cGMP modulation of ACh-stimulated exocytosis in guinea pig antral mucous cells

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Departments of 1Molecular Cell Physiology and 5Respiratory Molecular Medicine, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, and Departments of 2Physiology and 3Internal Medicine and 4Central Research Laboratory (Nakahari Project), Osaka Medical College, Takatsuki, Japan

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Saad, Adel H., Chikao Shimamoto, Takashi Nakahari, Shoko Fujiwara, Ken-ichi Katsu, and Yoshinori Marunaka. cGMP modulation of ACh-stimulated exocytosis in guinea pig antral mucous cells. Am J Physiol Gastrointest Liver Physiol 290: G1138–G1148, 2006. First published January 19, 2006; doi:10.1152/ajpgi.00357.2005.—In guinea pig antral mucous cells, ACh stimulates the Ca2+-regulated exocytosis, which has a characteristics feature: an initial transient phase followed by a sustained phase. The effects of cGMP on ACh-stimulated exocytosis were studied in guinea pig antral mucous cells using video microscopy. cGMP enhanced the frequency of ACh-stimulated exocytotic events, whereas cGMP alone did not induce any exocytotic events under the ACh-unstimulated condition. cGMP did not stimulate either Ca2+ mobilization or cAMP accumulation. The Ca2+-dose-response studies demonstrated that cGMP shifted the dose-response curve upward with no shift to the lower concentration. This indicates that cGMP increased maximal responsiveness of the Ca2+-regulated exocytosis, but not the Ca2+ sensitivity. Moreover, under a condition of ATP depletion by dinitrophenol (DNP) or anoxia (N2 bubbling), ACh evoked only a sustained phase in exocytotic events with no initial transient phase. However, ACh evoked an initial transient phase followed by a sustained phase with addition of cGMP after ATP depletion, whereas only a sustained phase was evoked in a case of cGMP addition after ATP depletion. Thus cGMP-induced enhancement in ACh-stimulated exocytotic events requires ATP, suggesting that cGMP modulates ATP-dependant priming of Ca2+-regulated exocytosis in antral mucous cells. In conclusion, cGMP increases the number of primed granules via acceleration of the ATP-dependent priming, which enhances the Ca2+-regulated exocytosis stimulated by ACh.

Gastric mucin secretion; guanosine 3′-cyclic monophosphate; exocytosis; acetylcholine; intracellular calcium concentration

cGMP is an established intracellular second messenger that is accumulated in the cell by hormones, peptides, neurotransmitters, and nitric oxide (NO; see Refs. 27 and 30). The accumulation of cGMP, which is synthesized by guanylate cyclase, activates cGMP-dependent protein kinase (19), leading to the phosphorylation of target proteins. Their phosphorylation modulates many cellular functions, including ion transport, protein synthesis, cellular metabolism, and the relaxation of smooth muscle. Moreover, cGMP increases Ca2+ influx in several cell types, including pancreatic acinar cells, submandibular acinar cells, and colonic epithelial cells (1, 2, 33), and also increases cAMP content by inhibiting cAMP breakdown in platelet and vascular tissues (5, 18).

NO/cGMP protects endothelial cells from damage in the pulmonary artery (24, 25). PGE2 increases intracellular cGMP content in rabbit gastric parietal cells, protecting the cells from injuries via activation of Cl− channels (26). cGMP also increases HCO3− secretion in bullfrog duodenum, which shows a protecting function from damage (8). These observations suggest that cGMP plays an important role in gastric cytoprotection.

Gastric mucin, which is secreted by exocytosis from mucous cells and covers mucosal surfaces, protects the mucosa from gastric acid. An increase in mucin secretion is believed to be important for gastric mucosal protection. PGE2, which is a key mediator of gastric cytoprotection, stimulates mucin exocytosis in antral mucous cells (22). Moreover, ACh-stimulated PGE2 release, which leads to cAMP accumulation via the prostaglandin EP4 receptor, is demonstrated to be essential for the maintenance of a large amount of mucin secretion in antral mucous cells (21, 22, 28). As mentioned above, the NO/cGMP pathway protects cells from damage in many tissues. cGMP also modulates exocytosis in salivary acinar cells, pancreatic acinar cells, HT-29 cells (human colonic mucus-secreting cells), and chromaffin cells (3, 12, 17, 34), leading us to an idea that cGMP may increase mucin exocytosis in antral mucous cells.

We have been studying exocytosis in isolated antral mucous cells by directly observing exocytotic events using video-enhanced microscopy. Our previous studies demonstrated that “Ca2+-regulated exocytosis” is the main mechanism for mucin release in antral mucous cells of the guinea pig (6, 7, 21, 22, 28). In the present study, we investigated whether cGMP enhances Ca2+-regulated exocytosis in antral mucous cells and clarified how cGMP modulates Ca2+-regulated exocytosis in antral mucous cells.

MATERIALS AND METHODS

Solutions and chemicals. Solution I contained (in mM): 121 NaCl, 4.5 KCl, 25 NaHCO3, 1 MgCl2, 1.4 CaCl2, 5 Na-HEPES, 5 H-HEPES, and 5 glucose. To prepare a Ca2+-free solution, CaCl2 was excluded from solution I, and 1 mM EGTA was added. The pH values of the solutions were adjusted to 7.4 by adding 1 M HCl. The solutions were gassed with 95% O2 and 5% CO2 at 37°C. A HCO3−-free solution, in which NaHCO3 of solution I was replaced with NaCl, was gassed with 100% O2 or 100% N2. H-89 and PKI-(6–22) were purchased from Biomol (Plymouth Meeting, PA), ionomycin (IM), dibutyryl-cGMP (DBcGMP), 8-bromo-cGMP (8-BrcGMP), and dinitro-
phenol (DNP) were purchased from Sigma (St. Louis, MO); ACh was from Daiichi Pharmaceuticals (Osaka, Japan); and collagenase (for cell dispersion, 180–220 U/mg) and BSA were from Wako (Osaka, Japan). All reagents were dissolved in DMSO and diluted to their final concentrations just before the experiments. The concentration of DMSO never exceeded 0.1%; at this concentration, DMSO does not affect exocytotic events in antral mucous cells (6, 7, 11, 21).

**Cell preparations.** Hartley strain male guinea pigs weighing ~250 g were purchased from Shimizu (Kyoto, Japan) and 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 were previously described in detail (6, 7, 21, 22, 28).

Briefly, the antrum was excised and the mucosal layer was stripped from the muscle layer in cooled saline (4°C), using glass slides. The stripped antral mucosa was minced and then incubated in solution I containing 0.1% collagenase and 2% BSA for 10 min at 37°C. The digested mucosa was then filtered through a nylon mesh with a pore size of 150-μm squares and washed three times. The cells were resuspended in solution I containing 2% BSA (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 cover slip precoated with neutralized Cell-Tak (Becton Dickinson Labware, Bedford, MA) for the firm attachment of cells. This cover slip was in a perfusion chamber mounted on the stage of a differential interference contrast microscope (BX50Wi; Olympus, Tokyo, Japan) connected to a video-enhanced contrast system (ARGUS-10; Hamamatsu Photonics, Hamamatsu, Japan; see Refs. 6, 7, 10, 11, 14, 20, 21, 28). Images were recorded continuously using 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 (4, 6, 21, 22, 28, 29), were counted in five to six cells every 30 s and normalized to the number per cell during a unit time period (events·cell⁻¹·30 s⁻¹). The frequencies of exocytotic events in four to seven experiments were expressed as

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**Fig. 1.** Effect of cGMP on ACh-stimulated exocytotic events in antral mucous cells. The ACh concentration was 1 μM. ACh (1 μM) alone-activated exocytotic events consisted of an initial phase followed by a sustained phase (n = 6). A: 8-bromo-cGMP (8-BrcGMP, 100 μM) alone did not induce exocytotic events. However, 8-BrcGMP added 5 min before ACh stimulation enhanced the initial phase and the sustained phase in the ACh-stimulated exocytotic events (n = 4). B: dibutyl-cGMP (DBcGMP, 200 μM) enhanced the frequency of ACh-stimulated exocytotic events similar to 8-BrcGMP (100 μM, n = 5). C: effect of 8-BrcGMP on the sustained phase in ACh-stimulated exocytotic events. 8-BrcGMP (100 μM) added 4 min after the ACh addition increased the sustained phase ~100% (n = 5).

The removal of 8-BrcGMP returned the sustained frequency to the control level immediately. Values marked by arrows are significantly different (P < 0.05). D: cells were stimulated with both 8-BrcGMP (200 μM) and ACh (1 μM) simultaneously. 8-BrcGMP added simultaneously enhanced the ACh-stimulated exocytotic events similarly to those shown in A. E: dose effect of 8-BrcGMP on the initial peak frequency of 1 μM ACh-stimulated exocytotic events. F: dose effect of 8-BrcGMP on the sustained phase frequency of 1 μM ACh-stimulated exocytotic events. 8-BrcGMP enhanced the initial phase and sustained phase in a dose-dependent manner. At 8-BrcGMP concentrations >50 μM, the frequencies of the initial and sustained phase reached plateau levels. One experiment was performed on 4–6 cover slips from 2–3 animals. *Significantly different from the control value (1 μM ACh alone), P < 0.05.
means ± SE. In statistically comparing the results obtained from the experiments, the initial peak frequency and sustained frequency were used. The initial peak frequency was the peak frequency within 2 min from the start of ACh stimulation, and the sustained frequency was obtained by averaging three frequencies at 3, 3.5, and 4 min after the start of ACh stimulation (21). One experiment was performed using four to seven cover slips obtained from two to four animals.
To calculate the intermediate concentration of a dose-response curve, a program for curve fitting (sigmoid curve) was used. The following is the equation used for curve fitting:

\[ y = (a - d)/(1 + (x/c)^b) + d, \]

where \(a-d\) are constant, \(x\) is a concentration used, and \(y\) is the frequency of exocytotic events; \(a\) is the maximum value, \(d\) is the minimum value, \(c\) is the intermediate concentration, and \(b\) is a constant.

**Intracellular Ca\(^{2+}\) concentration measurements.** The isolated antral mucous cells were incubated in solution I containing 2% BSA and 2.5 \(\mu\)M 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 on a cover slip precoated with neutralized Cell-Tak. These cover slips were set in a perfusion chamber, which was then mounted on the stage of an inverted microscope (IX70; Olympus) connected to an image analysis system (ARGUS/HiSCA; Hamamatsu Photonics; see Refs. 6, 7, 21, 22). All experiments were performed at 37°C. The volume of the perfusion chamber was \(\approx 80 \mu\)L, and the rate of perfusion was 500 \(\mu\)L/min. Fura 2 was excited at 340 and 380 nm, and the emission was measured at 510 nm. The fluorescence ratio [ratio of fluorescence at 340 nm to 380 nm (F340/F380)] was calculated and stored in an image analysis system. Solution II contained (in mM): 130 KCl, 20 NaCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES. To prepare the cell-free Ca\(^{2+}\) calibration solutions, an appropriate amount of CaCl\(_2\) (0.2–2 mM) calculated was standardized and stored in an image analysis system. Solution I contained (in mM): 130 KCl, 20 NaCl, 2 EGTA, and 10 HEPES. To prepare the cell-free Ca\(^{2+}\) calibration solutions, an appropriate amount of CaCl\(_2\) (0.2–2 mM) calculated was standardized and stored in an image analysis system. Solution II contained (in mM): 130 KCl, 20 NaCl, 2 EGTA, and 10 HEPES. To prepare the cell-free Ca\(^{2+}\) calibration solutions, an appropriate amount of CaCl\(_2\) (0.2–2 mM) calculated was standardized and stored in an image analysis system. Solution II contained (in mM): 130 KCl, 20 NaCl, 2 EGTA, and 10 HEPES.

**RESULTS**

In unstimulated antral mucous cells, no exocytotic events were observed. ACh (1 \(\mu\)M) activated exocytotic events, which consisted of two phases, an initial phase followed by a sustained phase (Fig. 1, A and C).

**Effects of cGMP on ACh-stimulated exocytotic events.** 8-BrcGMP (100 \(\mu\)M) enhanced the both phases of the 1 \(\mu\)M ACh-stimulated exocytotic events in antral mucous cells, whereas 8-BrcGMP (100 \(\mu\)M) alone induced no exocytotic events. 8-BrcGMP (100 \(\mu\)M) increased the initial peak frequency and the sustained phase frequency of ACh-stimulated exocytotic events \(\sim 100\%\) and \(80\%\), respectively (Fig. 2A). 8-BrcGMP (200 \(\mu\)M) also enhanced the frequency of the ACh-stimulated exocytotic events similar to 8-BrcGMP (Fig. 1B). 8-BrcGMP added 4 min after the start of ACh stimulation increased the frequency of exocytotic events in the sustained phase by \(\sim 100\%\), and 8-BrcGMP removal immediately diminished the elevated frequency to the control level with 1 \(\mu\)M ACh alone (Fig. 1C). 8-BrcGMP (200 \(\mu\)M) simultaneously added with ACh (1 \(\mu\)M) also enhanced the ACh-stimulated exocytotic events similarly to that added before ACh addition (Fig. 1D). The dose effects of 8-BrcGMP on 1 \(\mu\)M ACh-stimulated exocytotic events are shown in Fig. 1, E and F. The initial peak frequency and sustained phase frequency of the 1 \(\mu\)M ACh-stimulated exocytotic events increased as increment of 8-BrcGMP concentration from 5 to 50 \(\mu\)M, and they reached a plateau at concentrations \(\geq 100\) \(\mu\)M. Therefore, we used 200 \(\mu\)M 8-BrcGMP in the present study.

cGMP was reported to accumulate cAMP by inhibiting phosphodiesterase III (5, 18). In general, cAMP induces cellular actions by stimulating protein kinase A (PKA). Therefore, if cGMP accumulates cAMP, the cGMP action is blocked by inhibition of PKA. We examined the effect of PKI (a PKA inhibitor) on cGMP action. To inhibit PKA, cells were treated with PKI (1 \(\mu\)M) for 5 min, which eliminated the exocytotic events stimulated by 1 \(\mu\)M forskolin (Fig. 2C). As a control experiment, we applied 10 \(\mu\)M ACh, which evoked an initial phase followed by a sustained phase in the exocytotic events (Fig. 2A). The addition of 8-BrcGMP enhanced the initial peak frequency and the sustained phase frequency of ACh-stimulated exocytotic events (Fig. 2A). PKI (1 \(\mu\)M) added 5 min before ACh stimulation decreased the initial peak frequency of ACh-stimulated exocytotic events by \(\sim 60\%\) (Fig. 2A). Inhibition of PKA by H-89 (another PKA inhibitor) has been reported to decrease the initial peak frequency of ACh-stimulated exocytotic events similarly by inhibition of a PGE\(_2\)/cAMP-autocrine mechanism (28). 8-BrcGMP added 5 min before ACh stimulation still enhanced the initial peak frequency of
ACh-stimulated exocytotic events in PKI-treated cells (Fig. 2B). The initial peak frequencies of ACh-stimulated exocytotic events with or without 8-BrcGMP were plotted for PKI-treated cells and non-PKI-treated cells (Fig. 2D). 8-BrcGMP still increased the initial peak frequency of ACh-stimulated exocytotic events in the presence of PKI. Similar results were obtained by 20 μM H-89 (data not shown). Thus 8-BrcGMP increased the frequency of ACh-stimulated exocytotic events independent of PKA (i.e., not via accumulation of cAMP).

Effects of Ca^{2+} on cGMP-induced enhancement. Antral mucous cells were stimulated with 10 μM ACh in the Ca^{2+}-free solution. ACh induced a small initial phase without any sustained phase of exocytotic events. Further addition of 8-BrcGMP enhanced the small initial transient phase, but no sustained phase was detected (Fig. 3A). To completely chelate intracellular Ca^{2+}, antral mucous cells were incubated with 25 μM BAPTA-AM for 30 min at room temperature (21–23°C). In the BAPTA-loaded cells perfused with the Ca^{2+}-free solution, ACh (10 μM) induced no exocytotic events, and the further addition of 8-BrcGMP also induced no exocytotic events (Fig. 3B). In the presence of Ca^{2+} channel blocker (1 mM Ni^{2+} or 100 μM Gd^{3+}), ACh induced only a small initial phase, which was enhanced by the addition of 8-BrcGMP (Fig. 3, C and D).

The effects of 8-BrcGMP on the exocytotic events activated by 1 μM IM or 2 μM thapsigargin (TG) were examined. The compounds IM and TG stimulate the store-operated Ca^{2+} entry by depletion of the Ca^{2+} stores without muscarinic receptor stimulation. Cells were perfused with the Ca^{2+}-free solution containing 1 μM IM for 5 min, and then the perfusion solution was switched to the Ca^{2+}-containing solution (solution I), keeping the concentration of IM constant (reintroduction of Ca^{2+}). The reintroduction of Ca^{2+} induced an initial phase followed by a sustained phase of the exocytotic events in IM-treated cells, similar to ACh stimulation. 8-BrcGMP enhanced the IM-stimulated exocytotic events after the reintroduction of Ca^{2+}.

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**Fig. 5.** Dose effects of ACh on exocytotic events in 8-BrcGMP-treated cells (○) and non-8-BrcGMP-treated cells (○). The concentration of 8-BrcGMP used was 200 μM. A: 0.1 μM ACh. B: 4 μM ACh. C: 40 μM ACh. D: 100 μM ACh. E: dose effects of ACh on the initial peak frequencies of the exocytotic events in non-8-BrcGMP-treated cells (○) and 8-BrcGMP-treated cells (○). ACh increased the initial peak frequencies of exocytotic events in a dose-dependent manner. The IC_{50} were 8.9 μM in the absence of 8-BrcGMP and 5.4 μM in the presence of 8-BrcGMP. F: dose effects of ACh on the sustained phase of the exocytotic events in non-8-BrcGMP-treated cells (○) and 8-BrcGMP-treated cells (○). ACh increased the sustained phase frequencies of exocytotic events in a dose-dependent manner. The IC_{50} were 1.8 μM in the absence of 8-BrcGMP and 0.7 μM in the presence of 8-BrcGMP. *Significantly different from control value (ACh alone), P < 0.05.
duction of Ca²⁺ (Fig. 4A). Similar experiments were performed using 2 μM TG. In TG-treated cells, the reintroduction of Ca²⁺ also induced an initial phase followed by a sustained phase of the exocytotic events, and 8-BrcGMP enhanced the TG-stimulated exocytotic events (Fig. 4B). Thus 8-BrcGMP enhanced the frequency of exocytotic events stimulated by [Ca²⁺]i increase without ACh stimulation.

**Effects of cGMP on the ACh dose-response curve.** ACh (0.01 μM) induced no exocytotic events in antral mucous cells, and the further addition of 8-BrcGMP (200 μM) did not induce any exocytotic events (data not shown). ACh of 0.1 μM increased the frequency of exocytotic events slightly (0.8 events·cell⁻¹·30 s⁻¹), and the addition of 8-BrcGMP enhanced the frequency of exocytotic events stimulated by 0.1 μM ACh (1.5 events·cell⁻¹·30 s⁻¹; Fig. 5A). ACh of 4 μM induced an initial phase followed by a sustained phase of exocytotic events. The addition of 8-BrcGMP further increased the frequencies of both by ~100%. ACh of 40 or 100 μM induced an initial phase followed by a sustained phase, and further addition of 8-BrcGMP increased the initial peak frequency by ~30%, but it suppressed the sustained phase (Fig. 5, C and D). Because a high concentration of ACh, such as 1 mM, inhibits the initial phase of exocytotic events as shown in the ACh dose-response study (6), cGMP may enhance this inhibitory effect especially in the sustained phase. Moreover, a high concentration of ACh reduces the number of granules during the initial phase. This may also cause the exocytotic events in the sustained phase to decrease, especially in 8-BrcGMP-treated cells.

Results of the ACh dose-response study are summarized in Fig. 5, E and F. The initial peak frequency of ACh-stimulated exocytotic events increased with an increment of ACh concentration from 0.1 to 100 μM, although it decreased at the ACh concentration of 1 mM. The addition of 8-BrcGMP enhanced the initial peak frequency of the ACh-stimulated exocytotic events within the range of ACh concentration from 0.1 to 100 μM; however, the 8-BrcGMP treatment did not influence the initial peak frequency induced by 1 mM ACh (Fig. 5E). Thus
8-BrcGMP shifted the ACh dose-response curve upward within the range of ACh concentrations from 0.1 to 100 μM, whereas at a higher ACh concentration such as 1 mM, 8-BrcGMP appears to have no effects on the exocytotic responses in antral mucous cells. In the ACh dose-response curve of the initial peak frequency, the half-maximum concentrations (ED50) were 8.9 μM in the absence of 8-BrcGMP and 5.5 μM in the presence of 8-BrcGMP (Fig. 5E).

The sustained phase frequency of ACh-stimulated exocytotic events also increased with an increment in ACh concentration from 0.01 to 100 μM (Fig. 5F). However, the frequency decreased as increment of ACh concentration from 100 μM to 1 mM. 8-BrcGMP enhanced the sustained phase frequency of ACh-stimulated exocytotic events within the range of ACh concentration from 0.1 to 10 μM; however, it decreased the sustained phase frequency at concentrations of 40 and 100 μM. Thus 8-BrcGMP shifted the ACh dose-response curve upward within the range of concentrations from 0.01 to 10 μM (Fig. 5E). In the ACh dose-response curve of the sustained phase, the ED50 in the absence and presence of 8-BrcGMP were 1.8 and 0.65 μM, respectively.

Thus it remains uncertain whether or not 8-BrcGMP shifts the ACh dose-response curve toward the lower concentration side in the initial peak frequency or sustained phase frequency of exocytotic events. To confirm this, ACh (1 μM)-stimulated exocytotic events were measured at various [Ca2+]o, controlled by altering extracellular Ca2+ concentration ([Ca2+]o).

Effects of [Ca2+]o. [Ca2+]o used was within the range of concentrations from 10 μM to 1.5 mM. At 10 μM [Ca2+]o, 1 μM ACh induced the initial phase without any sustained phase in exocytotic events (Fig. 6A). The frequency of the initial peak and the sustained phase frequency of ACh-stimulated exocytotic events increased as an increment of [Ca2+]o from 0.2 to 1 mM (Fig. 6, B-D). 8-BrcGMP enhanced the frequencies of ACh-stimulated exocytotic events at all [Ca2+]o, except the sustained phase frequency at 10 μM [Ca2+]o. The initial peak frequency and the sustained phase frequency were plotted against [Ca2+]o (Fig. 6, E and F). 8-BrcGMP shifted the Ca2+ dose-response curves upward for the initial peak frequency and also for the sustained phase frequency. In the dose-response curve of the initial peak frequency, ED50 were 0.21 mM in the absence of 8-BrcGMP and 0.26 mM in the presence of 8-BrcGMP.
8-BrcGMP (Fig. 6E). In the dose-response curve of the sustained phase frequency, the ED50 in the absence and presence of 8-BrcGMP were 0.62 and 0.62 mM, respectively (Fig. 6F). Based on these observations, it is concluded that 8-BrcGMP does not affect the Ca^{2+} sensitivity in the ACh-stimulated exocytosis.

**Effects of DNP and anoxia.** The effects of cGMP on the ATP-dependent priming of Ca^{2+}-regulated exocytosis were examined by depleting ATP. To deplete intracellular ATP, DNP (an uncoupler of oxidative phosphorylation) and N2 bubbling (anoxia) were used. In these experiments, ACh used was 1 μM. Cells were pretreated with 100 μM DNP for 3 min before ACh stimulation. Stimulation with ACh induced only the sustained phase of the exocytotic events with no initial phase (Fig. 7A). When cells were treated with DNP (100 μM) for 3 min or longer, no initial phase of ACh-stimulated exocytotic events was detected. 8-BrcGMP added before DNP addition induced an initial phase followed by a sustained phase in the ACh-stimulated exocytotic events (Fig. 7C), whereas 8-BrcGMP added after the DNP addition induced only the sustained phase of ACh-stimulated exocytotic events, the frequency of which was enhanced (Fig. 7B). However, the initial peak frequency (7.4 ± 0.7 events·cell⁻¹·30 s⁻¹, n = 5) was small compared with that induced by 1 μM ACh alone (10.2 ± 1.2 events·cell⁻¹·30 s⁻¹, n = 5). Similar experiments were performed in cells perfused with HCO₃⁻-free solution bubbled with N₂. N₂ bubbling (anoxia) eliminated the initial transient phase with no effects on the sustained phase in ACh-stimulated exocytotic events (Fig. 7D). When cells were bubbled with N₂ for 5 min or longer, no initial phase of ACh-stimulated exocytotic events was detected. 8-BrcGMP added before the N₂ bubbling (anoxia) evoked an initial phase followed by a sustained phase in the ACh-stimulated exocytotic events (Fig. 7F), whereas 8-BrcGMP added after N₂ bubbling induced only the sustained phase, the frequency of which was enhanced (Fig. 7E). The initial peak frequency (9.7 ± 1.0 events·cell⁻¹·30 s⁻¹, n = 5) was small compared with that induced by ACh.

![Image](http://ajpgi.physiology.org/)

Fig. 8. Cells were restimulated with ACh (1 μM) after a brief recovery (5 min) and after a brief ACh stimulation (1 min). A: after a brief recovery in the control condition, the second ACh stimulation induced only a sustained phase without any initial phase. B: after a brief recovery with 200 μM 8-BrcGMP, the second ACh stimulation induced an initial phase followed by a sustained phase in the exocytotic events.

![Image](http://ajpgi.physiology.org/)

Fig. 9. [Ca^{2+}]i changes in ACh-stimulated antral mucous cells. The ratio of fura 2 [ratio of fluorescence at 340 nm to 380 nm (F340/F380)] was measured in antral mucous cells. ACh and 8-BrcGMP used were 1 and 200 μM, respectively. A: ACh increased F340/F380. B: 8-BrcGMP was added before the ACh addition. ACh increased F340/F380, which was similar to that induced by ACh alone. C: 8-BrcGMP added during ACh stimulation did not induce any further increase in F340/F380.
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alone (12.3 ± 1.0 events·cell⁻¹·30 s⁻¹, n = 5). Thus ATP depletion by DNP or anoxia eliminates the initial phase. 8-BrcGMP added before ATP depletion induces the initial phase, whereas 8-BrcGMP added after ATP depletion induced no initial phase. cGMP added in the presence of ATP appears to increase the number of the primed granules, which was maintained by cGMP even during depletion of ATP, although it decreases gradually.

Another test of the hypothesis that cGMP accelerates priming was performed by examining the ability of cGMP to enhance a second stimulation by ACh. Cells were restimulated with 1 μM ACh after a brief recovery in control condition (5 min) following a brief ACh stimulation (1 min). The second ACh stimulation induced only a sustained phase without any initial transient phase (Fig. 8A). Cells were also restimulated with 1 μM ACh after a brief recovery with 200 μM 8-BrcGMP (5 min) following a brief ACh stimulation (1 min). The second stimulation, however, induced an initial transient phase followed by a sustained phase (Fig. 8B). Thus a brief recovery with 8-BrcGMP recovered the number of primed granules, which were depleted by the first ACh stimulation. These suggest that 8-BrcGMP accelerates priming.

\[ \text{[Ca}^{2+}]_i \text{ measurement.} \] cGMP is reported to increase \([\text{Ca}^{2+}]_i\) in many cell types (1, 2, 33). We measured \([\text{Ca}^{2+}]_i\) to examine whether or not cGMP increases \([\text{Ca}^{2+}]_i\) influx in antral mucous cells. In antral mucous cells, stimulation with 1 μM ACh increased the fura 2 ratio (F340/F380) rapidly and sustained it at a level higher than basal (Fig. 9A). The addition of 8-BrcGMP alone did not increase F340/F380. Further stimulation with ACh increased the ratio to a level similar to that of the non-8-BrcGMP-treated cells (Fig. 9B). In the third experiment, cells were first stimulated with ACh, and then 8-BrcGMP was added. ACh increased F340/F380 rapidly and sustained it. The further addition of 8-BrcGMP induced no significant increase in F340/F380. Thus 8-BrcGMP did not increase \([\text{Ca}^{2+}]_i\) under the basal or the ACh-stimulated conditions.

DISCUSSION

The present study demonstrated that cGMP accumulation enhances the frequency of ACh-stimulated exocytotic events in antral mucous cells. ACh stimulates \([\text{Ca}^{2+}]_i\) influx from extracellular fluid, which increases \([\text{Ca}^{2+}]_i\). IM or TG also increases \([\text{Ca}^{2+}]_i\), similarly to ACh without any stimulation of muscarinic receptors (31). These increases in \([\text{Ca}^{2+}]_i\) induce an initial transient phase followed by a sustained phase in the frequency of exocytotic events in antral mucous cells. In contrast, the inhibition of \([\text{Ca}^{2+}]_i\) entry using a \([\text{Ca}^{2+}]_i\)-free solution or \([\text{Ca}^{2+}]_i\) channel blockers causes the ACh-stimulated exocytotic events to be few and transient. A small transient increase in the frequency of ACh-stimulated exocytotic events was induced by \([\text{Ca}^{2+}]_i\) released from intracellular stores. Moreover, ACh evoked no exocytotic events when intracellular \([\text{Ca}^{2+}]_i\) was chelated by BAPTA. Thus an initial phase followed by a sustained phase in the ACh-stimulated exocytotic events is a characteristic feature of \([\text{Ca}^{2+}]_i\)-regulated exocytosis in antral mucous cells. The ACh-, IM-, or TG-evoked exocytotic events enhanced by cGMP also showed an initial transient phase followed by a sustained phase in its frequency. cGMP alone caused no exocytotic events, and had no effect on the exocytotic events during ACh stimulation in BAPTA-loaded cells. These observations indicate that cGMP enhances \([\text{Ca}^{2+}]_i\)-regulated exocytosis in antral mucous cells.

cGMP accumulates cAMP by inhibiting phosphodiesterase III in platelets and vascular tissues (5, 13, 15, 18). The present study demonstrated that cGMP enhanced the frequency of ACh-stimulated exocytotic events independent of PKA inhibitors. Thus cGMP-induced enhancement of \([\text{Ca}^{2+}]_i\)-regulated exocytosis is not induced by cAMP accumulation.

In some exocrine cells, cGMP affects \([\text{Ca}^{2+}]_i\) influx or \([\text{Ca}^{2+}]_i\) release from stores (1, 2). However, \([\text{Ca}^{2+}]_i\) measurements using fura 2 fluorescence demonstrated that 8-BrcGMP induces no increase in \([\text{Ca}^{2+}]_i\) in unstimulated cells and did not enhance an increase in \([\text{Ca}^{2+}]_i\) in ACh-stimulated antral mucous cells. These observations indicate that cGMP does not stimulate \([\text{Ca}^{2+}]_i\) mobilization in antral mucous cells. Thus cGMP enhances the frequency of ACh-stimulated exocytotic events not via the accumulation of either cAMP or \([\text{Ca}^{2+}]_i\).

The \([\text{Ca}^{2+}]_i\)-regulated exocytosis in antral mucous cells consisted of two phases, as mentioned above. Similar biphasic responses in exocytosis were reported in pancreatic acinar cells, salivary acinar cells, and bullfrog peptic cells (4, 29, 35). In pancreatic acinar cells, exocytosis is composed of two biochemically distinct steps (32). The first step requires ATP, but not \([\text{Ca}^{2+}]_i\), and prims granules with exocytotic machinery. The second step requires \([\text{Ca}^{2+}]_i\), but not ATP, and triggers fusion of the granules to the apical membrane (23).

The present study demonstrated that depletion of ATP by DNP or anoxia abolished the initial phase in ACh-stimulated exocytotic events without any effect on the sustained phase. These observations are explained as follows: depletion of ATP

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**Fig. 10.** A possible model of cGMP modulation of \([\text{Ca}^{2+}]_i\)-regulated exocytosis in guinea pig antral mucous cells.
Inhibits priming and decreases the pool of primed granules, which results in no initial phase of ACh-stimulated exocytotic events. However, a small amount of ATP is supplied from the anaerobic glycolysis, and it primes granules with exocytotic machinery, which maintains the exocytotic events at the ACh-stimulated sustained phase. The anoxia (N₂ bubbling) has already been reported not to affect [Ca²⁺], in non-ACh-stimulated and ACh-stimulated antral mucous cells (21). Thus the initial phase is induced by the fusion of the pooled primed granules, and the sustained phase is induced by the recruitment of granules to the apical membrane and the repriming of the exocytotic apparatus, as previously reported for pancreatic acinar cells and antral mucous cells (21, 23). However, cGMP added before ATP depletion (by DNP or anoxia) induced an initial transient phase followed by a sustained phase in the ACh-stimulated exocytotic events. In contrast, cGMP added after ATP depletion induced only a sustained phase with no initial transient phase in the ACh-stimulated exocytotic events. These results suggest that 1) cGMP accelerates the ATP-dependent priming and increases the number of primed granules, and 2) cGMP maintains a pool of these primed granules, even in the absence of ATP. Under this condition, ACh triggers the fusion of the pooled primed granules, which evokes the initial phase. However, cGMP also enhanced the sustained phase even after the ATP depletion by DNP or anoxia. This suggests that cGMP still accelerates the priming step, which might be mediated by ATP supplied from the anaerobic glycolysis.

CGRP did not shift the Ca²⁺ dose-response curve toward the lower concentration. These observations indicate that cGMP did not increase the Ca²⁺ sensitivity of ACh-stimulated exocytotic events, that is, cGMP is unlikely to enhance Ca²⁺-dependent fusion of Ca²⁺-regulated exocytosis in antral mucous cells. Our previous study has demonstrated that cAMP increases the Ca²⁺ sensitivity of ACh-stimulated exocytosis in antral mucous cells, suggesting that cAMP enhances fusion (21). This indicates that cGMP modulates ACh-stimulated exocytosis differently from cAMP.

NO is reported to accumulate cGMP in gastric parietal cells and duodenal cells, and an increase in [Ca²⁺)], stimulates NO synthase in many cell types. In gastric mucosal cells, the NO/cGMP pathway is known to play an important role in mucosal protection. In antral mucous cells, NO/cGMP pathway may enhance Ca²⁺-regulated exocytosis, which may play an important role in mucosal protection. Further studies will be needed to clarify what stimulates the NO/cGMP pathway in antral mucous cells.

Figure 10 shows a possible model of the cGMP modulation of Ca²⁺-regulated exocytosis in antral mucous cells. The accumulation of cGMP accelerates priming of the Ca²⁺-regulated exocytosis, but not fusion. On the other hand, cAMP modulates both priming and fusion (21). The modulation by cAMP has already been reported to be essential in maintaining Ca²⁺-regulated exocytosis in antral mucous cells during massive mucous secretion, such as during a meal (21, 28). Although the cGMP-induced enhancement of Ca²⁺-regulated exocytosis is small compared with the cAMP-induced one, cGMP continuously increases the frequency of ACh-stimulated exocytotic events in the sustained phase. This continuous secretion appears to be of particular importance in mucosal protection.

In conclusion, cGMP enhances Ca²⁺-regulated exocytosis by accelerating the priming step in antral mucous cells.

REFERENCES


