-stimulated exocytotic events were examined. Cells were pretreated with ONO-8713 (100 μM) for 5 min. The subsequent addition of PGE2 increased the frequency of exocytotic events. The initial peak frequency was 21.3 ± 1.0 events·cell⁻¹·30 s⁻¹ (n = 4). Thus ONO-8713 (100 μM) decreased the frequency of ACh-stimulated exocytotic events by ~60% (Fig. 6A).
PGE₂ (1 μM) increased the frequency of exocytotic events only transiently, although PGE₂ (1 μM) sustained the frequency of exocytotic events without the pretreatment with ONO-8713 (Fig. 6B). Thus ONO-8713 (100 μM) inhibited EP₁ and EP₄ receptor subtypes in antral mucous cells.

Antral mucous cells were perfused with the Ca²⁺-free solution containing 1 μM ionomycin. The addition of 1 μM ionomycin induced a small transient increase in the frequency of exocytotic events during perfusion with the Ca²⁺-free solution. The perfusion solution was suddenly switched to the Ca²⁺-containing solution containing 1 μM ionomycin (reintroduction of Ca²⁺). The reintroduction of Ca²⁺ increased the frequency of the exocytotic events transiently, similar to ACh (Fig. 7). The initial peak frequency of the exocytotic events following the reintroduction of Ca²⁺ was 59.5 ± 3.4 events·cell⁻¹·30 s⁻¹ (n = 4). Similar experiments were performed in the presence of 10 μM IDM. The cells were perfused with the Ca²⁺-free solution containing 10 μM IDM for 5 min before ionomycin addition. Ionomycin (1 μM) did not induce any increase in the frequency of exocytotic events. The reintroduction of Ca²⁺ increased the frequency of exocytotic events; however, the initial peak frequency decreased by ~30%. The initial peak frequency of exocytotic events following the reintroduction of Ca²⁺ was 42.8 ± 3.9 events·cell⁻¹·30 s⁻¹ (n = 4). Thus IDM decreased the frequency of exocytotic events stimulated by ionomycin. These findings suggest that COX is stimulated by an increase in [Ca²⁺]ᵢ.
Effects of inhibitors of COX-1 and COX-2. Antral mucous cells were pretreated with 80 nM SC-560 (a specifically selective COX-1 inhibitor) and NS-398 (a specifically selective COX-2 inhibitor) for 2 min before ACh stimulation (Fig. 8). SC-560 (80 nM) induced small increases in the frequency of exocytotic events, similar to IDM, when cells were perfused with SC-560-containing solution for 5–7 min (data not shown). The subsequent ACh stimulation increased the frequency of exocytotic events transiently. However, the initial peak frequency decreased by ~30% (33.6 ± 2.5 events·cell⁻¹·30 s⁻¹; n = 5), compared with that in the control experiments (Fig. 8A). Similar experiments were performed using NS-398 (20 μM), NS-398 (20 μM) had no effects on unstimulated cells and ACh-stimulated cells (Fig. 8B). The initial peak frequency of ACh-stimulated exocytotic events was 45.3 ± 1.6 events·cell⁻¹·30 s⁻¹ (n = 4) in the presence of NS-398. These findings suggest that COX-1 generates PGE₂ from AA in guinea pig antral mucous cells.

[Ca²⁺]i measurement. Increases in [Ca²⁺]i were examined in both IDM-treated and non-IDM-treated antral mucous cells. Stimulation with ACh caused the fura-2 ratio (F340/F380) to increase rapidly and to be sustained in non-IDM-treated cells (Fig. 9A). Similar increases in F340/F380 were induced by ACh in IDM-treated cells (Fig. 9B). Thus, IDM, at least, did not decrease the [Ca²⁺]i during ACh stimulation.

cAMP content. The cAMP content was measured in the stripped antral mucosa, which was incubated for 5 min in the control or test solutions. The content of cAMP in the antral mucosa in the control solution was 3.8 ± 0.3 pmol/mg (n = 6). The results summarized in Table 1 show that cAMP content increased approximately twofold in cells stimulated with 10 μM ACh, 1 μM ionomycin, or 1 μM PGE₂ compared with those incubated with the control solution. However, in IDM-treated cells, ACh and ionomycin did not increase cAMP content. The increases in cAMP content evoked by 1 μM IBMX were ~15-fold greater than those evoked by 1 μM PGE₂.

PGE₂ release. The PGE₂ released from the stripped antral mucosa for 10 min was measured (Fig. 10). The amount of PGE₂ released for 10 min was 0.23 ± 0.03 μg/g mucosa (n = 3) in the control experiment. Addition of ASA (10 μM) or IDM (10 μM) decreased the spontaneous PGE₂ release by 30–50%. Stimulation with ACh (10 μM) or ionomycin (10 μM) increased PGE₂ release by 70–80%. IDM or ASA eliminated the increases in PGE₂ release induced by ACh or ionomycin in the antral mucosa. The values with agonists and inhibitors were significantly different from that of the control experiment (P < 0.05).

DISCUSSION

In antral mucous cells, ACh increased [Ca²⁺]i, which activates Cl⁻ and HCO₃⁻ secretion, cell shrinkage, and mucin exocytosis (8, 13, 16, 25). The present study, however, demonstrated that a PKA inhibitor (H-89) decreases the frequency of ACh-stimulated exocytotic events and that ACh increases the cAMP content in the antral mucosa. These suggest that ACh also stimulates cAMP accumulation in addition to [Ca²⁺]i increase in antral mucous cells. In our previous report, cAMP accumulation was shown to enhance the frequency of Ca²⁺-regulated exocytotic events by modulating priming and fusion steps, although the frequency of cAMP-regulated exocytotic events is <10% of that of Ca²⁺-regulated exocytotic events (8, 15–17). These observations indicate that cAMP accumulation induced by ACh stimulation enhances the frequency of Ca²⁺-regulated exocytotic events in antral mucous cells.
In antral mucous cells, however, an [Ca$^{2+}$]$_i$ increase is unlikely to directly stimulate adenyl cyclase, since IDM inhibited the increases in cAMP content and the frequency of exocytotic events during ACh stimulation. Moreover, ASA inhibited the frequency of ACh-stimulated exocytotic events, similar to H-89 (a PKA inhibitor). Thus mechanisms underlying Ca$^{2+}$-stimulated cAMP accumulation in antral mucous cells are not similar to those in salivary acinar cells.

On the other hand, IDM decreased the frequency of ACh-stimulated exocytotic events. However, the extent of inhibition induced by IDM was less than that induced by ASA or H-89 (a PKA inhibitor). As described in RESULTS, COX-1 and -2 generate 15R-HETE in addition to PGH. IDM inhibits the synthesis of both (4, 18, 24). ASA results in the synthesis of 15R-HETE, although it inhibits PGH synthesis (4, 18, 24). This suggests that IDM, unlike ASA, may induce the accumulation of AA (the substrate of COX). We examined the effects of IDM or AA on ASA-treated cells. The results showed that the addition of AA or IDM increased the initial peak frequency of ACh-stimulated exocytotic events in ASA-treated cells in a way similar to that observed in IDM-treated cells. Moreover, IDM increased the frequency of ACh-stimulated exocytotic events in H-89-treated cells. Thus AA increases the frequency of exocytotic events independently of cAMP. AA was reported to be a second messenger for mobilizing intracellular Ca$^{2+}$ (30). AA accumulation may modulate Ca$^{2+}$ mobilization in IDM-treated antral mucous cells.

It is known that two subtypes of COX (COX-1 and COX-2) exist in gastric mucosa. In antral mucous cells, the COX-1 inhibitor (SC-560) decreased the frequency of ACh-stimulated exocytotic events but the COX-2 inhibitor (NS-398) did not. The extent of inhibition induced by SC-560 was similar to that induced by IDM. This suggests that COX-1 regulates PGE$_2$ synthesis in antral mucous cells. The acetylated COX-1 by ASA was reported to synthesize 15R-HETE, similar to nonacetylated COX-1. The acetylation of COX-2 by ASA, however, was reported to enhance 15R-HETE synthesis by five- or sixfold (4, 18). If the conversion from AA to 15R-HETE is allowed in COX-1, the COX-1 inhibitors (NS-398) that inhibit AA metabolism are ineffective. On the other hand, IDM decreased the frequency of ACh-stimulated exocytotic events by 10.2 ± 0.3 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from

Table 1. cAMP contents in antral mucosal cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>cAMP Content</th>
<th>n</th>
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<tbody>
<tr>
<td>Control</td>
<td>3.8 ± 0.3</td>
<td>6</td>
</tr>
<tr>
<td>ACh (10 μM)</td>
<td>7.3 ± 0.5</td>
<td>6</td>
</tr>
<tr>
<td>Indomethacin + ACh (10 μM)</td>
<td>3.2 ± 0.3</td>
<td>6</td>
</tr>
<tr>
<td>Ionomycin (1 μM)</td>
<td>6.9 ± 0.7</td>
<td>6</td>
</tr>
<tr>
<td>IDM + ionomycin (1 μM)</td>
<td>3.7 ± 0.4</td>
<td>3</td>
</tr>
<tr>
<td>PGE$_2$ (1 μM)</td>
<td>7.4 ± 0.6</td>
<td>6</td>
</tr>
<tr>
<td>IBMX (1 mM)</td>
<td>103.4 ± 12.3</td>
<td>6</td>
</tr>
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</table>

Values are pmol/mg ± SE; n = number of cells. *P < 0.05 vs. control.
In unstimulated antral mucous cells, no exocytotic events occurred. Our previous reports showed that PGE2 (1 μM) induced a small increase in the frequency of exocytotic events (1–2 events·cell⁻¹·30 s⁻¹) (16, 17). The present study demonstrated that PGE2 release occurs continuously in unstimulated antral mucosal cells and that the amount of PGE2 released over 10 min is ~0.6 μmol/kg tissue. The half-life of PGE2 is known to be rapid (~30 s in the circulatory system) (6). If PGE2 is assumed to be replaced with new PGE2 within 1 min and extracellular fluid volume is assumed to be 30%, the PGE2 concentration in the antral mucosa is estimated to be 0.1 μM. In our previous report, PGE2 (0.1 μM) activated the exocytotic events in unstimulated antral mucous cells (1 events·cell⁻¹·30 s⁻¹) (16, 17). The PGE2 release in unstimulated antral mucosa appears to maintain the resting mucin release. In our experimental system, however, PGE2 released from antral mucous cells is immediately diluted by a high perfusion rate, which may not activate exocytotic events.

On the other hand, PGE2 release in ACh (10 μM) stimulated mucosa was twofold greater than that in unstimulated mucosa. The concentration of PGE2 also appears to increase to 0.2 μM. A small increase in cAMP, which does not activate exocytotic events, still enhances Ca²⁺-regulated exocytotic events in antral mucous cells, as shown in our previous report (15). In our experimental system, the concentration of PGE2 during ACh stimulation, especially near the cell surface, appears to increase sufficiently to enhance Ca²⁺-regulated exocytotic events in antral mucous cells. Therefore, when the gastric branches of the vagus are active, ACh released from the gastric branches stimulates PGE2 synthesis and release mediated via [Ca²⁺], increases in antral mucous cells. The PGE2 release stimulated by ACh appears to maintain mucin release in large amounts mediated by cAMP accumulation.

In conclusion, the results of the present study are summarized in Fig. 11. ACh stimulation increases [Ca²⁺], via Ca²⁺ release from its stores followed by the store-operated Ca²⁺ entry (14, 15, 30). A [Ca²⁺] increase directly stimulates exocytosis in antral mucous cells, and it also stimulates...
COX-1, which results in PGE2 synthesis and PGE2 release. The released PGE2 stimulates cAMP accumulation mediated by the EP4 receptor in antral mucous cells (16, 17), which potentiates Ca2+-regulated exocytosis. However, NSAIDs, such as IDM or ASA, inhibit COX-1, which in turn inhibits PGE2 synthesis and the subsequent cAMP accumulation in antral mucous cells. Consequently, NSAIDs decrease the frequency of ACh-stimulated exocytotic events, because the cAMP modulation of Ca2+-regulated exocytosis is eliminated (15).

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