[Cl\textsuperscript{−}]\texttextsubscript{i} modulation of Ca\textsuperscript{2+}-regulated exocytosis in ACh-stimulated antral mucous cells of guinea pig

Chikao Shimamoto,1,2 Eiji Umegaki,1,2 Ken-ichi Katsu,1,2 Masumi Kato,1,3 Shoko Fujiwara,1,3 Takahiro Kubota,1,3 and Takashi Nakahari1,3

1Central Research Laboratory (Nakahari Project), 2Department of Internal Medicine (Division II), and 3Department of Physiology, Osaka Medical College, Takatsuki, Japan

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Shimamoto C, Umegaki E, Katsu K, Kato M, Fujiwara S, Kubota T, Nakahari T, [Cl\textsuperscript{−}], modulation of Ca\textsuperscript{2+}-regulated exocytosis in ACh-stimulated antral mucous cells of guinea pig. Am J Physiol Gastrointest Liver Physiol 293: G824–G837, 2007. First published August 2, 2007; doi:10.1152/ajpgi.00125.2007.—The effects of intracellular Cl concentration ([Cl\textsuperscript{−}]\texttextsubscript{i}) on acetylcholine (ACh)-stimulated exocytosis were studied in guinea pig antral mucous cells by video microscopy. ACh activated Ca\textsuperscript{2+}-regulated exocytosis (an initial phase followed by a sustained phase). Bumetanide (20 μM) or a Cl\textsuperscript{−}-free solution (NO3\textsuperscript{−}) solution enhanced it; in contrast, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, a Cl\textsuperscript{−} channel blocker) decreased it and eliminated the enhancement induced by bumetanide or NO3\textsuperscript{−} solution. ACh and Ca\textsuperscript{2+} dose-response studies demonstrated that NO3\textsuperscript{−} solution does not shift their dose-response curves, and ATP depletion studies by dinitrophenol or anoxia demonstrated that exposure of NO3\textsuperscript{−} solution prior to ATP depletion induced an enhanced initial phase followed by a sustained phase, whereas exposure of NO3\textsuperscript{−} solution after ATP depletion induced only a sustained phase. Intraacellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\texttextsubscript{i}) measurements showed that bumetanide and NO3\textsuperscript{−} solution enhanced the ACh-stimulated [Ca\textsuperscript{2+}]\texttextsubscript{i} increase. Measurements of [Cl\textsuperscript{−}]\texttextsubscript{i}, revealed that ACh decreases [Cl\textsuperscript{−}]\texttextsubscript{i}, and that bumetanide and NO3\textsuperscript{−} solution decreased [Cl\textsuperscript{−}]\texttextsubscript{i} and enhanced the ACh-evoked [Cl\textsuperscript{−}]\texttextsubscript{i} decrease; in contrast, NPPB increased [Cl\textsuperscript{−}]\texttextsubscript{i}, and inhibited the [Cl\textsuperscript{−}]\texttextsubscript{i} decrease induced by ACh, bumetanide, or NO3\textsuperscript{−} solution. These suggest that [Cl\textsuperscript{−}]\texttextsubscript{i} modulates [Ca\textsuperscript{2+}]\texttextsubscript{i} increase and ATP-dependent priming. In conclusion, a decrease in [Cl\textsuperscript{−}]\texttextsubscript{i} accelerates ATP-dependent priming and [Ca\textsuperscript{2+}]\texttextsubscript{i} increase, which enhance Ca\textsuperscript{2+}-regulated exocytosis in ACh-stimulated antral mucous cells.

gastric antrum; mucin exocytosis; acetylcholine; intracellular Cl concentration

ACETYLCHOLINE (ACh) activates Ca\textsuperscript{2+}-regulated exocytosis in guinea pig antral mucous cells, which is the main mechanism for mucin release (6, 15, 20). Ca\textsuperscript{2+}-regulated exocytosis is enhanced by many substances, such as PGE\textsubscript{2}, cAMP, cGMP, and arachidonic acid (7, 15, 20, 25, 27, 28). Moreover, Ca\textsuperscript{2+}-regulated exocytosis in antral mucous cells is enhanced by isosmotic cell shrinkage (6).

Various agonists induce isosmotic cell shrinkage by activating K\textsuperscript{+} and Cl\textsuperscript{−} channels in epithelial cells, such as salivary acinar cells (5, 18), lung cells (11, 14, 16, 17, 23), sweat gland cells (30), and bronchiolar ciliary cells (26). The cell shrinkage modulates some cellular functions, such as ion transport (23, 32), apoptosis (13), ciliary beat frequency (26), and exocytosis (6). Isosmotic cell shrinkage decreases intracellular Cl concentration ([Cl\textsuperscript{−}]\texttextsubscript{i}) (4, 14, 23, 32), which also modulates cellular functions, such as Na\textsuperscript{+}-permeable channels in fetal lung cells (32) and salivary duct cells (3), Na\textsuperscript{+}/K\textsuperscript{+}/2Cl\textsuperscript{−} cotransporters (NKCCs) in tracheal epithelial cells (9) and squid giant axons (2), G proteins (10), cell cycle (29), and the exocytosis of β-cells (1) and melanotrophs (24, 31).

In guinea pig antral mucous cells, ACh-induced cell shrinkage enhances Ca\textsuperscript{2+}-regulated exocytosis, as previously reported (6). Moreover, hypoosmotic stress and bumetanide (an inhibitor of NKCC), which appear to decrease [Cl\textsuperscript{−}]\texttextsubscript{i}, enhance ACh-stimulated exocytosis in antral mucous cells (6). Moreover, [Cl\textsuperscript{−}] is reported to modulate Ca\textsuperscript{2+}-regulated exocytosis and granular maturation (priming) in β-cells (1) and melanotrophs (31). These findings suggest that [Cl\textsuperscript{−}] may also modulate Ca\textsuperscript{2+}-regulated exocytosis in antral mucous cells.

We have studied the regulation of mucin release from isolated antral mucous cells by directly observing exocytotic events using videomicroscopy (6, 7, 15, 20, 25, 26). In this study, we examined the effects of bumetanide, NO3\textsuperscript{−} solution (a Cl\textsuperscript{−}-free solution), and a Cl\textsuperscript{−} channel blocker [5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB)] on ACh-stimulated exocytotic events in antral mucous cells. Bumetanide or NO3\textsuperscript{−} solution inhibits NKCC, which decreases [Cl\textsuperscript{−}]\texttextsubscript{i}, and, moreover, NO3\textsuperscript{−} solution may enhance decreases in [Cl\textsuperscript{−}]\texttextsubscript{i} by replacing Cl\textsuperscript{−} with NO3\textsuperscript{−} via Cl\textsuperscript{−} channels (21, 22). In contrast, NPPB may increase [Cl\textsuperscript{−}], by inhibiting Cl\textsuperscript{−} efflux.

In the present study, we used bumetanide, Cl\textsuperscript{−}-free solution, and NPPB as tools to control [Cl\textsuperscript{−}]\texttextsubscript{i} and examined the effects of [Cl\textsuperscript{−}] on Ca\textsuperscript{2+}-regulated exocytosis in antral mucous cells. The goal of this study is to confirm that an [Cl\textsuperscript{−}] decrease enhances Ca\textsuperscript{2+}-regulated exocytosis in antral mucous cells. If so, which step of the exocytotic cycle, the ATP-dependent step (priming) or the Ca\textsuperscript{2+}-dependent step (fusion), is modulated by intracellular Cl\textsuperscript{−}?

MATERIALS AND METHODS

Solutions and chemicals. Solution 1 contained (in mM) 121 NaCl, 4.5 KCl, 25 NaHCO\textsubscript{3}, 1 MgCl\textsubscript{2}, 1.5 CaCl\textsubscript{2}, 5 NaHEPES, 5 HHEPES, and 5 glucose. To prepare a Ca\textsuperscript{2+}-free solution, CaCl\textsubscript{2} was excluded from solution 1 and EGTA (1 mM) was added. To prepare a Cl\textsuperscript{−}-free solution, Cl\textsuperscript{−} in solution 1 was replaced with NO3\textsuperscript{−} (NO3\textsuperscript{−} solution) or gluconate (gluconate solution). The pHs of the solutions were adjusted to 7.4 by adding 1 M HCl, HNO\textsubscript{3}, or gluconic acid, as appropriate. The solutions were gassed with 95% O\textsubscript{2} and 5% CO\textsubscript{2} at 37°C. A HCO\textsubscript{3}\textsuperscript{−}-free solution, in which NaHCO\textsubscript{3} of solution 1 was replaced

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Address for reprint requests and other correspondence: T. Nakahari, Dept. of Physiology, Osaka Medical College, 2-7 Daigaku-cho, Takatsuki 569-8686, Japan (e-mail: takan@art.osaka-med.ac.jp).
with NaCl, was gassed with 100% O₂ or 100% N₂. The following reagents were purchased: dinitrophenol (DNP) and NPPB from Sigma (St. Louis, MO); acetylcholine chloride (ACh) from Daiichi Pharmaceuticals (Osaka, Japan); and collagenase (for cell dispersion, 180–220 U/mg) and bovine serum albumin (BSA) from Wako Pure Chemical Industries (Osaka, Japan). All the reagents were dissolved in dimethyl sulfoxide (DMSO) and diluted to their final concentrations immediately before the experiments. The DMSO concentration did not exceed 0.1%. At this concentration, DMSO has no effect on cell volume and exocytotic events (6, 7, 11, 15, 19, 20, 25–28).

Cell preparation. Male guinea pigs (Hartley) weighing ~250 g were purchased from SLC-Japan (Hamamatsu, Japan) and maintained on standard pellet food and water. The guinea pigs were anesthetized by an intraperitoneal injection of pentobarbital-Na (Nembutal, 60–70 mg/kg). After removal of the stomach, they were euthanized 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 procedure for cell preparation was previously described in detail (6). Briefly, the antrum was excised and the mucosal layer was stripped from the muscle layer in cooled saline (4°C) by using glass slides. The stripped antral mucosa was minced with fine forceps and then incubated in

solution 1 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 1 containing 2% BSA (4°C). The cells were stored at 4°C and used in the experiments within 3 h.

Fig. 1. Effect of bumetanide (Bum) on ACh-stimulated exocytotic events in antral mucous cells. The ACh concentration was 1 μM. In the control experiment, the cells were stimulated with ACh (1 μM) alone. ACh (1 μM) evoked a biphasic increase in the frequency of exocytotic events: an initial phase was followed by a sustained phase. A: bumetanide (20 μM) alone induced no exocytotic events. However, the addition of bumetanide 3 min before ACh stimulation enhanced the initial and sustained phases of ACh-stimulated exocytotic events. *Significantly different from control (1 μM ACh alone), P < 0.05. B: addition of bumetanide (20 μM) 2 min after the ACh stimulation increased the sustained phase transiently, and the frequency was sustained. The frequency of the sustained phase was approximately twice higher than that of ACh alone. *Significantly different from control (1 μM ACh alone), P < 0.05.

Fig. 2. Effect of Cl⁻-free solution on ACh-stimulated exocytotic events in antral mucous cells. A: NO₃⁻ solution. NO₃⁻ solution alone induced no exocytotic events. However, the addition of NO₃⁻ solution 3 min before ACh stimulation enhanced the initial and sustained phases of ACh-stimulated exocytotic events. B: addition of NO₃⁻ solution 2 min after the ACh stimulation increased the sustained frequency transiently, and the frequency was sustained. The sustained frequency was approximately twice higher than that of ACh alone. *Significantly different from control (1 μM ACh alone), P < 0.05.

Observation of exocytosis. Isolated antral mucous cells were mounted on a coverslip precoated with neutralized Cell-Tak (Becton Dickinson Labware, Bedford, MA) for the firm attachment of the cells. The coverslip was set 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) (6). Images were recorded continuously with a video recorder. The experiments were performed at 37°C. The volume of the perfusion chamber
was ~20 μl and the rate of perfusion was 200 μl/min. Exocytotic events, which were detected as rapid changes in the light intensity of granules (6), were counted in five to six cells every 30 s and normalized to the number of cells (events per cell per 30 s). The frequencies of exocytotic events in three to seven experiments were expressed as means ± SE.

**Intracellular Ca2+ measurement.** Isolated antral mucous cells were incubated in solution 1 containing 2% BSA and 2.5 μM fura 2-acetoxy-methyl ester (fura 2-AM, Dojindo, Kumamoto, Japan) for 25 min at room temperature (22–24°C). They were then washed three times with solution 1 containing 2% BSA. Fura 2-loaded cells were resuspended and stored in solution 1 containing 2% BSA at 4°C and then mounted on a coverslip precoated with neutralized Cell-Tak. These coverslips were set in a perfusion chamber, which was then mounted on the stage of an inverted microscope (IX70, Olympus, Tokyo, Japan) connected to an image analysis system (model ARGUS/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan) (6, 19). All the experiments were performed at 37°C. The volume of the perfusion chamber was ~80 μl and the rate of perfusion was 500 μl/min. Fura 2 was excited at 340 and 380 nm, and emission was measured at 510 nm. Fluorescence ratio (F340/F380) was calculated and stored in an image analysis system. The calibration curve was obtained from the F340/F380 of the cell-free Ca2+ calibration solutions containing 10 μM fura 2. Solution 2 contained (in mM) 130 KCl, 20 NaCl, 2 EGTA, and HEPES 10. To prepare the cell-free Ca2+ calibration solutions, an appropriate amount of CaCl2 (0.2–2 mM) calculated by a computer program was added to solution 2. The pH was adjusted to 7.05 by adding 1 M KOH. The dissociation constant of Ca2+ and EGTA used was 214 nM (37°C, pH 7.05) (12). One experiment used five to six coverslips, and the F340/F380s of seven cells on two to three coverslips were expressed as means ± SE.

**[CI]− measurement.** Isolated antral mucous cells were incubated in solution 1 containing 2% BSA and 5 mM N-ethoxycarbonylmethyl-6-methoxyquinolinium bromide (MQAE, Dojindo, Kumamoto, Japan) at 30°C for 50 min. They were then washed three times with solution 1 containing 2% BSA. MQAE-loaded cells were resuspended and stored in solution 1 containing 2% BSA at room temperature (22–24°C), and then mounted on a coverslip precoated with neutralized Cell-Tak.

![Fig. 3. Dose effects of ACh in NO3 solution (•) and in the control solution (○). A: ACh (0.1 μM). B: ACh (0.4 μM). C: ACh (4 μM). D: ACh (40 μM). NO3 solution enhanced the initial peak frequencies of ACh-stimulated exocytotic events (initial phase). E: effects of NO3 solution and bumetanide on the initial phase. NO3 solution and bumetanide shifted the ACh dose-response curve upward. IC50 were shifted to the low concentration by bumetanide or NO3 solution; however, their shifts were not significant. *Significantly different from control (ACh alone), P < 0.05.](http://ajpgi.physiology.org/)
Cell-Tak. MQAE was excited at 355 nm, and emission was measured at 510 nm. The fluorescence intensity measured (F) was stored in an image analysis system. To compare among experiments, the ratio (F/F₀) was calculated, where F₀ is the fluorescence intensity at time 0. One experiment used five to six coverslips from two to three guinea pigs. The F/F₀s of seven cells on two to three coverslips were expressed as means ± SE.

To calculate the intermediate concentration (IC₅₀) of a dose-response curve, a program for curve fitting (Delta Graph 4.5, SPSS) was used. The following is the equation used for curve fitting: \( y = \frac{a-d}{1 + (x/c)^b} + d \) (1) where a–d are constants, 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, b is a constant.

After linearization of Eq. 1 by logarithm, the significance of the difference in IC₅₀s was assessed by Student’s t-test, and that between means was assessed using paired or unpaired Student’s t-test, as appropriate. Differences were considered significant at \( P < 0.05 \).

RESULTS

In unstimulated antral mucous cells, no exocytotic events were observed. ACh (1 μM) induced a biphasic increase in the frequency of exocytotic events: an initial phase followed by a sustained phase (Figs. 1, A and B). In comparing the experiments, we used the initial peak frequency, which was the maximum frequency within 2 min from the start of ACh stimulation. The ACh concentration used was 1 μM or 10 μM.

Effects of bumetanide and Cl⁻-free solutions on ACh-stimulated exocytotic events. Bumetanide (20 μM), added for 3 min, induced no exocytotic events in most of antral mucous cells; however, in some cells (2 of 13 experiments), a longer exposure, such as 5 min, induced 0.5–1 events·cell⁻¹·min⁻¹ as previously reported (6). However, it enhanced the frequency of ACh-stimulated exocytotic events. Bumetanide (20 μM) increased the initial peak frequency of ACh-stimulated exocytotic events by ~100% (Fig. 1A). The effects of bumetanide on the sustained phase were examined. The addition of bumetanide 2 min after the start of ACh stimulation increased the frequency of exocytotic events transiently and plateaued (Fig. 1B).

Similar experiments were performed using Cl⁻-free solutions, in which Cl⁻ was replaced with NO₃⁻ or gluconate. NO₃⁻ solution added for 3 min activated no exocytotic events in most of antral mucous cells, however, in some cells (3/14 experiments), a longer exposure of NO₃⁻ solution, such as 5 min, activated 0.5–1 events·cell⁻¹·min⁻¹. The further addition of ACh induced an enhanced frequency of exocytotic events (approximately by 200%) (Fig. 2A). NO₃⁻ solution added in the sustained phase induced a biphasic increase in the sustained frequency, similarly to that of bumetanide (Fig. 2B). Experiments were also performed using gluconate solution. Gluconate solution also enhanced ACh-stimulated exocytotic events by ~100% (Fig. 2C). A similar enhancement was observed when glutamate was used instead of gluconate (data not shown). In this study, we used NO₃⁻ solution for the Cl⁻-free experiments.

In these experiments, the cells were treated with bumetanide or NO₃⁻ solution for 20 or 30 min prior to ACh stimulation. The frequencies of ACh-stimulated exocytotic events following the 20-min and 30-min pretreatment, however, were similar to that following the 3-min pretreatment. In this study, the period of bumetanide or NO₃⁻ solution treatment prior to ACh stimulation was 3–5 min throughout the experiments.

The dose effects of ACh were examined during perfusion with NO₃⁻ solution. ACh (0.01 μM) activated no exocytotic events during perfusion with solution I or NO₃⁻ solution. ACh (0.1 μM) increased the frequency of exocytotic events only transiently, and NO₃⁻ solution enhanced it (Fig. 3A). ACh (0.4 μM) induced a biphasic increase in the frequency of exocytotic events (an initial phase followed by a sustained phase) and NO₃⁻ solution enhanced the frequency of the initial phase by ~150%, but the enhancement of the sustained phase was uncertain (Fig. 3B). ACh (1 μM) induced a biphasic increase in

Fig. 4. Effects of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). A: ACh (10 μM) induced a biphasic increase in the frequency of exocytotic events. NPPB (2 μM) decreased the initial peak frequency slightly but significantly (\( P < 0.05 \)). B: NO₃⁻ solution enhanced the frequency of exocytotic events at 10 μM ACh. However, NPPB did not induce any enhancement of the frequency of ACh-stimulated exocytotic events induced by NO₃⁻ solution. C: effects of NPPB on the initial peak frequencies in the control solution and NO₃⁻ solutions. NPPB eliminated the enhancement induced by NO₃⁻ solution. *Significantly different from control (10 μM ACh alone), \( P < 0.05 \).
the frequency of exocytic events and NO$_3^-$ solution enhanced the frequency of exocytic events markedly, as shown in Fig. 2A. Stimulation with 4 μM ACh (Fig. 3C) and 40 μM ACh (Fig. 3D) also induced a transient increase in the frequency of exocytic events, and NO$_3^-$ solution enhanced it. The initial peak frequency enhanced by NO$_3^-$ solution was 100–150% of the control experiments. Similar experiments were performed using bumetanide (20 μM) instead of NO$_3^-$ solution. The results are summarized in Figs. 3E. The initial peak frequency was plotted against ACh concentration (Fig. 3, E and F). In the ACh dose-response curves of the initial peak frequency, bumetanide (20 μM) and NO$_3^-$ solution shifted the maximum responsiveness upward by ~50 and 100%, respectively. The half-maximal concentrations (IC$_{50}$s) of the ACh dose-response curve were 5.9 μM in the control experiments and were shifted to the low concentration by bumetanide (1.9 μM) and NO$_3^-$ solution (2 μM). The statistical analysis, however, demonstrated that bumetanide or NO$_3^-$ solution induces no significant shift in the ACh dose-response curve (P < 0.05). In the experiments of bumetanide or NO$_3^-$ solution, the enhancement of the sustained phase was unclear.

**Effects of NPPB.** The effects of a Cl$^-$ channel blocker, 2 μM NPPB, were examined, because NPPB may increase [Cl$^-$]o by inhibition of Cl$^-$ release via Cl$^-$ channels. NPPB (2 μM) alone activated no exocytic events, and further stimulation with 10 μM ACh induced a biphasic increase in the frequency of exocytic events. However, the initial peak frequency of ACh-stimulated exocytic events decreased by 20% (Fig. 4A), and, moreover, in the presence of 2 μM NPPB, NO$_3^-$ solution did not enhance the initial peak frequency (Fig. 4B). Thus NPPB decreased the frequency of ACh-stimulated exocytic events and eliminated the enhancement of ACh-stimulated exocytic events induced by NO$_3^-$ solution (Fig. 4C). Similar results were obtained by 20 μM NPPB, whereas NPPB (0.2 μM) induced no significant decrease in the frequency of 10 μM ACh-stimulated exocytic events (data not shown).

Cell volume changes were also measured. The method has already been described in detail in the previous reports (6, 33). To calculate relative cell volume [V/V$_0$ = (A/A$_0$)$^{1/3}$], cellular area (A) was measured by tracing on the video screen (6, 33). The subscript 0 shows time = 0. The addition of bumetanide or NO$_3^-$ solution induced cell shrinkage [V/V$_0$ = 0.84 ± 0.03 (n = 4)] at 2 min after the stimulation, as previously reported (6). The addition of NPPB (2 μM) increased volume of antral mucous cells [V/V$_0$ = 1.13 ± 0.02 (n = 4)], the further addition of NO$_3^-$ solution does not induce cell shrinkage [V/V$_0$ = 1.15 ± 0.03 (n = 4) at 3 min after exposure of the NO$_3^-$ solution], and stimulation with 1 μM ACh induced no significant cell shrinkage [V/V$_0$ = 1.16 ± 0.03 (n = 4) at 2 min after ACh stimulation].

**Effects of [Cl$^-$]o.** The effects of extracellular Cl$^-$ concentration ([Cl$^-$]o) on ACh-stimulated exocytic events were examined. [Cl$^-$]o was varied by mixing appropriate amounts of solution I and NO$_3^-$ solution. The initial peak frequencies were 42 ± 2.2 events·cell$^{-1}$·30 s$^{-1}$ (n = 9) at 0 mM [Cl$^-$]o, 29 ± 1.5 events·cell$^{-1}$·30 s$^{-1}$ (n = 4) at 33 mM [Cl$^-$]o (Fig. 5A), 18 ± 0.3 events·cell$^{-1}$·30 s$^{-1}$ (n = 4) at 65 mM [Cl$^-$]o (Fig. 5B), 13 ± 0.8 events·cell$^{-1}$·30 s$^{-1}$ (n = 4) at 98 mM [Cl$^-$]o (Fig. 5C), and 11 ± 0.7 events·cell$^{-1}$·30 s$^{-1}$ (n = 9) at 130

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**Fig. 5.** Effects of extracellular Cl$^-$ concentration ([Cl$^-$]o). NO$_3^-$ solution ([Cl$^-$]o = 0 mM) enhanced the frequency of ACh-stimulated exocytic events (dotted line). A: 33 mM [Cl$^-$]o, B: 65 mM [Cl$^-$]o, C: 98 mM [Cl$^-$]o. *Significantly different from corresponding value of NO$_3^-$ solution experiment, P < 0.05. D: the initial peak frequency was plotted against [Cl$^-$]o. With an increment of [Cl$^-$]o, the initial peak frequency decreased. †Significantly different from control, P < 0.05.
mM [Cl\(^{-}\)]_o. The initial peak frequency of ACh-stimulated exocytotic events was plotted against [Cl\(^{-}\)]_o (Fig. 5D). With an increase in [Cl\(^{-}\)]_o, the initial peak frequency of ACh-stimulated exocytotic events decreased. Thus a decrease in [Cl\(^{-}\)]_o, which is caused by [Cl\(^{-}\)]_o decrease, appears to enhance the frequency of ACh-stimulated exocytotic events.

Changes in [Cl\(^{-}\)]. The change in [Cl\(^{-}\)]_i was measured by using MQAE fluorescence ratio (F/F\(_0\)), where F\(_0\) is the fluorescence intensity at time 0 (excitation 355 nm and emission 510 nm). In this figure, the ordinate (F/F\(_0\)) is reversed (the upward direction indicates an increase in [Cl\(^{-}\)]_i), and conversely a downward direction shows a decrease in [Cl\(^{-}\)]_i. The effects of [Cl\(^{-}\)]_o on F/F\(_0\)_s used were 131 mM, 65 mM, 33 mM, and 0 mM. As [Cl\(^{-}\)]_o decreased, F/F\(_0\)_s increased. The addition of bumetanide (1 mM) further increased F/F\(_0\) (Fig. 6). Thus both ACh and bumetanide enhanced the ACh-stimulated decrease in [Cl\(^{-}\)]_i.

Similar experiments were performed using NO\(_3^-\) solution. NO\(_3^-\) solution enhanced the ACh-stimulated increase in F/F\(_0\) (Fig. 7C). The addition of NO\(_3^-\) solution decreased F/F\(_0\) and plateaued within 2 min. Further stimulation with ACh increased F/F\(_0\) (Fig. 7D). However, the increase in F/F\(_0\) induced by NO\(_3^-\) solution is lower than that induced by bumetanide (Fig. 7).

The effects of a Cl\(^{-}\) channel blocker, 2 \(\mu\)M NPPB, were examined. The addition of NPPB decreased F/F\(_0\) and plateaued within 3 min. Further stimulation with ACh induced no increase in F/F\(_0\) (Fig. 8A). The effects of bumetanide (2 \(\mu\)M) were examined in the NPPB-treated cells. In the presence of NPPB, the addition of bumetanide induced no increase in F/F\(_0\), whereas the addition of Bumetanide further induced F/F\(_0\). Thus ACh decreased [Cl\(^{-}\)]_i, which was enhanced by bumetanide. B: addition of bumetanide increased F/F\(_0\), which reached a plateau within 1 min. ACh further increased F/F\(_0\). Bumetanide decreased [Cl\(^{-}\)]_i, and ACh further increased [Cl\(^{-}\)]_i. C: ACh (1 \(\mu\)M) increased F/F\(_0\) immediately and the addition of NO\(_3^-\) solution further increased F/F\(_0\), which reached a plateau within 2 min but still remained at a much higher level than before stimulation. Then, ACh induced a further increase in F/F\(_0\). Thus NO\(_3^-\) solution decreased [Cl\(^{-}\)]_i, which is much lower than that of bumetanide experiments. The F/F\(_0\)_s of 7 cells on 2–3 coverslips were expressed as means ± SE.
and further stimulation with ACh also induced no increase in F/F₀ (Fig. 8B). Similar experiments were performed using NO₃⁻ solution instead of bumetanide. In the NPPB-treated cells, NO₃⁻ solution induced no increase in F/F₀ with or without ACh stimulation (Fig. 8C). Thus, in the presence of NPPB, bumetanide or NO₃⁻ solution with and without ACh stimulation induced no increase in F/F₀; NPPB increased [Cl⁻]; and inhibited the decreases in [Cl⁻] induced by ACh, bumetanide, and NO₃⁻ solution.

**Effects of ionomycin, BAPTA, PKI, and Rp8BrPETcGMPS.** ACh actions are induced by an increase in intracellular Ca²⁺ concentration ([Ca²⁺]). To increase [Ca²⁺], without ACh stimulation, ionomycin was used. The cells were first perfused with a Ca²⁺-free solution, and then ionomycin (1 μM) was added. During perfusion with the Ca²⁺-free solution, the addition of 1 μM ionomycin did not increase the frequency of exocytotic events except for a transient increase (1–2 events·cell⁻¹·30 s⁻¹) within the first minute of ionomycin addition. This small increase in the frequency of exocytotic events was induced by Ca²⁺ release from internal stores induced by ionomycin. The perfusion solution was suddenly switched from Ca²⁺-free solution to solution 1 (1.5 mM Ca²⁺), to reintroduce Ca²⁺. The reintroduction of Ca²⁺ increased the frequency of exocytotic events similarly to that of 10 μM ACh. Similar experiments were performed using NO₃⁻ solution. NO₃⁻ solution enhanced ionomycin-stimulated exocytotic events following the reintroduction of Ca²⁺ by ~100% (Fig. 9A). Thus ionomycin mimicked the ACh actions, and NO₃⁻ solution enhanced the frequency of ionomycin-stimulated exocytotic events.

**Fig. 9. Effects of ionomycin and BAPTA-AM.** A: cells were first stimulated with 1 μM ionomycin in a Ca²⁺-free solution, and then Ca²⁺ (1.5 mM) was added (reintroduction of Ca²⁺). The reintroduction of Ca²⁺ induced a transient increase in the frequency of exocytotic events, similarly to that of ACh (10 μM). The same experiments were performed using NO₃⁻ solution. Exposure of the NO₃⁻ solution enhanced a transient increase in the frequency of exocytotic events induced by the reintroduction of Ca²⁺. B: the cells were loaded with BAPTA-AM (25 μM) for 30 min at 30°C and then were perfused with NO₃⁻ solution containing no Ca²⁺ for 3 min prior to 10 μM ACh stimulation. ACh stimulation increased a small transient increase in the frequency of exocytotic events in the NO₃⁻ solution. *Significantly different from corresponding value, P < 0.05.
To completely chelate intracellular Ca\(^{2+}\), cells were incubated with BAPTA-AM (25 μM) for 30 min at 30°C. When BAPTA-loaded cells were perfused with the Ca\(^{2+}\)-free solution, ACh evoked a small transient increase in ACh-stimulated exocytotic events. Moreover, when BAPTA-loaded cells were perfused with Ca\(^{2+}\)-free NO\(_3\) solution, ACh evoked only a small transient increase in exocytotic events (Fig. 9B). After chelation of intracellular Ca\(^{2+}\) by BAPTA, NO\(_3\) solution did not enhance ACh-stimulated exocytotic events.

The effects of PKI (1 μM, an inhibitor of PKA) and guanosine 3',5'-cyclic monophosphorothate, β-phenyl-1, N\(^2\) -etheno-8-bromo-Rp isomer (Rp8BrPETcGMPS; 500 nM, a PKG inhibitor) were examined (Fig. 10). In antral mucous cells, ACh accumulates cAMP via PGE\(_2\) production (34). PKI (1 μM) decreased the frequency of ACh-stimulated exocytotic events by ~50%, as previously reported (8, 32, 34) (Fig. 10A). In the presence of 1 μM PKI, NO\(_3\) solution still enhanced the frequency of ACh-stimulated exocytotic events by ~200% (Fig. 10A).

Our previous report demonstrated that cGMP also enhances the frequency of ACh-stimulated exocytotic events (25). We examined the effects of a PKG inhibitor, Rp8BrPETcGMPS (500 nM). Rp8BrPETcGMPS (500 nM) decreased the initial peak frequency by 30%, suggesting that 10 μM ACh stimulation accumulates cGMP in antral mucous cells. However, NO\(_3\) solution still enhanced the frequency of ACh-stimulated exocytotic events by 150% during inhibition of PKG (Fig. 10B).

Thus NO\(_3\) solution enhanced Ca\(^{2+}\) actions independent of PKA and PKG.

Effects of DNP and anoxia. The effects of NO\(_3\) solution on the ATP-dependent priming of Ca\(^{2+}\)-regulated exocytosis were examined by use of DNP (an uncoupler of oxidative phosphorylation) and N\(_2\) bubbling (anoxia). The cells were pretreated with 100 μM DNP prior to ACh stimulation. ACh activated exocytotic events, but it induced only a sustained phase with no initial transient phase (Fig. 11A). In the next experiment, cells were first treated with 100 μM DNP and then with NO\(_3\) solution. Further stimulation with ACh also induced only a sustained phase, the frequency of which was enhanced (Fig. 11B). In contrast, the first cells were perfused with NO\(_3\) solution and then 100 μM DNP was added. Under these conditions, however, ACh induced an initial transient phase followed by a sustained phase (Fig. 11C). Similar experiments were performed in the cells bubbled with 100% N\(_2\). In this experiment, a HCO\(_3\)\(^{-}\)-free solution was used. During 100% N\(_2\) bubbling, ACh induced only a sustained phase with no initial transient phase (Fig. 11D). In the next experiment, cells were perfused with HCO\(_3\)\(^{-}\)-free solution, which was bubbled with 100% N\(_2\) and then with HCO\(_3\)\(^{-}\)-free NO\(_3\) solution bubbled with 100% N\(_2\). Further stimulation with ACh also induced only a sustained phase, the frequency of which was enhanced (Fig. 10E). In contrast, the cells were first perfused with HCO\(_3\)\(^{-}\)-free NO\(_3\) solution bubbled with 100% O\(_2\) and then the gas was switched to 100% N\(_2\). Further stimulation with ACh evoked an initial transient phase followed by a sustained phase in the frequency of exocytotic events. Thus ATP depletion by DNP or anoxia (100% N\(_2\) bubbling) eliminates the initial transient phase, and exposure of NO\(_3\) solution prior to ATP depletion maintains the initial transient phase. Exposure of the NO\(_3\) solution prior to ATP depletion is suggested to accelerate the ATP-dependent step (priming) and to increase the number of the primed granules. Under this condition, ATP depletion does not cause reduction of the primed granules; that is, the primed granules are maintained, which induces the initial transient phase even during the ATP depletion.

Another test of the hypothesis that exposure of the NO\(_3\) solution accelerates priming was performed by examining the ability of NO\(_3\) solution to enhance a second stimulation by ACh. The cells were first stimulated with ACh for 1 min and then restimulated with 1 μM ACh after a 7-min recovery with control solution. The second ACh stimulation induced only a sustained phase with no initial transient phase (Fig. 12A). In the next experiment, the cells were first stimulated with ACh for 1 min and then the cells were also restimulated with 1 μM ACh after 7-min recovery with NO\(_3\) solution. The second stimulation, however, induced an initial transient phase followed by a sustained phase (Fig. 13B). Thus a brief recovery with NO\(_3\) solution led to the recovery in the number of primed granules, which were depleted by the first ACh stimulation. These results suggest that exposure of the NO\(_3\) solution accelerates priming.

Effects of extracellular Ca\(^{2+}\) concentration. The final step of Ca\(^{2+}\)-regulated exocytosis consists of two biochemically distinct steps, ATP-dependent priming and Ca\(^{2+}\)-dependent fu-
As shown in the ACh dose-response studies (Fig. 3), no significant shift of the ACh dose-response curve was detected in the bumetanide experiments or the NO3/H11002 solution experiments. To confirm whether NO3/H11002 solution increases Ca2+/H11001 sensitivity, the initial peak frequencies of exocytotic events for 1M ACh stimulation were measured at various extracellular Ca2+/H11001 concentrations ([Ca2+/H11001]o). At 10 M [Ca2+/H11001], ACh (1 M) transiently increased the frequency of exocytotic events, but no sustained phase was noted (Fig. 13A). The initial frequency of ACh-stimulated exocytotic events increased as [Ca2+/H11001]o increased from 0.1 to 1.5 mM (Fig. 13, B–D). NO3/H11002 solution enhanced the initial peak frequency of ACh-stimulated exocytotic events at every [Ca2+/H11001]o, except 10 M [Ca2+/H11001]. The initial peak frequencies were plotted against [Ca2+/H11001]os (Fig. 13D). NO3/H11002 solution induced an upward shift of the Ca2+/H11001 dose-response curve. IC50s in control solution and NO3 solution were 0.08 and 0.18 mM, respectively, which were not significantly different (P < 0.05). Thus NO3 solution did not significantly shift the Ca2+/H11001 dose-response curve. The effects of [Ca2+/H11001]o on fura 2 fluorescence ratio (F340/F380, [Ca2+/H11001]i) were examined (Fig. 13E). However, NO3/H11002 solution enhanced ACh-stimulated increases in [Ca2+/H11001]i (F340/F380s). Thus the results of fura 2 experiments (Fig. 13E) appear to be inconsistent with those of the Ca2+/H11001 dose-response curve (Fig. 13D). This suggests that “priming” may be a rate-limiting step of the exocytotic cycle (priming and fusion).

[G832] [Cl−]: MODULATION OF MUCIN EXOCYTOSIS

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cellular Cl\(^{-}\) with that during perfusion with control solution (Fig. 14A). ACh increased F340/F380, which was slightly high compared to the frequency of exocytotic events. *Significantly different from corresponding second stimulation induced an initial phase followed by a sustained phase in the first stimulation, cells were recovered with NO\(_3\)solution. The first stimulation induced only a sustained phase with no initial phase.

The present study demonstrated that intracellular Cl\(^{-}\) modulates ACh-stimulated exocytosis in antral mucous cells; that is, a decrease in [Cl\(^{-}\)]\(i\) enhances ACh-stimulated exocytosis, and in contrast an increase in [Cl\(^{-}\)]\(i\) suppresses it. The intracellular Cl\(^{-}\) modulation was independent of accumulation of cAMP or cGMP, but it required an increase in [Ca\(^{2+}\)]\(i\). In antral mucous cells, Ca\(^{2+}\)-regulated exocytosis is the main mechanism for mucin release (cAMP-regulated exocytosis is less than 10%) (6, 20). On the basis of these observations, intracellular Cl\(^{-}\) directly modulates Ca\(^{2+}\)-regulated exocytosis during ACh stimulation in antral mucous cells.

Ca\(^{2+}\)-regulated exocytosis in antral mucous cells is composed of two biochemically distinct steps, as previously reported (15, 25, 34). The first step requires ATP, but not Ca\(^{2+}\), and primes granules with the exocytotic machinery. The second step requires Ca\(^{2+}\), but not ATP, and triggers the fusion of granules (33). Depletion of ATP by DNP or anoxia inhibits priming and decreases the pool of primed granules, which results in no initial transient phase of Ca\(^{2+}\)-regulated exocytosis. However, a small amount of ATP is supplied by anaerobic glycolysis, which primes granules with the exocytotic machinery; this induces a sustained phase of Ca\(^{2+}\)-regulated exocytotic events. Thus the initial phase is induced by the fusion of primed granules, and the sustained phase is induced by the recruitment of granules to the apical membrane and the repriming of the exocytotic machinery (15, 25, 34). Moreover, anoxia (N\(_2\) bubbling) has already been reported to have no effect on the ACh-stimulated [Ca\(^{2+}\)]\(i\) increase in antral mucous cells (15), indicating that ATP depletion does not affect [Ca\(^{2+}\)] mobilization.

However, exposure of NO\(_3\) solution prior to ATP depletion (by DNP or N\(_2\) bubbling) induced an initial transient phase followed by a sustained phase in the Ca\(^{2+}\)-regulated exocytosis. In contrast, exposure of NO\(_3\) solution following the ATP depletion induced only a sustained phase without any initial phase in the Ca\(^{2+}\)-regulated exocytosis. These results suggest that a decrease in [Cl\(^{-}\)]\(i\) accelerates ATP-dependent priming and increases the pool of primed granules, which is maintained even during ATP depletion. Under this condition, ACh triggers the fusion of the primed granules in the pool, which induces an initial transient phase. Moreover, NO\(_3\) solution also enhanced the sustained phase even following the ATP depletion by DNP or anoxia. This suggests that a decrease in [Cl\(^{-}\)]\(i\) still accelerates the priming maintained by ATP supplied by anaerobic glycolysis.

On the other hand, NO\(_3\) solution increased [Ca\(^{2+}\)]\(i\), and enhanced ACh-stimulated [Ca\(^{2+}\)]\(i\) increases. Thus a decrease in [Cl\(^{-}\)]\(i\), also enhances [Ca\(^{2+}\)]\(i\), increases in antral mucous cells. Enhancement of the ACh-stimulated [Ca\(^{2+}\)]\(i\), increase appears to cause dose-response curve of ACh or Ca\(^{2+}\) to shift to the low concentration. However, no shift of the ACh dose-response curve or the Ca\(^{2+}\) dose-response curve was noted. Thus the results of dose-response studies are inconsistent with those of [Ca\(^{2+}\)]\(i\) measurements. Similar inconsistency was also shown in Fig. 13; the frequencies of ACh-stimulated exocytotic events were similar (\(\sim 10\) events.cell\(^{-1}\).30 s\(^{-1}\)) as increment of [Ca\(^{2+}\)]\(i\), from 0.5 to 1.5 mM, although F340/F380 ([Ca\(^{2+}\)]\(i\)) increased from 2.1 to 2.4. The fusion step is a continuous reaction following the priming step in the exocytotic cycle. A possible explanation is that the pool size of primed granules may be limited, which may cause a similar initial frequency at different [Ca\(^{2+}\)]\(i\).s. The initial peak frequency is determined by the number of the primed granules (pool size). When the pool size is limited, the maximum frequency is also limited. This limitation appears to cause a similar initial peak frequency, even during acceleration of

![Fig. 12. Restimulation of ACh (1 \(\mu\)M) after a brief recovery. A: control solution. Cells were stimulated with ACh for 1 min, and then the second ACh stimulation was performed after 7-min recovery. The first stimulation induced a transient increase in the frequency of exocytotic events, and the second stimulation induced only a sustained phase with no initial phase. B: NO\(_3\) solution. Similar experiments were performed using NO\(_3\) solution. Following the first stimulation, cells were recovered with NO\(_3\) solution for 7 min. The second stimulation induced an initial phase followed by a sustained phase in the frequency of exocytotic events. *Significantly different from corresponding value, \(P < 0.05\).](http://ajpgi.physiology.org/content/293/10/G833/F1)
Ca\textsuperscript{2+}-dependent fusion induced by an enhanced [Ca\textsuperscript{2+}]\textsubscript{i} increase. Thus, in antral mucous cells, it seems probable that an [Cl\textsuperscript{-}]\textsubscript{i} decrease accelerates the Ca\textsuperscript{2+}-dependent fusion in the exocytotic cycle. However, it still remains uncertain whether an [Cl\textsuperscript{-}]\textsubscript{i} decrease increases the Ca\textsuperscript{2+} sensitivity of the fusion.

MQAE fluorescence measurements revealed that both bumetanide and NO\textsubscript{3} solution decreased [Cl\textsuperscript{-}]\textsubscript{i}. Bumetanide or NO\textsubscript{3} solution inhibits NKCCs, which decreases [Cl\textsuperscript{-}], by inhibition of Cl\textsuperscript{-} entry. A decrease in [Cl\textsuperscript{-}]\textsubscript{i} induced by NO\textsubscript{3} solution, however, is much greater than that induced by bumetanide. In NO\textsubscript{3} solution, intracellular Cl\textsuperscript{-} was replaced with NO\textsubscript{3} via Cl\textsuperscript{-} channels, because many Cl\textsuperscript{-} channels are highly permeable to NO\textsubscript{3} (21, 22). Thus NO\textsubscript{3} solution more effectively decreases [Cl\textsuperscript{-}]\textsubscript{i} than bumetanide. Moreover, ACh activates NKCC and also activates Ca\textsuperscript{2+}-activated K\textsuperscript{+} and Cl\textsuperscript{-} channels and increases KCl release, which decreases [Cl\textsuperscript{-}]\textsubscript{i} and also cell volume. Therefore, bumetanide, which inhibits Cl\textsuperscript{-} entry via NKCCs, enhances an ACh-stimulated [Cl\textsuperscript{-}] decrease, and NO\textsubscript{3} solution, which inhibits Cl\textsuperscript{-} entry via NKCCs and accelerates replacement of Cl\textsuperscript{-} with NO\textsubscript{3} via Cl\textsuperscript{-} channels, also enhances an ACh-stimulated [Cl\textsuperscript{-}] decrease.

In contrast, NPPB increased [Cl\textsuperscript{-}]\textsubscript{i} and eliminated decreases in [Cl\textsuperscript{-}]\textsubscript{i} induced by ACh, bumetanide, and NO\textsubscript{3} solution. NPPB inhibits Cl\textsuperscript{-} efflux via Cl\textsuperscript{-} channels maintaining Cl\textsuperscript{-} entry via NKCCs, which increases [Cl\textsuperscript{-}], and moreover, during inhibition of Cl\textsuperscript{-} efflux by NPPB, inhibition of Cl\textsuperscript{-} entry via NKCC does not cause [Cl\textsuperscript{-}] to decrease. [Cl\textsuperscript{-}]\textsubscript{i}s are maintained at an increased level.

Our previous report exhibited that cell shrinkage enhances Ca\textsuperscript{2+}-regulated exocytosis in antral mucous cells. The present study demonstrated that cell shrinkage induced by ACh is coincided with [Cl\textsuperscript{-}] decrease by activation of KCl release. On the other hand, NPPB induced cell swelling in antral mucous cells.
mucous cells. A similar cell swelling was reported in alveolar type II cells (33). NPPB (2 μM) also inhibited cell shrinkage induced by NO3− solution and ACh. NPPB, which increased [Cl−]i and cell volume, decreased the frequency of the initial phase as shown in Fig. 4. These observations indicate that the isosmotic cell shrinkage decreases [Cl−]i (14). Thus the enhancement of Ca2+−regulated exocytosis induced by cell shrinkage is caused by the [Cl−]i decrease in antral mucous cells, although we never neglect the direct effects of cell volume.

This study also showed that a decrease in [Cl−]i increases [Ca2+]i, and enhances the ACh-stimulated [Ca2+]i increase, as mentioned above. However, the addition of bumetanide or NO3− solution during ACh stimulation increased [Ca2+]i gradually and plateaued within 5 min, whereas the addition of bumetanide or NO3− solution in the sustained phase immediately induced a transient increase in the frequency of ACh-stimulated exocytosis in guinea pig antral mucous cells.
stimulated exocytotic events (within 1 min). The transient enhancement of the sustained phase is unlikely to be induced by the gradual [Ca\(^{2+}\)] increase following [Cl\(^{-}\)] decrease, and it appears to be caused by an acceleration of priming, which increases in the number of the primed granules and induces a transient increase in the frequency of exocytotic events.

Intracellular Cl\(^{-}\) modulates many cellular functions, such as nonselective cation channels in fetal lung cells (32) and salivary duct cells (3), NKCCs in squid axons and tracheal cells (2, 9), cell proliferation in human gastric cancer cells (29), G proteins (10), and exocytosis. In insulin-secreting pancreatic β cells, the priming of granules requires granular Cl\(^{-}\) uptake, suggesting that Cl\(^{-}\) modulates the priming (1). In melanotrophs, an increase in [Cl\(^{-}\)] increases exocytosis via G proteins and intracellular Cl\(^{-}\) plays an important role in granule maturation (24, 31). Moreover, in rat lactotrophs, a low [Cl\(^{-}\)] decreases Ca\(^{2+}\) entry via voltage-gated Ca\(^{2+}\) channels by modulating G proteins (8). Thus intracellular Cl\(^{-}\) also modulates exocytosis in endocrine cells. However, the effects of intracellular Cl\(^{-}\) on exocytosis and Ca\(^{2+}\) channels in antral mucous cells (exocrine cells) are opposite to those in endocrine cells (such as insulin-secreting pancreatic β cells, lactotrophs, and melanotrophs); a decrease in [Cl\(^{-}\)] enhances exocytosis and Ca\(^{2+}\) channels in antral mucous cells, whereas an increase in [Cl\(^{-}\)] enhances them in endocrine cells. The modulation mechanisms of Cl\(^{-}\) in antral mucous cells may be different from those in endocrine cells; for example, Cl\(^{-}\) modulates stimulatory G proteins in endocrine cells, whereas it modulates inhibitory G proteins in antral mucous cells.

Figure 16 shows the Cl\(^{-}\) modulation of Ca\(^{2+}\)-regulated exocytosis in antral mucous cells. Intracellular Cl\(^{-}\) modulates ATP-dependent priming; that is, an [Cl\(^{-}\)] decrease accelerates priming, which increases primed granules. Moreover, intracellular Cl\(^{-}\) also modulates [Ca\(^{2+}\)]; that is, an [Cl\(^{-}\)] decrease increases [Ca\(^{2+}\)], which accelerates fusion of the primed granules. Both actions enhance Ca\(^{2+}\)-regulated exocytosis in antral mucous cells.

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