Vol. 176, No. 1, 1991
April 15, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 466-472

EXOCYTOSIS IN COLONIC GOBLET CELLS VISUALIZED BY VIDEO-ENHANCED LIGHT MICROSCOPY

Susumu Terakawa[1] and Yuichi Suzuki[2]

[1]Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki, 444 Japan

[2]Department of Physiology, Yamagata University School of Medicine, Yamagata, 990-23, Japan

Received March 8, 1991

SUMMARY: In order to develop a method to quantify the mucus secretion, we observed the mucus epithelium of the rabbit colon under a video-enhanced differential interference contrast microscope. Upon stimulation with muscarinic agonists, secretory granules in individual goblet cells were found to undergo a rapid light intensity change. Simultaneously, the lumen was widened and filled with a cloudy material. In each cell, many of such responses were followed by formation of a large cavity which could be recovered after removal of the stimulant. We infer that the light intensity change of a granule arises from exocytosis. Direct counting of the frequency of these quantal responses would be very useful to monitor the secretory activity of single cell in real time at a high sensitivity.

Secretory activity occurs in a wide variety of cells and has been the subject of extensive and continuing investigations. Particularly, the intracellular mechanism underlying its final step called exocytosis is still ambiguous and controversial (1-3). Measurement of the capacitance of the plasma membrane with a patch pipette (4) has been the only technique previously available for analysis of exocytosis in the time domain. However, this is technically difficult, inapplicable to cells surrounded by an intact tissue, and complicated by problems caused by membrane retrieval and other morphological changes of the cell. Thus, we employed the technique based on light microscopy (5,6) and video image processing (7-10). By using recently developed video apparatus, we improved the quality of the video-microscopic image considerably. As a result, this has become an

Abbreviations: CCh, carbachol; DIC, differential interference contrast.

0006-291X/91 $1.50

alternative and more powerful method for dynamic studies of the secretory process at the level of a single granule. Here, we present new findings obtained by applying this method to colonic goblet cells without isolating them from the associated epithelial tissue.

MATERIALS AND METHODS

Mucus epithelium was obtained from distal colons of common rabbits. After removal of the underlying connective tissue and muscle layers, the colonic mucosa was sliced into small pieces containing 5 to 10 crypts. These pieces were placed in a narrow space between a slide glass and a coverslip, and observed under a differential interference contrast (DIC) microscope (Optiphot, Nikon, Tokyo). The DIC image was detected with a CCD (charge coupled device) camera (TI-23P, NEC, Tokyo), and the video signal of the camera was contrast-enhanced with a high speed digital image processor (PIP-4000, ADS, Osaka), monitored at magnifications of 4,500 to 9,000X, and recorded in videotape using an S-VHS video recorder (AG-7500, Panasonic, Osaka). The preparation was perfused continuously at a flow rate of 100 µl/min with a solution containing (in mM) NaCl, 135; KCl, 5.4; $CaCl_2$, 1.2; $MgCl_2$, 1.2; glucose, 10; Na-phosphate buffer, 3 (pH adjusted to 7.3). This solution was oxygenated and warmed so as to maintain the temperature of the preparation at 30 to 36°C. Goblet cells of the colonic crypt were identified by their large group of granules (0.3 to 2 µm in diameter) in the apical pole.

RESULTS AND DISCUSSION

Secretory granules in goblet cells were found to be quiescent during continuous perfusion of the crypt with normal solution. When a cholinergic agonist, carbachol (CCh) or acetylcholine, was added to the perfusion solution at a concentration of 1 to 100 µM, these granules abruptly changed in brightness one after another and turned into a small 'blister' or caveole (Fig. 1). When a clean crypt lumen was obtained, these rapid optical changes were sometimes accompanied by a discharge of smoke-like viscous materials which were immediately carried away by water flowing in the lumen. Many of these responses appeared intermittently in individual cells so that a large concave surface gradually formed at their apical pole. We infer that these rapid optical responses arise from exocytosis of mucus-containing granules. These observations are quite consistent with and complementary to the results of electron microscopic studies (11-15).

The light intensity in the DIC image is primarily determined by the difference in the refractive indices of the object and its surrounding.

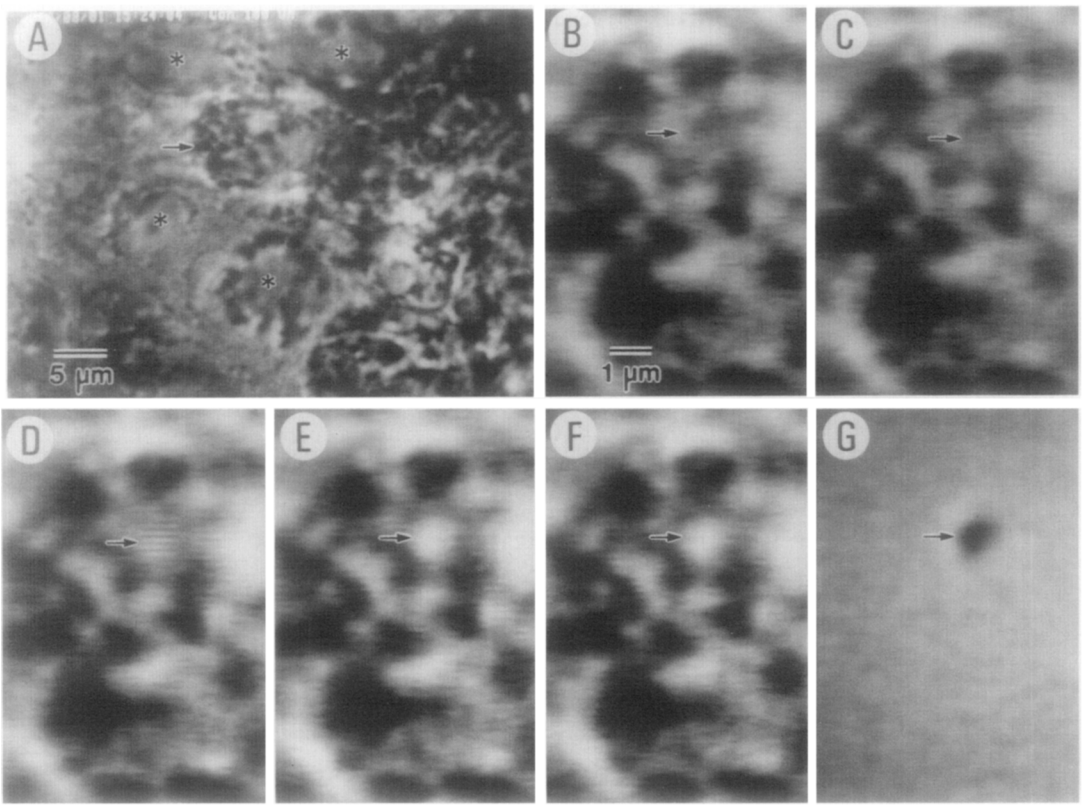

Fig. 1. DIC images of exocytosis in a colonic goblet cell reproduced from sequential video frames. **A**; Part of a crypt observed by optical sectioning close to the lumen at 5 min after application of 100 μM CCh. Many goblet cells (e.g., arrow) containing secretory granules are shown with their apicobasal axis perpendicular to the focal plane. Several cells are seen to form a cavity (asterisk) as a result of many exocytotic responses. **B – F**; Sequential video images showing an enlarged view of the cell indicated by the arrow in A. The interval between each frame is 33 ms. A granule abruptly changed in brightness (arrow). **G**; Difference image obtained by subtracting image E from image C and by adding a proper offset value. This clearly reveals the rapid brightness change of a granule (arrow).

Therefore, any rapid change in the light intensity from mucus-containing granules can be ascribed to changes in their refractive index. The most granules can be ascribed to changes in their refractive index. The most simple, plausible interpretation of the refractive index change is that the granule contents are rapidly released and replaced with a material of lower refractive index, i.e., the external medium. We exclude the possibility that translational movement of the granule caused the brightness change, because we saw no rapid movement in any directions along the focal plane even when side and top views of many responding cells were clearly visible.

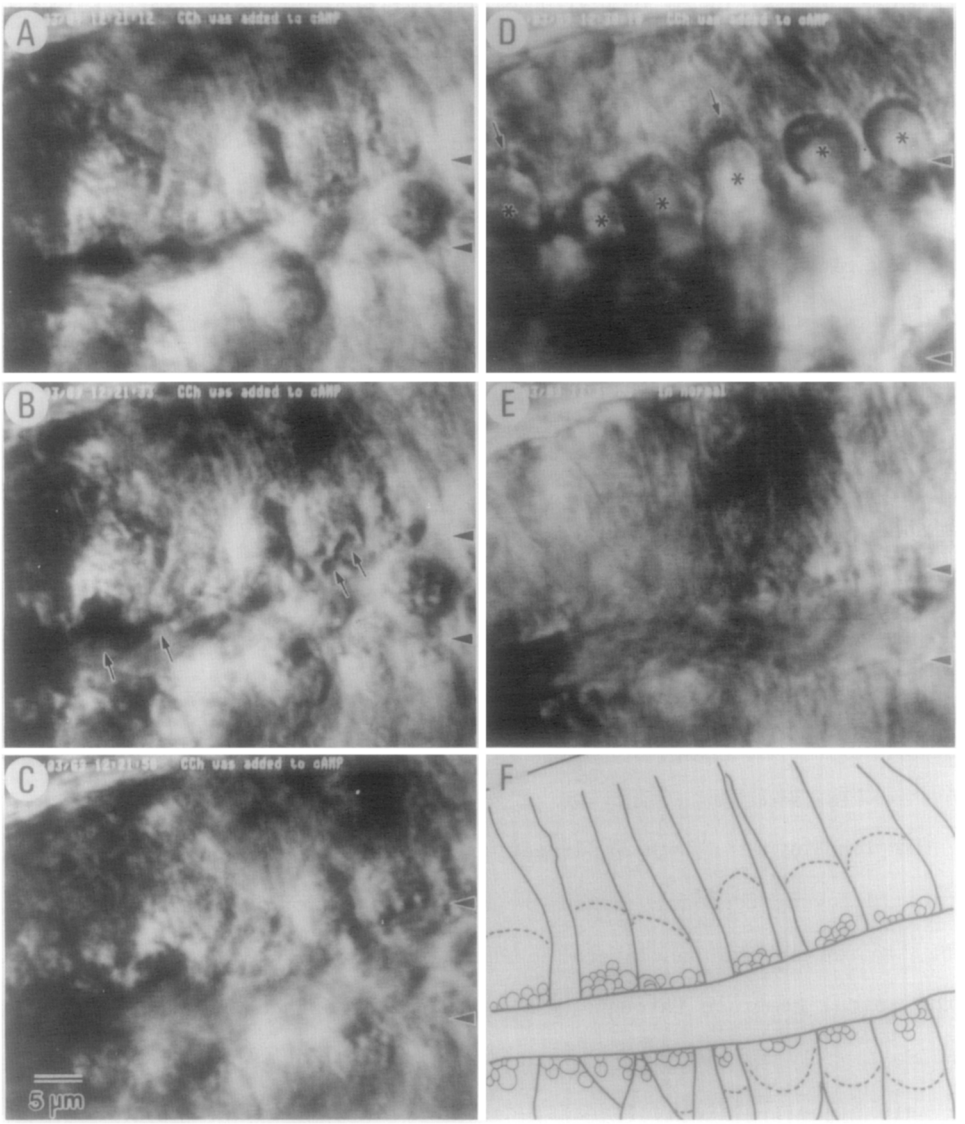

Fig. 2. Morphological changes of goblet cells as revealed by DIC microscopy. A crypt was optically sectioned along the lumen. The border of the lumen is indicated by arrow heads. **A**; DIC image obtained 3 s before application of CCh. **B**; Image obtained 18 s after application of CCh (100 μM CCh was applied in the presence of 1 mM 8Br-cyclic AMP to obtain the maximal response). Many granules showed an abrupt brightness change and formed a small cavity (arrow). **C**; Image obtained 35 s after arrival of CCh. As a result of many exocytotic responses, the lumen was widened and the apical membrane became very rough. **D**; Image obtained 17 min after arrival of CCh. Large cavities (asterisk) formed in the individual goblet cells, leaving few granules underneath (arrow). **E**; Image of the same preparation 50 min after removal of CCh. **F**; Traces of granules showing abrupt brightness change during an initial 45 s period of CCh stimulation. The bottom border for aggregation of granules in each cell is indicated by a curved broken line.

The sites of exocytotic responses in each goblet cell could be clearly determined when the cell was optically sectioned along the apicobasal axis.

Initially, the sites of exocytotic optical changes were distributed in limited areas exactly aligned along the luminal border of the crypt (Fig. 2); later, they gradually spread toward the goblet cell bases. These responses were never seen at an isolated site deep inside the aggregation of secretory granules. This indicates that granules fuse only with the apical membrane, rather than with other granules discontinuous to the apical membrane. Once a granule fused with the apical membrane to form a caveole, another granule was able to fuse with this caveole within a few hundred milliseconds. Often, three or four granules fused successively in this manner; this may correspond to the process previously referred to as 'compound exocytosis' from electron microscopic studies (17). When CCh was applied for a long period of time, almost all granules in the goblet cell were depleted after their exocytotic responses, leaving a large cavity in the apical pole as shown in Fig. 2D. About 1 h after removal of the stimulant, the cell recovered its initial volume without producing new secretory granules (Fig. 2E).

Our finding of a lack of direct granule-granule fusion is significant for the following reasons. Since the receptors to most agonists are likely to be located on the basolateral membrane of the goblet cell, the intra- to the apical membrane through narrow cytoplasmic spaces between secretory granules tightly apposed to each other (13-15). Our observation indicates that this signal, while in transit, cannot initiate membrane fusion between granules. It is very likely that the membrane of the secretory granule acquires the ability to fuse with neighboring granules only after fusion with the apical membrane. The apical membrane may possess a special class of fusion-initiating molecules, and they may be transferred to a granule membrane by lateral diffusion following the fusion response.

Each exocytotic event in an individual goblet cell was easily captured by eye on real-time playback of the videotape, and was delivered to a computer through key board operation. Frequency histograms (Fig. 3) were made from the data files thus stored. Although there was a probabilistic variation in the shape of histograms obtained from individual cells, 100 μM

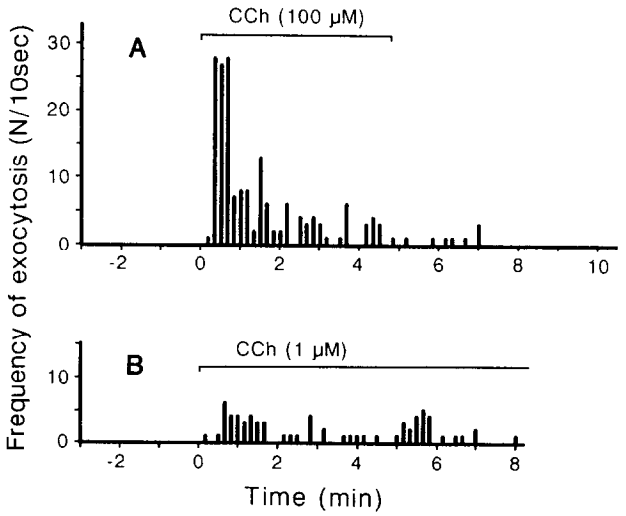

Fig. 3. Frequency of exocytotic response (abrupt brightness change of granules) in a single goblet cell stimulated by 100 μM CCh (A) and in another cell stimulated by 1 μM CCh (B). The number of quantal responses in every 10 s period was counted and expressed as histograms. CCh was applied for the period indicated by horizontal bars.

CCh always induced a large peak of secretory activity during an initial period of a few minutes (Fig. 3A). Then, after a short silence, a moderate activity persisted for 20 to 40 min as application of the stimulant was continued. At lower concentrations of CCh (1 - 10 μM), the frequency of exocytotic events was much lower, and usually no clear peak was formed in the initial phase (Fig. 3B). Since all of these responses were suppressed completely by 10 μM atropine, we ascertained that the muscarinic receptors are involved in the CCh-induced response.

Through frequency determination of these quantal responses, we have developed a precise method for real-time estimation of secretory activity. This quantal analysis attains ultimate sensitivity and improves facility for long-period assay, and thus reveals dynamic characteristics of the goblet cell which can not be studied otherwise (16). Dynamic aspects of exocytosis of a single granule will also be studied in detail by analyzing the light intensity change of the granule recorded in the videotape (17,18). Studies of various exocrine (17), endocrine (18) and neurosecretory cells using the same technique are currently in progress in our laboratory.

ACKNOWLEDGMENTS

We wish to thank Ms. R. Sakurai and Mr. M. Ohara for their technical assistance. Preliminary results were reported in the Gumma Symposia on Endocrinology, 1988 at Gumma University, Maebashi, Japan.

REFERENCES

1. Burgoyne, R.D. & Cheek, T.R. (1987) Nature 326, 448.
2. Green, D.P.L. (1989) Trends Neurosci. 12, 285.
3. Knight, D.E., von Grafenstein, H. & Athayde, C.M. (1989) Trends Neurosci. 12, 451-458.
4. Penner, R & Neher, E. (1989) Trends Neurosci. 12, 159-163.
5. McCuseky, R.S. & Chapman, T.M. (1969) Am. J. Anat. 126, 395-408.
6. Kanno, T. (1974) In: Secretory Mechanism of Exocrine Glands (ed.) N.A. Thorn & O.H. Petersen, Munksgaard, Copenhangen pp. 278-292.
7. Inoue, S. (1981) J. Cell Biol. 89, 346-356.
8. Allen, R.D., Allen, N.S. & Travis, J.L. (1981) Cell Motilty 1, 291-302 (1981).
9. Edwards, C., Englert, D., Latshow, D. & Ye, H.-Z. (1984) Cell Motility 4, 297-303.
10. Allen, R.D. (1985) Ann. Rev. Biophys. Biophys. Chem. 14, 265-290.
11. Neutra, M.R., O'Malley, L.J. & Specian, R.D. (1982) Am. J. Physiol. 242, G380-G387.
12. Neutra, M.R. & Forstner, J.F. (1987) In: Physiology of Gastrointestinal Tract (Second Ed.) ed. by L.R. Johnson. Raven Press, New York, N.Y. pp. 975-1009.
13. Neutra, M.R. & Shaffer, S.F. (1977) J. Cell Biol. 74, 983-991.
14. Specian, R.D. & Neutra, M.R. (1980) J. Cell Biol. 85, 626-640.
15. Ichikawa, M., Ichikawa, A. & Kidokoro, S. (1987) J. Electron Microsc. 36, 117-127.
16. Specian R.D. & Neutra, M.R. (1982) Am. J. Physiol. 242, G370-G379.
17. Segawa, A., Terakawa, S. Yamashina, S. & Hopkins, C.R. (1991) Eur. J. Cell Biol. 46. in press.
18. Terakawa, S., Fan, J.-H., Kumakura, K. & Ohara-Imaizumi, M. (1991) Neurosci. Lett. in press.