PPARα autocrine regulation of Ca$^{2+}$-regulated exocytosis in guinea pig antral mucous cells: NO and cGMP accumulation

Saori Tanaka,1,4 Nanae Sugiyama,1,4 Yuko Takahashi,1,5 Daiki Mantoku,1,4 Yukinori Sawabe,1,6 Hiroko Kuwabara,1,2 Takashi Nakano,1,3 Chikao Shimamoto,4 Hitoshi Matsumura,4 Yoshinori Marunaka,1,6 and Takashi Nakahari1,6

1Nakahari Project of Central Research Laboratory, 2Department of Pathology, and 3Department of Microbiology and Infection Control, Osaka Medical College, Takatsuki, Japan, 4Laboratory of Pharmacotherapy, Osaka University of Pharmaceutical Sciences, Takatsuki, Japan, 5Mechanobiology Laboratory, Graduate School of Medicine, Nagoya University, Nagoya, Japan, and 6Department of Molecular Cell Physiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan

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THE GASTRIC MUCOSAL SURFACE is covered with mucins secreted from mucous cells. Mucins, which are high-molecular-weight glycoproteins, are synthesized in the endoplasmic reticulum, stored in intracellular granules, and secreted into the lumen by exocytosis. In antral mucous cells, ACh activates Ca$^{2+}$-regulated exocytosis, which is the main mechanism for mucin release (6, 14, 20). ACh-stimulated exocytosis has a characteristic feature in its frequency: a peak in exocytotic events that declines rapidly (initial phase) followed by a second slower decline (late phase) lasting during ACh stimulation (6, 14, 20, 24).

In ACh-stimulated antral mucous cells, two autocrine mechanisms modulate the Ca$^{2+}$-regulated exocytosis, resulting in the enhancement of initial phase (7, 21). One is the prostaglandin E2 (PGE2)/EP4 receptor mechanism (12, 21, 22), and the other is the arachidonic acid (AA)/peroxisome proliferation activation receptor α (PPARα) agonist mechanism (7, 21). In both mechanisms, AA is the key substance, because AA is the substrate for PGE2 synthesis and a ligand for PPARα (13). AA is produced from membrane lipids via phospholipase A2 (PLA2) stimulated by an increase in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]i). In the PGE2/EP4 receptor mechanism, PGE2 stimulates cAMP accumulation, which enhances the initial phase. However, in the AA/PPARα mechanism, we do not know how PPARα enhances the initial phase.

On the other hand, in many tissues and cells, there are reports showing that AA and PPARα stimulate nitric oxide (NO) accumulation (10, 18, 21, 25, 27, 28). NO, which is a mucosal defense factor in the gastric mucosa, is an intracellular or intercellular messenger that accumulates cGMP (1, 11, 29). NO, which is a transient, increase in the ACh-stimulated late phase, and it also induced enhancement. However, GW6471 produced the delayed, but transient, increase in the ACh-stimulated late phase, and it also induced enhancement. However, GW6471 produced the delayed increase in the late phase. Moreover, GW6471 and ACh stimulated NO production and cGMP accumulation in antral mucosae, which was abolished by GW6471-induced-enhancement of ACh-stimulated initial phase but produced the delayed increase in the late phase. However, in the presence of N-PLA, an NO donor or 8BrGMP enhanced the ACh-stimulated initial phase and abolished the delayed increase in the late phase. Moreover, GW6471 and ACh stimulated NO production and cGMP accumulation in antral mucosae, which was inhibited by GW6471 or N-PLA. Western blotting and immunohistochemistry revealed that NOS1 and PPARα colocalize in antral mucous cells. In conclusion, during ACh stimulation, a PPARα autocrine mechanism, which accumulates NO via NOS1 leading to cGMP accumulation, is an intracellular mucosal defense factor in the gastric mucosa.
activation of PPARα produces NO leading to cGMP accumulation, which enhances the initial phase of the Ca^{2+}-regulated exocytosis in ACh-stimulated antral mucous cells. The goal of the present study is to confirm this hypothesis.

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. This solution, pH of which was adjusted to 7.4 by adding HCl (1 M) before the start of experiments, was aerated with a gas mixture (95% O_2 and 5% CO_2) at 37°C, and the final pH during aeration was 7.4. Acetylated chloride (ACh) was purchased from Daiichi Pharmaceuticals (Osaka, Japan). Collagenase (for cell dispersion, 180–220 ml units/mg), bovine serum albumin (BSA), AA, arachidonyl trifluoromethyl ketone (AACOCF_3), N-(4-aminomethylbenzyl)acetamidine, (i-Nor-A, N-(2S)-(−))-(1Z)-1-methyl-3-oxo-3-(4-trifluoromethyl)phenyl)propl-enyl)-amino)-3-(4-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy)phenyl)propyl)-propanamide (GW6471, Tocris), 2-[4-[2-[(cyclohexylamino)-carbonyl]-(4-cyclohexylbutyl)]amino]-3-[ethyl]-4-oxo-4H-pyridin-2-yl]-2-methyl-propanoic acid (GW7647, Tocris), N^4-nitro-[l]-arginine methyl ester hydrochloride ([l-NNAME], Tocris), N-[aminopropylamino)methyl]-l-ornithine dihydrochloride (N^L5,OD, Tocris), and 1-hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-l-triazeno (NOC 12, Dojindo) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The 8-bromo-cGMP (8Br-cGMP) and Rp8BrPETcGMPS were purchased from Sigma (St. Louis, MO). All reagents were dissolved in dimethyl sulfoxide (DMSO) and were diluted to their final concentrations immediately before the experiments. The final DMSO concentration did not exceed 0.1%, and this concentration had no effect on cell volume, [Ca^{2+}]_i, intracellular Cl^- concentration ([Cl^-]), or the frequency of exocytotic events in antral mucous cells (6, 7, 14, 15, 20–24).

**Cell preparations.** Hartley strain male guinea pigs weighing ~450 g were purchased from SLC (Shizuoka, Japan) and fed standard pelleted food and water. Guinea pigs were anesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg), after which they were euthanized by cervical dislocation. The procedures for the cell preparation have been previously described (6, 7, 14, 15, 20, 22–24). Briefly, the gastric antrum was excised, and the mucosal layer was stripped from the muscle layer in cold phosphate-buffered saline (4°C) by using glass slides. The stripped antral mucosa was suspended in solution I containing 4% BSA (4°C) and then incubated in solution I containing 0.1% collagenase and 4% BSA for 12 min at 37°C. The digested mucosa was filtered through nylon mesh with a pore size of 300 μm² and washed three times. The cells were resuspended in solution I containing 4% BSA (4°C). The cell suspension was stored at 4°C and used in experiments within 3 h.

**Measurement of NO and cGMP.** The stripped antral mucosa was fixed in 10% formalin buffered with 150 mM phosphate for 24 h, dehydrated in a graded series of ethanol concentrations and embedded in paraffin, in accordance with a standard protocol. Some sections were then stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) reagent, or 4'-6-diamidino-2-phenylindole (DAPI) reagent. Triple immunofluorescence staining was performed using PPARα monoclonal mouse, 1:25; LifeSpan BioSciences, NOS1 polyclonal rabbit, 1:25; LifeSpan BioSciences, and DAPI (Dojindo, Osaka, Japan). Sections were incubated with PPARα for 1 h, and Alexa Fluor 488 (1:50; Invitrogen, Carlsbad, CA) was used. The sections were subsequently incubated with NOS1 for 1 h, and Alexa Fluor 546 (1:50; Invitrogen) was used. And they were incubated with DAPI for 3 min. Finally, the sections were examined by use of a Leica TCS SP8 laser scanning confocal microscope (Leica, Wetzlar, Germany).

**Histological examination.** The stripped antral mucosa was fixed in 10% formalin with 150 mM phosphate for 24 h, dehydrated in a graded series of ethanol concentrations and embedded in paraffin, in accordance with a standard protocol. Some sections were then stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) reagent, or 4'-6-diamidino-2-phenylindole (DAPI) reagent. Triple immunofluorescence staining was performed using PPARα monoclonal mouse, 1:25; LifeSpan BioSciences, NOS1 polyclonal rabbit, 1:25; LifeSpan BioSciences, and DAPI (Dojindo, Osaka, Japan). Sections were incubated with PPARα for 1 h, and Alexa Fluor 488 (1:50; Invitrogen, Carlsbad, CA) was used. The sections were subsequently incubated with NOS1 for 1 h, and Alexa Fluor 546 (1:50; Invitrogen) was used. And they were incubated with DAPI for 3 min. Finally, the sections were examined by use of a Leica TCS SP8 laser scanning confocal microscope (Leica, Wetzlar, Germany).
−80°C for 24 h. The lyophilized antral mucosae were weighed and their cGMP contents were measured by use of a cGMP EIA kit (no. 581021, Cayman Chemical). The cGMP content was expressed as picomoles per gram of dry tissue weight (14, 15). The n showed the number of animals.

Measurement of $[\text{Ca}^{2+}]$. The isolated antral mucus cells were incubated in solution I containing 4% BSA and 5 μM fura 2-acetoxyethyl ester (fura-2 AM, Dojinbo, Kumamoto, Japan) for 30 min at room temperature (22–24°C). They were then washed with solution I containing 4% BSA. Fura-2-loaded cells were resuspended, stored in solution I containing 4% BSA at 4°C, and then mounted on a coverslip precoated with neutralized Cell-Tak. The coverslip was set in a perfusion chamber mounted on the stage of an inverted microscope (TE2000, Nikon, Tokyo, Japan), which was connected to an image-analysis system (Acua Cosmos, Hamamatsu Photonics) (7, 14, 20, 22–24). All the experiments were carried out at 37°C. The volume of the perfusion chamber was ~20 μl, and the perfusion rate was 200 μl/min. Fura-2 was excited at 340 and 380 nm, and the emission was measured at 510 nm. The fluorescence ratio ($F_{340}/F_{380}$) was calculated and stored in the image-analysis system. Experiments were carried out using five or six coverslips from two or three animals; a typical response of the typical response of the

RESULTS

The ACh concentration used for stimulation was 1 μM throughout the experiments. In unstimulated antral mucous cells, no exocytotic events were noted. The ACh-stimulated exocytotic events were characterized by a peak in exocytotic events that declined rapidly (initial phase) followed by a second slower decline (late phase) lasting during ACh stimulation (6, 7, 14, 15, 20, 21–24). The frequency of the initial phase (1 min after the start of stimulation) was $11.2 \pm 0.4$ events·cell$^{-1}$·30 s$^{-1}$ ($n = 11$), and that of the late phase (4 min after the start of stimulation) was $1.8 \pm 0.3$ events·cell$^{-1}$·30 s$^{-1}$ ($n = 11$) (Fig. 1A).

Effects of PPARα agonist and antagonist on ACh-stimulated exocytotic events. A previous study demonstrated that a PPARα agonist (WY14643) enhances the initial phase of ACh-stimulated exocytotic events in antral mucous cells (21). In the present study, GW7647 (50 nM) and GW6471 (5 μM) were used, because they are a specifically selective agonist and antagonist for PPARα, respectively. Data sheet from Tocris shows EC$50$ of GW7647 and IC$50$ of GW6471 are 6 nM and 0.24 μM, respectively. We repeated the experiments using 50 nM GW7647, and the experimental protocol is shown in Fig. 1A. GW7647 alone did not activate any exocytotic events, but it enhanced the ACh-stimulated initial phase in antral mucous cells (Fig. 1A). The frequencies of ACh-stimulated initial and late phases were $18.4 \pm 1.0$ and $17.7 \pm 0.3$ events·cell$^{-1}$·30 s$^{-1}$ ($n = 5$), respectively. In the next experiments, we examined the effects of 5 μM GW6471 on the exocytotic events stimulated by ACh. The initial phase frequency was reduced by 64% in antral mucous cells: an initial phase (delayed increase) in the exocytotic events stimulated by GW7647 plus ACh. GW6471 (5 μM) abolished the enhancement of initial phase of exocytotic events and decreased the frequency to 43% of that enhanced by GW7647 plus ACh; rather, the frequency was reduced to 71% of that increased by ACh alone. However, the addition of GW6471 produced the delayed, but transient, increase in the late phase (delayed increase) in the exocytotic events stimulated by GW7647 plus ACh. PKI abolished the GW6471-induced delayed increase in the exocytotic events stimulated by GW7647 plus ACh. D: effects of PKI-amine (1 μM, a PKA inhibitor) on the GW6471-induced delayed increase in the exocytotic events stimulated by GW7647 plus ACh. PKI abolished the GW6471-induced delayed increase in the exocytotic events stimulated by GW7647 plus ACh. GW6471 decreased the frequency of the ACh-stimulated initial phase to 76% of that increased by ACh alone and produced the delayed increase in the exocytotic events stimulated by ACh alone. *Significantly different from the corresponding values and ‡significantly different from the value 3.5 min from the start of ACh stimulation ($P < 0.05$).
lated by GW7647 plus ACh, and the experimental protocol is shown in Fig. 1B. GW6471 abolished the GW7647-induced enhancement of ACh-stimulated initial phase and decreased the frequency of initial phase to 43% (7.9 ± 0.4 events·cell⁻¹·30 s⁻¹, n = 6) of that enhanced by GW7647 plus ACh; rather, the frequency of ACh-stimulated initial phase was reduced to 71% of that increased by ACh alone, suggesting that ACh alone activates PPARα. However, GW6471 produced the delayed, but transient, increase in the late phase (delayed increase) in the exocytotic events stimulated by GW7647 plus ACh (the frequency of delayed increase was 2.9 ± 0.2 events·cell⁻¹·30 s⁻¹, n = 6). A similar delayed increase in the ACh-stimulated exocytotic events has been reported to be evoked by a PKG inhibitor, Rp8BrPETcGMPS, which accumulates cAMP via inhibition of cGMP-dependent PDE2 (26).

We examined the effects of a PKA inhibitor (1 μM, PKI-amide) on the GW6471-induced delayed increase in the exocytotic events stimulated by GW7647 plus ACh, and the experimental protocol is shown in Fig. 1C. PKI-amide abolished the GW6471-induced delayed increase in the exocytotic events stimulated by GW7647 plus ACh (1.0 ± 0.1 events·cell⁻¹·30 s⁻¹, n = 6), although it did not affect the initial phase (7.9 ± 0.4 events·cell⁻¹·30 s⁻¹, n = 6) (Fig. 1C). Thus, as shown in Fig. 1C, the delayed increase produced by GW6471 is PKI-amide blockable, suggesting that GW6471 induces cAMP accumulation. GW6471 may inhibit cGMP accumulation leading to a decrease in PDE2 activities (26).

We also examined the effects of GW6471 on the exocytotic events stimulated by ACh alone (Fig. 1D), because ACh alone appears to activate PPARα (Fig. 1B). GW6471 decreased the frequency of ACh-stimulated initial phase to 76% of that increased by ACh alone (8.5 ± 0.1 events·cell⁻¹·30 s⁻¹, n = 10) and produced the delayed increase in the exocytotic events stimulated by ACh alone (3.0 ± 0.1 events·cell⁻¹·30 s⁻¹, n = 10) (Fig. 1D). Thus ACh alone activates the signaling pathway inhibited by GW6471 (an inhibitor of PPARα) in antral mucous cells.

Effects of a PKG inhibitor on ACh-stimulated exocytotic events enhanced by GW7647. The previous study demonstrated that, during stimulation with 8BrcGMP plus ACh, 500 nM Rp8BrPETcGMPS, similarly to GW6471, completely abolishes the enhancement of initial phase and produces the delayed increase (26). We examined the effects of Rp8BrPETcGMPS on the exocytotic events stimulated by GW7647 plus ACh, and the experimental protocol is shown in Fig. 2A. Rp8BrPETcGMPS abolished the enhancement of initial phase and produced the delayed increase in the exocytotic events stimulated by GW7647 plus ACh. The frequencies of the initial phase and the delayed increase were 8.3 ± 0.3 events·cell⁻¹·30 s⁻¹ (n = 6, 45% of that enhanced by GW7647 plus ACh) and 2.9 ± 0.2 events·cell⁻¹·30 s⁻¹, respectively (Fig. 2A).

In the next experiment, we used 8BrcGMP instead of GW6471, and the experimental protocol is shown in Fig. 2B. The 8BrcGMP concentration used was 100 μM, which maximally enhanced the ACh-stimulated initial phase (20). Even in the GW6471-treated cells, 8BrcGMP enhanced the initial phase (17.7 ± 0.9 events·cell⁻¹·30 s⁻¹, n = 10), and abolished the delayed increase in the exocytotic events stimulated by ACh (1.6 ± 0.2 events·cell⁻¹·30 s⁻¹, n = 10). Thus the signal pathway mediated via PPARα appears to stimulate cGMP accumulation, if we assume that GW6471 or GW7647 only inhibit or activate the PPARα-mediated pathway leading to cGMP accumulation.

Effects of NOS inhibitors on ACh-stimulated exocytotic events enhanced by GW7647. In many cell types, PPARs are reported to stimulate NO synthesis (10, 12), and NO is known to stimulate cGMP accumulation (1, 11, 12). We examined the effects of NOS inhibitors on the exocytotic events stimulated by GW7647 plus ACh in antral mucous cells, and the experimental protocol is shown in Fig. 3A. We, first, used a nonspecific inhibitor of NOS, 100 μM L-NAME. The addition of 100 μM L-NAME abolished the enhancement of initial phase and produced the delayed increase in the exocytotic events stimulated by GW7647 plus ACh. The frequencies of the initial phase and the delayed increase were 7.6 ± 0.4 events·cell⁻¹·30 s⁻¹ (n = 6, 41% of that enhanced by GW7647 plus ACh) and 3.0 ± 0.3 events·cell⁻¹·30 s⁻¹, respectively (Fig. 3A).

To determine the NOS isoforms involved in NO production of antral mucous cells, we used three specifically selective inhibitors: 1 μM N-PLA for NOS1, 50 nM 1400 W for NOS2, and 2 μM LN5-OD for NOS3. The data sheets from Tocris

Fig. 2. Effects of a PKG inhibitor [guanosine 3',5'-cyclic monophosphorothioate, β-phenyl-1, N'-etheno-8-bromo-, Rp-isomer, sodium salt (Rp8BrPETcGMPS)] on the frequency of ACh-stimulated exocytotic events enhanced by GW6477. A: effects of Rp8BrPETcGMPS on the exocytotic events stimulated by GW7647 plus ACh. Rp8BrPETcGMPS (500 nM) abolished the enhancement of initial phase of exocytotic events (the frequency to 45% of that enhanced by GW7647 plus ACh) and produced the delayed increase in the exocytotic events stimulated by GW7647 plus ACh. B: effects of 8-bromo-cGMP (8BrcGMP) on the exocytotic events stimulated by GW6471 plus ACh. 8BrcGMP (100 μM) enhanced the initial phase and abolished the delayed increase in the ACh-stimulated exocytotic events of GW6471-treated cells. *Significantly different from the corresponding values and †significantly different from the values 3.5 min from the start of ACh stimulation (P < 0.05).
show that $K_i$ values of N-PLA for NOS1, 1400W for NOS2, and LN5-OD for NOS3 are 57, 7, and 500 nM, respectively. Experiments were carried out by using three inhibitors instead of L-NAME, with the same protocol (Fig. 3B). N-PLA (1 μM) abolished the enhancement of initial phase ($7.7 \pm 0.3$ events·cell$^{-1}$·30 s$^{-1}$, $n = 5$) and produced the delayed increase in the exocytotic events stimulated by GW7647 plus ACh ($3.2 \pm 0.2$ events·cell$^{-1}$·30 s$^{-1}$) (Fig. 3B).

Antral mucous cells were treated with 1400W and LN5-OD instead of N-PLA, as shown in Fig. 3, C and D. 1400W and LN5-OD did not affect the exocytotic events stimulated by GW7647 plus ACh. These results suggest that GW7647 stimulates NOS1 to produce NO in antral mucous cells. We also examined the effects of N-PLA on the exocytotic events stimulated by ACh alone. N-PLA decreased the initial phase and produced the delayed increase in the exocytotic events stimulated by ACh alone (data not shown).

Effects of NO, l-Arg, and cGMP on Ach-stimulated exocytotic events in the presence of N-PLA. We examined the effects of NO on the exocytotic events stimulated by GW7647 plus ACh in N-PLA-treated cells using NOC12 (an NO donor); the experimental protocol is shown in Fig. 4A. In the presence of N-PLA, NOC12 enhanced the initial phase ($19.2 \pm 0.4$ events·cell$^{-1}$·30 s$^{-1}$, $n = 5$), and abolished the delayed increase in the exocytotic events stimulated by GW7647 plus ACh ($0.4 \pm 0.2$ events·cell$^{-1}$·30 s$^{-1}$, $n = 5$) (Fig. 4A). Experiments, the protocol of which was the same as Fig. 4A, were also carried out using l-Arg or 8BrcGMP instead of NOC12. In the presence of N-PLA, l-Arg, or 8BrcGMP also enhanced the initial phase and abolished the delayed increase in the exocytotic events stimulated by GW7647 plus ACh (Figs. 4, B and C). The frequency of the initial phase enhanced by l-Arg or 8BrcGMP was $19.3 \pm 0.8$ ($n = 5$) or $18.7 \pm 1.3$ events·cell$^{-1}$·30 s$^{-1}$ ($n = 5$), and the frequency of the delayed increase was $1.9 \pm 0.6$ ($n = 5$) or $1.6 \pm 0.2$ events·cell$^{-1}$·30 s$^{-1}$ ($n = 5$), respectively.

In the absence of GW7647, NOC12, l-Arg, or 8BrcGMP similarly enhanced the initial phase and abolished the delayed increase in the exocytotic events stimulated by ACh alone in N-PLA-treated cells (data not shown). We also examined the effects of NOC12 using GW6471 instead of N-PLA. NOC12 enhanced the initial phase and abolished the delayed increase in the exocytotic events stimulated by ACh alone (data not shown). The results with 8BrcGMP instead of NOC12 have already shown in Fig. 2B.

Thus the signal pathway mediated via PPAR$\alpha$ appears to stimulate NO production via NOS1, if we assume that GW6471 or GW7647 only inhibit or activate the PPAR$\alpha$-mediated pathway leading to NO production. NO appears to stimulate cGMP accumulation in antral mucous cells.

Effects of GW6471 on Ach-stimulated exocytotic events enhanced by AA. AA (1 μM), which is a natural ligand for PPAR$\alpha$ (13), enhances the Ach-stimulated initial phase in antral mucous cells (Fig. 5A), as previously reported (7, 21). The enhancement of initial phase induced by AA is similar to that induced by GW7647 (Figs. 1A and 5A), and the frequencies of Ach-stimulated initial and late phases were $18.4 \pm 0.5$ and $1.2 \pm 0.4$ events·cell$^{-1}$·30 s$^{-1}$ ($n = 5$), respectively. When antral mucous cells were treated with GW6471 prior to the addition of 1 μM AA, GW6471 abolished the enhancement of

Effect of removing or blocking the PPAR$\alpha$ pathway. Experiments were carried out using L-Arg or 8BrcGMP instead of NOC12, as already shown in Fig. 2B.
to no endogenous AA production and resulting in no production of PGs, leukotrienes, and lipoxins from the AA cascade. AACOCF₃ also enhanced the ACh-stimulated initial phase followed by slower decline ACh-stimulated exocytotic events. The frequencies of the initial and late phases were 17.2 ± 0.9 and 0.9 ± 0.05 events·cell⁻¹·30 s⁻¹ (n = 6), respectively (Fig. 5B). GW6471 abolished the enhancement of initial phase (8.3 ± 0.5 events·cell⁻¹·30 s⁻¹, n = 5) and produced the delayed increase in the exocytotic events stimulated by AACOCF₃ plus ACh (3.2 ± 0.4 events·cell⁻¹·30 s⁻¹, n = 5) (Fig. 5B).

NO release and cGMP content. Figure 6 shows the amounts of NO released from and cGMP content of the stripped antral mucosae, which were stimulated with ACh, GW7647, GW7647 plus ACh, AACOCF₃, or AACOCF₃ plus ACh. Experiments were also carried out using stripped antral mucosa treated with GW6471 or N-PLA.

In the control experiment, stripped antral mucosa was incubated with DMSO (vehicle control) for 10 min. The amounts of NO released from the stripped antral mucosa for 2 and 10 min

initial phase (8.1 ± 0.6 events·cell⁻¹·30 s⁻¹, n = 8) and produced the delayed increase in the exocytotic events (2.8 ± 0.2 events·cell⁻¹·30 s⁻¹, n = 8) (Fig. 5A). Experiments were carried out using 1 μM AACOCF₃ (arachidonyl trifluoromethyl ketone), using the same protocol (Fig. 5B). AACOCF₃, which is a nonmetabolic analog of AA, inhibits PLA₂, leading

![Fig. 4. Effects of NO and cGMP in the presence of a NOS1 inhibitor, N-PLA, on the ACh-stimulated exocytotic events enhanced by GW7647. N-PLA abolished the enhancement of initial phase and produced the delayed increase in the exocytotic events stimulated by GW7647 plus ACh. In the presence of N-PLA, NOC12, l-arginine (l-Arg), and 8BrcGMP enhanced the initial phase and abolished the delayed increase in the exocytotic events stimulated by GW7647 plus ACh. A: NOC12 (500 μM, an NO donor). B: l-Arg (5 mM). C: 8BrcGMP. *Significantly different from the corresponding values (P < 0.05).](http://ajpgi.physiology.org)
were 0.02 ± 0.27 and 0.31 ± 0.16 nmol/g tissue \((n = 5)\), respectively. GW6471 and N-PLA slightly decreased the amounts of NO released from the stripped antral mucosa for 10 min compared with those of the vehicle control, but the decrease was not significant. ACh (1 \(\mu\)M) increased the amounts of NO released from stripped antral mucosa, and the amounts of NO released from the stripped antral mucosa for 2 and 10 min were 2.2 ± 0.3 and 3.8 ± 0.6 nmol/g tissue \((n = 3)\), respectively. Moreover, GW7647 or GW7647 plus ACh also increased the amounts of NO released from the stripped antral mucosa \([3.8 ± 0.8\) and 4.5 ± 0.2 nmol/g tissue for 2 min \((n = 4)\) or 4.9 ± 0.4 and 5.6 ± 0.4 nmol/g tissue for 10 min \((n = 4)\)]. Experiments were also carried out using GW6471 or N-PLA-treated antral mucosa. GW6471 or N-PLA inhibited these increases in the amounts of NO released during 10-min incubation with ACh, GW7647, and GW7647 plus ACh (Fig. 6A). The experiments were also carried out using AACOCF3 or AACOCF3 plus ACh. AACOCF3 or AACOCF3 plus ACh increased the amounts of NO released from the stripped antral mucosa, and the amounts of NO released from the stripped antral mucosa for 2 and 10 min were 2.6 ± 1.4 and 5.0 ± 0.7 nmol/g tissue \((n = 3)\) or 6.3 ± 1.2 and 7.2 ± 1.3 nmol/g tissue \((n = 4)\), respectively. GW6471 or N-PLA inhibited these increases in the amounts of NO released during 10-min incubation with AACOCF3 and AACOCF3 plus ACh (Fig. 6A). Moreover, the amounts of NO released from the stripped antral mucosa stimulated with PPAR\(\alpha\) agonists were enhanced by ACh. During incubation with GW6471 and N-PLA, the concentration of NO sometimes decreased compared with that before the stimulations as shown in AACOCF3 experiments. A small amount of NO may diffuse to air from the incubation solution, because no NO was supplied from cells to incubation solution.

Upon incubation with vehicle control for 10 min, the cGMP contents of the stripped antral mucosa was 25.6 ± 2.3 pmol/g tissue \((n = 7)\). GW6471 and N-PLA did not change cGMP contents compared with that of the control experiments. Stimulation with ACh for 10 min increased the cGMP content to 40.8 ± 6.8 pmol/g tissue \((n = 5)\). Stimulation with GW7647 or GW7647 plus ACh for 10 min also increased the cGMP content to 34.8 ± 1.3 pmol/g tissue \((n = 9)\) or 50.5 ± 7.7 pmol/g tissue \((n = 9)\), respectively. GW6471 or N-PLA inhibited these increases in cGMP content (Fig. 6B). Stimulation with AACOCF3 or AACOCF3 plus ACh for 10 min increased the cGMP content to 55.4 ± 8.5 pmol/g tissue \((n = 5)\) or 66.5 ± 5.2 pmol/g tissue \((n = 9)\), respectively. GW6471 or N-PLA inhibited these increases in cGMP content (Fig. 6B).

Thus PPAR\(\alpha\) agonists, GW7647 and AACOCF3, stimulate the NO production and cGMP accumulation in the antral mucosa.

We examined the effects of increase in the NO concentration ([NO]) on cGMP accumulation in antral mucosa (Table 1). Antral mucosa was incubated in solution I (3 ml) containing DMSO or NOC12 (500 \(\mu\)M) for 10 min. In the control experiment, vehicle control (2.5 \(\mu\)M DMSO) increased [NO] of incubation solution from 2.99 ± 0.59 \(\mu\)M to 3.17 ± 0.66 \(\mu\)M (\(\Delta[NO] = 0.17 ± 0.15 \mu\)M, \(n = 3\)). In the NOC12 experiments, [NO] of incubation solution increased from 3.87 ± 1.60 to 4.02 ± 0.33 \(\mu\)M (\(\Delta[NO] = 0.15 ± 0.26 \mu\)M, \(n = 3\)).

Table 1. The NO concentration and the cGMP content

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Final [NO], (\mu)M</th>
<th>(\Delta[NO]), (\mu)M</th>
<th>cGMP contents, pmol/g tissue</th>
<th>(n)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>3.17 ± 0.66</td>
<td>(0.17 ± 0.15)</td>
<td>27.27 ± 3.86</td>
<td>3</td>
</tr>
<tr>
<td>NOC12 500 (\mu)M</td>
<td>44.74 ± 1.07 *</td>
<td>(40.86 ± 2.49 *)</td>
<td>76.27 ± 9.23</td>
<td>3</td>
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An increase in [NO] of the incubation solution by 1-hydroxy-2-oxo-3-(\(N\)-ethyl-2-aminomethyl)-3-ethyl-1-triazene (NOC12) cause cGMP to accumulate in the antral mucosa. Values are expressed as means ± SE; \(n\), number of experiments. *\(P < 0.05\) vs. Control.
μM to 44.74 ± 1.07 μM (Δ[NO] = 40.86 ± 2.49 μM, n = 3). The cGMP content of the antral mucosa incubated with the vehicle control was 27.27 ± 3.86 pmol/g tissue (n = 3), and that with NOC12 was 76.27 ± 9.23 pmol/g tissue (n = 3). Thus increases in [NO] cause cGMP to accumulate in the antral mucosa.

Expression and localization of NOS1 and PPARα in antral mucosa. NOS1 and PPARα proteins expression in the antral mucosa were examined by Western blotting, using an anti-NOS1 antibody (LS-B3272, LifeSpan BioSciences) and anti-PPARα antibody (LS-B2560, LifeSpan BioSciences). The NOS1 band (160 kDa) and the PPARα band (52 kDa) were detected in the antral mucosa (Figs. 7, A and B).

Histological examination with H&E staining and PAS staining demonstrated that antral mucous cells exist in the upper 1/3 to 1/2 of the antral mucosa (Fig. 8, A and B) (14, 23). Antral mucous cells were positively stained for NOS1 (Fig. 8C) and PPARα (Fig. 8D). NOS1 exists in the cytoplasm of antral mucous cell (Fig. 8C) and PPARα exists in the cytoplasm and nucleus (Fig. 8D). The DAPI staining demonstrated that the nucleus exists in the basolateral side of antral mucous cells (Fig. 8E). Fig. 8F, which merged Fig. 8, C–E, demonstrated that the cytoplasm of the antral mucous cell is positively stained for both NOS1 and PPARα (Fig. 8F).

We also examined the expression of NOS2 and NOS3 using the Western blotting and immunohistochemistry. In Western blot analysis of the antral mucosae, a band of NOS3 was the Western blotting and immunohistochemistry. In Western blot analysis of the antral mucosae, a band of NOS3 was detected in the antral mucosa (Figs. 7, A and B).

Effects of GW7647 on [Ca²⁺]. Stimulation with ACh increased [Ca²⁺], immediately and then decreased gradually to a plateau within 3 min, which was maintained during ACh stimulation, as previously reported (6, 14, 20, 21, 22, 24) (Fig. 9A). We examined the effects of GW7647 on increases in [Ca²⁺], stimulated by ACh. GW7647 added in the plateau did not enhance increases in [Ca²⁺], stimulated by ACh (Fig. 9A). In another experiment, antral mucous cells were treated with 50 nM GW7647 for 5 min prior to ACh stimulation. GW7647 alone did not induce any increase in [Ca²⁺], and the further stimulation with ACh immediately increased [Ca²⁺], and then decreased to a plateau within 3 min. Increases in [Ca²⁺], stimulated with ACh plus GW7647 were similar to those stimulated with ACh alone (Fig. 9, A and B). Thus GW7647 did not affect [Ca²⁺], with or without ACh stimulation. Our previous reports have exhibited that 8BrGMP or Rp8BrPETcGMPS does not affect [Ca²⁺], with or without ACh stimulation (20, 26).

DISCUSSION

This study demonstrated that the AA/PPARα autocrine mechanism stimulates NO production via NOS1 leading to cGMP accumulation, which enhances the initial phase of ACh-stimulated exocytotic events in antral mucous cells. The previous study showed that the ACh-stimulated exocytosis is maintained by an autocrine mechanism via AA/PPARα in antral mucous cells (7, 21). However, the signals activated by PPARα were unknown.

On the other hand, PPARs and AA have already been reported to stimulate NO production in many cell types (10, 18, 25, 27, 28), and AA has also been reported to stimulate cGMP accumulation via NO production in vascular smooth muscle cells (12).

In antral mucosa, the measurement of cGMP contents demonstrated that PPARα agonists (GW7647 and AACOCF3) increase cGMP contents. Moreover, in ACh-stimulated exocytotic events, the GW6471 (a PPARα antagonist) blocked the actions of PPARα agonists (GW7647, AA, and AACOCF3); that is, it decreases the initial phase and produces the delayed increase, which is PKI-amide blockable. The results obtained by GW6471 were the same as those obtained by 8BrGMP. These findings indicate that PPARα agonists stimulate cGMP accumulation in antral mucous cells.

Moreover, the present study demonstrated that the cGMP accumulation is mediated via NO in antral mucous cells. Western blotting and immunofluorescence demonstrated that NOS1 and PPARα exist and colocalize in antral mucous cells, and the measurements of NO revealed that the PPARα agonists increase NO production via NOS1. The measurement of cGMP and exocytotic events in antral mucous cells by use of N-PLA (a selective inhibitor of NOS1) exhibited that PPARα agonists stimulate NO production via NOS1. Thus, in antral mucous cells, PPARα activation stimulates NOS1, leading to NO production followed by cGMP accumulation, which enhances the initial phase of ACh-stimulated exocytotic events.

![Fig. 7. Expression of NOS1 or PPARα in antral mucous cells. Western blot analysis for NOS1 (A) and PPARα (B). The NOS1 band (160 kDa) and PPARα band (52 kDa) were detected in the antral mucosa.](http://ajpgi.physiology.org/)

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The PPARs, including PPAR\textsubscript{H9251}, are nuclear receptors that modulate the expression of specific target genes (5, 8, 9, 16, 17). However, in antral mucous cells, a PPAR\textsubscript{H9251} agonist immediately increased the frequency of ACh-stimulated exocytotic events (initial phase) via NO production. Immediate increases in the exocytotic events and the NO production indicate that the PPAR\textsubscript{H9251} actions in this study are nongenomic. Some reports have already shown that PPAR\textsubscript{H9251} has nongenomic actions (2–4, 19).

There are many reports showing that AA is a natural ligand for PPAR\textsubscript{H9251} (5, 8, 9, 16, 17). The present study demonstrated that AA stimulates PPAR\textsubscript{H9251} in antral mucous cells, as shown in a previous report (21). However, AA has another role, because AA is the key substance for products of the AA cascade, such as PGs, leukotrienes, and lipoxins. These products may affect the ACh-stimulated exocytotic events in antral mucous cells. However, AACOCF\textsubscript{3}, which is a nonmetabolic analog of AA, still enhanced the initial phase of the ACh-stimulated exocytotic events mediated via PPAR\textsubscript{H9251}, as previously reported (21), and stimulated NO production and cGMP accumulation in antral mucosae mediated via PPAR\textsubscript{H9251}. AACOCF\textsubscript{3}, which is an inhibitor of PLA\textsubscript{2}, inhibits endogenous AA production, resulting in no production of PGs, leukotrienes, and lipoxins from the AA cascade. Thus, under physiological conditions, AA, not products of the AA cascade, stimulates PPAR\textsubscript{H9251}.

ACh stimulates an increase in $[\text{Ca}^{2+}]_i$ and the increase in $[\text{Ca}^{2+}]_i$ is essential to activate exocytotic events in antral mucous cells (6, 14, 20). This indicates that ACh activates the $\text{Ca}^{2+}$-regulated exocytosis. Moreover, the frequency of cAMP-regulated exocytosis is 5–10% of that of $\text{Ca}^{2+}$-regulated exo-

![Fig. 8. Localization of NOS1 or PPAR\textsubscript{H9251} in antral mucous cells.](http://ajpgi.physiology.org/)

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![Fig. 9. Effects of GW7647 on ACh-stimulated increases in intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$).](http://ajpgi.physiology.org/)

Fig. 8. Localization of NOS1 or PPAR\textsubscript{H9251} in antral mucous cells. A: hematoxylin and eosin (H&E) staining. B: periodic acid-Schiff (PAS) staining. C: immunofluorescence for NOS1. The cytoplasm of the antral mucous cell was positively stained for NOS1. D: immunofluorescence for PPAR\textsubscript{H9251}. The cytoplasm and the nucleus of the antral mucous cell were positively stained for PPAR\textsubscript{H9251}. E: 4′,6-diamidino-2-phenylindole (DAPI) staining. F: merged C–E. NOS1 and PPAR\textsubscript{H9251} were colocalized in the cytoplasm of the antral mucous cell. Scale bars represent 50 μM (A and B) and 75 μM (C–F). Arrows show the antral mucous cells (A and B).

Fig. 9. Effects of GW7647 on ACh-stimulated increases in intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$). A: ACh (1 μM) immediately increased $[\text{Ca}^{2+}]_i$ and then decreased to a plateau within 3 min. The further addition of GW7647 did not affect ACh-stimulated increases in $[\text{Ca}^{2+}]_i$. The cessation of ACh stimulation immediately decreased $[\text{Ca}^{2+}]_i$. B: the addition of GW7647 (50 nM) did not increase $[\text{Ca}^{2+}]_i$ and then stimulation with ACh immediately increased $[\text{Ca}^{2+}]_i$, and then decreased to a plateau within 3 min. Increases in $[\text{Ca}^{2+}]_i$ stimulated by GW7647 plus ACh were similar to those stimulated by ACh alone.
cytosis in antral mucous cells (14, 15) and 8BrcGMP alone never activates exocytotic events (20). Thus the previous studies clearly showed that the Ca$^{2+}$-regulated exocytosis activated by ACh is the main mechanism for mucin secretion in antral mucous cells. The PPAR$\alpha$ is a modulator of the Ca$^{2+}$-regulated exocytotic events.

On the other hand, the present study demonstrated that ACh stimulation alone activates PPAR$\alpha$ in antral mucous cells; that is, a PPAR$\alpha$ inhibitor, GW6471, decreased the frequency of the initial phase and produced the delayed increase in the exocytotic events stimulated by ACh alone. In the stripped antral mucosa, ACh alone stimulates NO production via NOS1 leading to cGMP accumulation. Our previous report also demonstrated that 1 $\mu$M ACh stimulates cGMP accumulation (26). These results indicate that the AA/PPAR$\alpha$ autocrine mechanism plays an important role in maintaining mucin secretion during 1 $\mu$M ACh stimulation; that is, AA produced by ACh stimulation stimulates PPAR$\alpha$ in antral mucous cells.

A previous study showed that 10 $\mu$M ACh activates another autocrine mechanism via the PGE$_2$/EP4 receptor, which enhances the initial phase of ACh-stimulated exocytotic events via cAMP accumulation in antral mucous cells (22). Thus, in antral mucous cells, Ca$^{2+}$-regulated exocytosis is modulated by two autocrine mechanisms during ACh stimulation (7, 21, 22). These two mechanisms appear to have different roles. A low concentration of ACh, such as 1 $\mu$M, does not increase PGE$_2$ production (22, 26). This indicates that 1 $\mu$M ACh does not activate the PGE$_2$/EP4 receptor autocrine mechanism. On the other hand, during 1 $\mu$M ACh stimulation, the AA/PPAR$\alpha$ autocrine mechanism stimulates cGMP accumulation by reducing cAMP actions via cGMP-dependent PDE2, which enhances Ca$^{2+}$-regulated exocytosis in antral mucous cells (7, 20, 21). Although physiological roles of the two autocrine mechanisms in antral mucous cells are still controversial, the AA/PPAR$\alpha$ autocrine mechanism may play a role in maintaining a continuous low-level mucin secretion under a physiological condition and the PGE$_2$/EP4 receptor autocrine mechanism plays a role under a pathophysiological condition, such as infection or inflammations, under which a large amount of mucin secretion is required.

This study demonstrated that, during 1 $\mu$M ACh stimulation, PPAR$\alpha$ stimulates NO production via NOS1. However, it remains unknown how PPAR$\alpha$ activates NOS1. Further studies are required to clarify how PPAR$\alpha$ activates NOS1 in antral mucous cells.

In the present study, we used many drugs to examine the signals activated by PPAR$\alpha$. The effects of drugs are not as specific as they are assumed. However, for example, the concentration-response studies exhibited that GW7647 or GW6471 concentration dependently increased the frequency of ACh-stimulated initial phase or abolished its frequency enhanced by GW7647 (data not shown). We also examined the concentration-dependent effects of N-PLA and L-NAME, and they concentration dependently abolished the enhancement of ACh-stimulated initial phase (data not shown). The concentration-response studies of AACOCF$_3$, 8BrcGMP, and Rp8BrPETcGMPS have already been shown in the previous studies (20, 21, 26). Thus GW7647 or GW6471, at least, stimulates or inhibits PPAR$\alpha$, which activates the NOS1/NO/cGMP signal, although they may have some effects on signaling pathways other than PPAR$\alpha$.

In conclusion, the AA/PPAR$\alpha$ autocrine modulation of Ca$^{2+}$-regulated exocytosis is shown in Fig. 10. Stimulation with 1 $\mu$M ACh increases [Ca$^{2+}$], which stimulates AA accumulation via PLA$_2$. Then AA stimulates PPAR$\alpha$, which induces NO accumulation via NOS1 leading to cGMP accumulation, which, finally, increases the frequency of the initial phase in the Ca$^{2+}$-regulated exocytotic events of antral mucous cells. Moreover, the accumulation of cGMP decreases cAMP.

![Fig. 10. AA/PPAR$\alpha$ autocrine modulation of Ca$^{2+}$-regulated exocytosis in guinea pig antral mucous cells.](image-url)
accumulation by activating PDE2 (26) and thereby maintains a low frequency of mucin exocytosis during the late phase.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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