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

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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 E\(_2\) (PGE\(_2\)/EP4 receptor mechanism (12, 21, 22), and the other is the arachidonic acid (AA)/peroxisome proliferation activation receptor α (PPAR\(\alpha\)) agonist mechanism (7, 21). In both mechanisms, AA is the key substance, because AA is the substrate for PGE\(_2\) synthesis and a ligand for PPAR\(\alpha\) (13). AA is produced from membrane lipids via phospholipase A\(_2\) (PLA\(_2\)) stimulated by an increase in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). In the PGE\(_2\)/EP4 receptor mechanism, PGE\(_2\) stimulates cAMP accumulation, which enhances the initial phase. However, in the AA/PPAR\(\alpha\) mechanism, we do not know how PPAR\(\alpha\) enhances the initial phase.

On the other hand, in many tissues and cells, there are reports showing that AA and PPAR\(\alpha\) stimulate nitric oxide (NO) accumulation (10, 18, 21, 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). Moreover, AA has been reported to stimulate cGMP accumulation via NO production in vascular smooth muscle cells (12). NO is synthesized by nitric oxide synthase (NOS), which is subdivided into three isoforms: NOS1, NOS2, and NOS3. Two isoforms that are Ca\(^{2+}\) dependent are constitutively expressed and localize to neurons in the brain (NOS1) and to vascular endothelial cells (NOS3). One isoform that is Ca\(^{2+}\) independent and inducible is expressed in many cell types (NOS2). At present, in antral mucous cells, we do not know which isoform produces NO and whether PPAR\(\alpha\) involves NO production.

We examined the effects of PPAR\(\alpha\) agonists on ACh-stimulated exocytosis in antral mucous cells. An inhibitor of PPAR\(\alpha\) (MK886) produced the delayed increase in the late phase, although it abolished the enhancement of ACh-stimulated initial phase induced by WY14643 (a PPAR\(\alpha\) agonist) (21). A similar delayed increase in the ACh-stimulated late phase has already been reported to be induced by a PKG inhibitor [guanosine 3\',5'-cyclic monophosphorothioate, β-phenyl-1, N\(^2\)-etheno-8-bromo-, Rp-isomer, sodium salt (RpBrPcGMP)], which induces cAMP accumulation by inhibiting cGMP-dependent PDE2 in antral mucous cells (26).

On the basis of these observations, we hypothesized that an
activation of PPARα produces NO leading to cGMP accumulation, which enhances the initial phase of the Ca\textsuperscript{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\textsubscript{3}, 1 MgCl\textsubscript{2}, 1.5 CaCl\textsubscript{2}, 5 K-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\textsubscript{2} and 5% CO\textsubscript{2}) at 37°C, and the final pH during perfusion was 7.4. Acetylcholine chloride (ACH) was purchased from Daichi Pharmaceuticals (Osaka, Japan). Collagenase (for cell dispersion, 180–220 units/mg), bovine serum albumin (BSA), AA, arachidonyl trifluoromethyl ketone (AAOCF\textsubscript{3}), L-arginine (L-Arg), (N-(2S)-2-((11Z)-1-methyl-3-oxo-3-(4-trifluoromethyl)phenyl)prop-1-enylamino)-3-(4-(2-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy)phenylpropyl)propanamide (GW6741, Tocris), 2-[4-[[2-[(cyclohexylamino)carbonyl][4-cyclohexylbutyl]amino]ethyl]phenyl][thio]-2-methylpropanoic acid (GW7647, Tocris), N\textsuperscript{3}-nitro-L-arginine methyl ester (L-NAME), N\textsuperscript{3}-[aminopropylaminomethyl]-l-ornithine hydrochloride (N-PLA, Tocris), and N\textsuperscript{3}-(aminomethylbenzyl)acetamidine, 2HCl (1400 W, Wako), (S)-5-(1-iminoethyl)-L-ornithine dihydrochloride (L5N-OD, Tocris), and 1-hydroxy-2-oxo-2-(N-ethyl-2-aminoethyl)-3-ethyl-l-triazene (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 to their final concentrations immediately before the experiments. The experiments obtained from two or three animals. In the statistical comparisons of the experiments, the frequencies of the initial and late phases were used. For the frequency of the initial phase, the frequency at 1 min from the start of ACh stimulation was used. For the frequency of the late phases, in the control experiment, the frequency 4 min after the ACh stimulation was used, whereas in the experiments using GW6471, Rp8BrPETcGMPs, L-NAME, or N-PLA, the highest frequency within 4–5 min from the start of ACh stimulation was used.

**Western blot analysis.** Western blot analysis for NO, NOS1, NOS3, and PPARα were carried out by using antral mucosa of guinea pigs. The procedures for protein extraction and Western blotting have already been published in our previous reports (21, 26). The antibodies used for detection were an anti-NOS1 antibody (LS-B3272, LifeSpan BioSciences), an anti-NOS2 antibody (no. 06-573, Millipore), an anti-NOS3 antibody (no. 07-520, Millipore), and an anti-PPARα antibody (LS-B2560, LifeSpan BioSciences). The protein band was visualized by use of an enhanced chemiluminescence reagent (WSE-7120 EzWestLumi plus, ATTO, Tokyo, Japan) and captured via a Lumino-image analyzer (LAS 3000; Fuji Film, Tokyo, Japan).

**Histological examination.** 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 NO1 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).

**Measurement of NO and cGMP.** The stripped antral mucosa from an animal was cut into three samples. For measuring the amount of NO released, the stripped mucosa was incubated in solution I containing 4% BSA (4°C) and then incubated for an additional 10 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.

The procedures and protocols for these experiments were reviewed and approved by the Animal Research Committee of Osaka Medical College, and the experiments were performed in accordance with the Guidelines of this Committee and the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences (Physiological Society of Japan).

**Observation of exocytosis.** The isolated antral mucous cells were mounted on a coverslip precoated with neutralized Cell-Tak (Becton Dickinson Labware, Bedford, MA) for firm attachment of the cells. The coverslip with the cells was set in a perfusion chamber mounted on the stage of a differential interference contrast microscope (BX50WI, Olympus, Tokyo, Japan), which was connected to a video- enhanced contrast system (ARGUS-20, Hamamatsu Photonics, Hamamatsu, Japan) (6, 7, 14, 15, 20, 22–24). Images were recorded continuously with a video recorder. The experiments were carried out 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 the granules (6, 7, 14, 15, 20, 22–24), were counted in two to four cells every 30 s and were normalized to the number of cells (events-cell\textsuperscript{−1}·30 s\textsuperscript{−1}). The frequency of exocytotic events measured from 5–11 coverslips from two or three animals was expressed as mean ± SE based on the number of experiments, and number, n, showed the number of experiments obtained from two or three animals. In the statistical comparisons of the experiments, the frequencies of the initial and late phases were used. For the frequency of the initial phase, the frequency at 1 min from the start of ACh stimulation was used. For the frequency of the late phases, in the control experiment, the frequency 4 min after the ACh stimulation was used, whereas in the experiments using GW6471, Rp8BrPETcGMPs, L-NAME, or N-PLA, the highest frequency within 4–5 min from the start of ACh stimulation was used.
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 ± 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 ± 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 EC50 of GW7647 and IC50 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 ± 0.9 and 1.7 ± 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. GW6471 decreased the frequency 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.
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 PKI 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 GW6476, 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, 11), 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. 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 1400W for NOS2, and 2 μM L-N5-OD for NOS3. The data sheets from Tocris
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 PPARγ appears to stimulate NO production via NOS1, if we assume that GW6471 or GW7647 only inhibit or activate the PPARγ-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 PPARγ (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
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...
were $0.02 \pm 0.27$ and $0.31 \pm 0.16$ 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 μM) 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 $2.2 \pm 0.3$ and $3.8 \pm 0.6$ 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 PPARα 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 $25.6 \pm 2.3$ 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 $40.8 \pm 6.8$ pmol/g tissue ($n = 5$). Stimulation with GW7647 or GW7647 plus ACh for 10 min also increased the cGMP content to $34.8 \pm 1.3$ pmol/g tissue ($n = 9$) or $50.5 \pm 7.7$ 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 $55.4 \pm 8.5$ pmol/g tissue ($n = 5$) or $66.5 \pm 5.2$ pmol/g tissue ($n = 9$), respectively. GW6471 or N-PLA inhibited these increases in cGMP content (Fig. 6B).

Thus PPARα 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 μM) for 10 min. In the control experiment, vehicle control (2.5 μM DMSO) increased [NO] of incubation solution from 2.99 ± 0.59 μM to 3.17 ± 0.66 μM (Δ[NO] = 0.17 ± 0.15 μM, $n = 3$). In the NOC 12 experiments, [NO] of incubation solution increased from 3.87 ± 1.60 nmol/g tissue to 3.91 ± 1.24 nmol/g tissue ($n = 3$). Furthermore, NOC12 (500 μM) slightly decreased the [NO] of incubation solution by 10.22 ± 0.33 μM.

- **Table 1. The NO concentration and the cGMP content**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Final [NO], μM</th>
<th>Δ[NO], μM</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 μM</td>
<td>44.74 ± 1.07</td>
<td>* (40.86 ± 2.49)</td>
<td>76.27 ± 9.23</td>
<td>3</td>
</tr>
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</table>

An increase in [NO] of the incubation solution by 1-hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazeno (NOC12) cause cGMP to accumulate in the antral mucosa. Values are expressed as means ± SE; $n$, number of experiments. *P < 0.05 vs. Control.
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 mucosae. 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. 8G).

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 detected, but immunohistochemistry revealed that vascular endothelial cells, not antral mucous cells, were stained for NOS3 (data not shown). NOS2 was not detected in the antral mucosa in both Western blotting and immunohistochemistry (data not shown).

Effects of GW7647 on [Ca2+]i. Stimulation with ACh increased [Ca2+]i, 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+]i stimulated by ACh. GW7647 added in the plateau did not enhance increases in [Ca2+]i 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+]i, and the further stimulation with ACh immediately increased [Ca2+]i, and then decreased to a plateau within 3 min. Increases in [Ca2+]i stimulated with ACh plus GW7647 were similar to those stimulated with ACh alone (Fig. 9, A and B). Thus GW7647 did not affect [Ca2+]i, with or without ACh stimulation. Our previous reports have exhibited that 8BrcGMP or Rp8BrPETcGMPS does not affect [Ca2+]i, 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, PPARα 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 antagonistic) 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 Rp8BrPETcGMPS (an inhibitor of PKG) (26). Moreover, the inhibitory actions of GW6471 were rescued by 8BrcGMP. 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 cGMP accumulation, leading to NO production followed by cGMP accumulation, which enhances the initial phase of ACh-stimulated exocytotic events.
The PPARs, including PPAR/γ, are nuclear receptors that modulate the expression of specific target genes (5, 8, 9, 16, 17). However, in antral mucous cells, a PPAR/γ 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/γ actions in this study are nongenomic. Some reports have already shown that PPAR/γ has nongenomic actions (2–4, 19).

There are many reports showing that AA is a natural ligand for PPAR/γ (5, 8, 9, 16, 17). The present study demonstrated that AA stimulates PPAR/γ 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, AACOCF₃, which is a nonmetabolic analog of AA, still enhanced the initial phase of the ACh-stimulated exocytotic events mediated via PPAR/γ, as previously reported (21), and stimulated NO production and cGMP accumulation in antral mucosae mediated via PPAR/γ. AACOCF₃, which is an inhibitor of PLA₂, 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/γ.

ACh stimulates an increase in [Ca²⁺]ᵢ and the increase in [Ca²⁺]ᵢ is essential to activate exocytotic events in antral mucous cells (6, 14, 20). This indicates that ACh activates the Ca²⁺-regulated exocytosis. Moreover, the frequency of cAMP-regulated exocytosis is 5–10% of that of Ca²⁺-regulated exo-

Fig. 8. Localization of NOS1 or PPAR/γ 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/γ. The cytoplasm and the nucleus of the antral mucous cell were positively stained for PPAR/γ. E: 4′,6-diamidino-2-phenylindole (DAPI) staining. F: merged C–E. NOS1 and PPAR/γ 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 Ca²⁺ concentration ([Ca²⁺]ᵢ). A: ACh (1 µM) immediately increased [Ca²⁺]ᵢ, and then decreased to a plateau within 3 min. The further addition of GW7647 did not affect ACh-stimulated increases in [Ca²⁺]ᵢ. The cessation of ACh stimulation immediately decreased [Ca²⁺]ᵢ. B: the addition of GW7647 (50 nM) did not increase [Ca²⁺]ᵢ, and then stimulation with ACh immediately increased [Ca²⁺]ᵢ, and then decreased to a plateau within 3 min. Increases in [Ca²⁺]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 \([Ca^{2+}]_i\), 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

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**Fig. 10.** AA/PPAR\(\alpha\) autocrine modulation of Ca\(^{2+}\)-regulated exocytosis in guinea pig antral mucous cells.
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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