

MECHANISM OF RAPID MUCUS SECRETION IN GOBLET CELLS STIMULATED BY ACETYLCHOLINE

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ABSTRACT

The parasympathetic control of goblet cell secretion and the membrane events accompanying accelerated mucus release were studied in large intestinal mucosal biopsies maintained in an organ culture system. The secretory response of individual goblet cells to 10^{-6} M acetylcholine chloride with 3×10^{-3} M eserine sulfate (a cholinesterase inhibitor) was assessed by light microscopy and autoradiography, by scanning and transmission electron microscopy, and by freeze-fracture. Goblet cells on the mucosal surface are unaffected by acetylcholine. In crypt goblet cells, acetylcholine-eserine induces rapid fusion of apical mucous granule membranes with the luminal plasma membrane (detectable by 2 min), followed by sequential, tandem fission of the pentalaminar, fused areas of adjacent mucous granule membranes. These events first involve the most central apical mucous granules, are then propagated to include peripheral granules, and finally spread toward the most basal granules. By 60 min, most crypt cells are nearly depleted. The apical membrane, although greatly amplified by these events, remains intact, and intracellular mucous granules do not coalesce with each other. During rapid secretion, membrane-limited tags of cytoplasm are observed attached to the cavitated apical cell surface. These long, thin extensions of redundant apical membrane are rapidly lost, apparently by being shed into the crypt lumen.

Intestinal goblet cells synthesize and secrete mucins, the high molecular weight glycoproteins that provide a viscous, presumably protective blanket on the mucosal surface (15, 18, 19, 31). Under normal conditions, the mucus blanket is maintained and renewed by the slow, continuous release of mucin from individual goblet cells throughout the epithelium (42, 43). This "baseline" secretion occurs by conventional exocytosis: the intermittent fusion of a single mucous granule membrane and the apical plasma membrane (34, 47, 58). The factors that control baseline mucus secretory rates are unknown. It is known, however, that a variety of intraluminal irritants are capable of inducing a rapid emptying of goblet cells, a

reaction that apparently provides an emergency blanket of fresh mucus on the threatened epithelial surface (9, 13, 16, 19, 41, 55, 59). Previous reports have described such massive mucus secretion as "apocrine," involving disruption of the apical plasma membrane and outpouring of mucous granules and cytoplasmic fragments (20, 29, 33).

Other types of secretory cells (serous exocrine, endocrine, mast cells) accomplish rapid secretion in response to secretagogues by a modification of conventional exocytosis. In these cells, fusion of an initial secretion granule membrane and the plasma membrane may be rapidly followed by tandem fusion of subjacent secretion granule membranes, allowing the contents of many gran-

ules to exit at a single surface site without interrupting the continuity of the plasma membrane (2, 3, 5, 8, 11, 25, 30, 33, 35, 37). During the secretory response, adjacent secretion granule membranes have been reported to form transient pentalaminar fusion sites just before fission or breakdown (3, 5, 8, 35, 37, 47).

In this report, the pentalaminar profiles that appear when two unit membranes are in such close contact that their abutting cytoplasmic leaflets merge to form a single dense line are considered images of membrane "fusion." The term implies a specific interaction, which may be accompanied, at least in aldehyde-fixed cells, by lateral displacement of intramembrane particles. Membrane "fission" (for lack of a better term) refers to the poorly understood molecular rearrangements through which one membrane becomes continuous with the other (37, 47).

An ultrastructural and freeze-fracture study of unstimulated, aldehyde-fixed intestinal goblet cells showed that even in the absence of a secretory response, the limiting membranes of adjacent mucous granules are fused to form pentalaminar, particle-poor contact areas (44). Because these areas appear to be potential sites of membrane fission, we thought that the appropriate stimulus might trigger a rapid breakdown of these sites throughout the cell, allowing massive secretion of mucus without rupture of the apical plasma membrane.

This hypothesis could only be tested under conditions in which goblet cell secretion could be reproducibly accelerated, inhibited, and followed in time. Because the parasympathetic neurotransmitter, acetylcholine (ACh), is thought to affect mucus release from the mammalian colonic mucosa (9, 16, 39), we investigated the secretory response of rabbit large intestinal goblet cells to ACh in an organ culture system. The secretory response of individual cells and the membrane events that accompany accelerated secretion were assessed by autoradiography, by light, transmission, and scanning electron microscopy, and by freeze-fracture.

MATERIALS AND METHODS

Mucosal samples 2–4 mm in diameter were excised from the descending colon of New Zealand white rabbits under nembutal or urethane anesthesia. Samples of untreated, control mucosa were immediately immersed in the appropriate fixative as discussed below. Samples for *in vitro* morphological studies were briefly immersed in oxygenated culture medium consisting of 90% chloromycetin-free Trowell T8 medium and 10% fetal calf

serum (both from Grand Island Biological Co., Grand Island, N. Y.), to which 100 µg/ml gentamicin and 60 U/ml Mycostatin (Squibb & Sons, Inc., Princeton, N. J.) had been added. The samples were then mounted mucosal side up on stainless steel wire screens in plastic organ culture dishes (Falcon Plastics Co., Div. of B-D Laboratories, Inc., Los Angeles, Calif.) as previously described (12, 39, 42). The screens were floated on the same culture medium containing acetylcholine chloride (1×10^{-6} M) and eserine sulfate (3×10^{-3} M), atropine sulfate (1×10^{-6} M), a combination of all three, or no additives, for 2, 5, 10, 20, 30, or 60 min. Covered culture dishes were incubated at 37°C in an atmosphere of 95% O₂ and 5% CO₂.

To follow the intrinsic rates of intracellular transport and release of glycoprotein secretory products in normal rabbit colon, mucosal samples were pulse-labeled by immersion for 30 min at 37°C in organ culture medium that contained [³H]-D-glucosamine hydrochloride (200 µCi/ml, specific activity 18.8 Ci/mM, New England Nuclear, Boston, Mass.). In these experiments, both the holding and the "pulse" media consisted of glucose-free, chloromycetin-free Trowell T8 medium with fetal calf serum and antibiotics, as described above. After the pulse period, the samples were mounted in organ culture dishes and maintained on nonradioactive culture medium (containing glucose, 4 g/liter) for 20 min to 8 h.

To assay the ACh-induced secretory response, samples were pulse-labeled as described above, maintained on chase medium for 3 h, and then transferred to fresh organ culture dishes and floated on medium that contained acetylcholine chloride and eserine sulfate for 2–60 min. Control samples were incubated with ACh, eserine, and atropine sulfate, with atropine sulfate alone, or without additives. Samples for autoradiography were fixed in potassium dichromate-buffered 1% OsO₄, postfixed in sodium phosphate-buffered formalin, and embedded in epoxy resin. 1-µm sections were stained with periodic acid-Schiff and iron hematoxylin (51), coated for autoradiography by dipping in Ilford K5 or L4 photographic emulsion (Ilford Ltd., Basildon, Essex, England) diluted 1:1, exposed at 4°C for 4–16 wk, and developed at 18°C in Kodak D19 developer for 4 min.

For transmission electron microscopy (TEM), mucosal slices were fixed for 3 h at 23°C in 0.1 M sodium cacodylate-buffered 2% formaldehyde–2.3% glutaraldehyde with 0.4% CaCl₂ at pH 7.4 (32), postfixed for 1 hr at 4°C in 0.1 M sodium cacodylate-buffered OsO₄ at pH 7.4, stained *en bloc* for 4 h at 4°C in 1% uranyl acetate in sodium acetate buffer, and embedded in Epon-Araldite. Some samples of untreated and stimulated mucosa were fixed for 2 h at 4°C in 0.1 M sodium cacodylate-buffered 2.5% glutaraldehyde and 1% OsO₄ (28) and stained *en bloc* with uranyl acetate as described above. In addition, samples of uncut rabbit colonic mucosa were mounted on aluminum disks mucosa side up and rapidly frozen against a copper block cooled to liquid helium temperature (–269°C) using the device and method developed by Heuser and colleagues (27). Frozen samples were transferred to liquid nitrogen, freeze-substituted with OsO₄ in acetone, stained *en bloc* with 1% uranyl acetate, and embedded in Epon-Araldite. Thin sections were stained with uranyl acetate and lead citrate and were examined with JEOL 100B, 100S, or 100CX electron microscopes.

For scanning electron microscopy, biopsies were fixed with aldehydes and OsO₄ as described above (but not stained *en bloc*), dehydrated to 100% ethanol, and critical-point dried from ethanol using liquid CO₂ in a SAMDRI PVT-3 critical-point apparatus (Biodynamics Research Corp., Rockville, Md.). Specimens were coated with gold-palladium in a Hummer II sputter coating apparatus (Technics Inc., Alexandria, Va.) and examined in a JEOL JSM-35 or JEOL 100CX scanning electron microscope.

For freeze-fracture, mucosal slices were fixed for 30–45 min at 23°C in the aldehyde fixative described for TEM, trimmed, and equilibrated with 20% glycerol in 0.1 M sodium cacodylate buffer for 1–2 h. Tissue slices were mounted on gold disks, rapidly frozen in the liquid phase of partially solidified Freon 22 (Virginia Chemicals, Inc., Portsmouth, Va), and stored in liquid nitrogen. Specimens were fractured in a Balzers freeze-etch device (Model BA 360, Balzers AG, Balzers, Liechtenstein) at a stage temperature of -115°C , replicated with platinum-carbon without etching, cleaned with methanol and household bleach, and mounted on 300-mesh copper grids. Replicas were examined with a JEOL 100CX electron microscope. Illustrations of freeze-fracture specimens are presented with the shadow direction approximately from bottom to top.

RESULTS

Goblet cells of the rabbit colonic epithelium are recognized in 1- μm , periodic acid-Schiff (PAS)-iron hematoxylin-stained sections by their densely stained nucleus and cytoplasm and by the PAS reactivity of their tightly packed apical mucous granules (Figs. 1 and 2). They differentiate deep in the crypt and migrate upward, separated from one another by columnar absorptive cells. The immature columnar cells in the crypts are filled with electron-lucent, membrane-bounded vacuoles, which commonly rupture during fixation, and which are secreted and lost as the cells emerge onto the mucosal surface (Fig. 1). Goblet cells elongate and change shape as they emerge onto the mucosal surface, but the size and the ultrastructural features of their stored mucous secretion granules do not change. With the fixation procedures used in our study, mucous granule membranes are intact, and adjacent intracellular granules do not coalesce.

Mucosal biopsies cultured on medium that contained [^3H]glucosamine for 30 min and subsequently maintained on cold chase medium for 20 min before fixation and autoradiography show radioactive mucus localized in the supranuclear region of both crypt and surface goblet cells, as previously reported in studies of rabbits and other species (39, 42). When the period on cold chase medium is extended to 2 h labeled mucous granules are still located in or near the supranuclear region, but by 4 h they have migrated into the

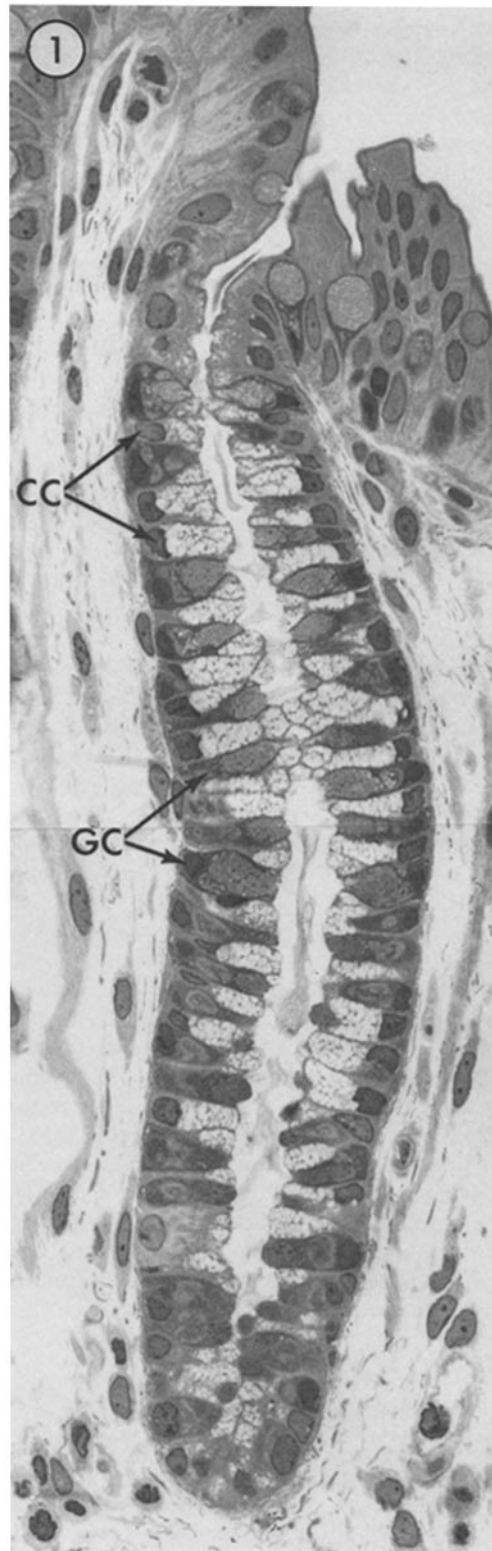
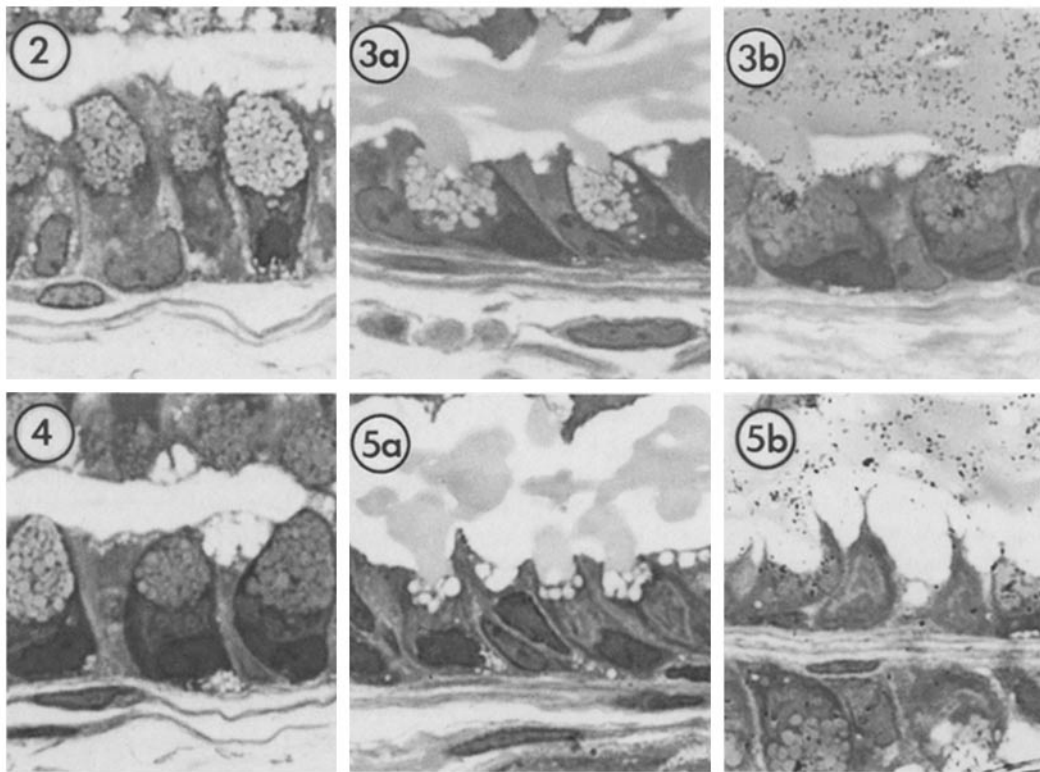


FIGURE 1 A crypt in the colonic mucosa of a rabbit, sectioned longitudinally. The dense, grey mucous granules distinguish the goblet cells (GC) from the immature columnar cells (CC), which are filled with clear vacuoles. At the mouth of the crypt, columnar cells lose their vacuoles and assume the morphological characteristics of mature surface columnar cells. 1- μm plastic section stained with PAS-iron hematoxylin. $\times 600$.



FIGURES 2-5 1- μ m plastic sections (*left*) and autoradiographs (*right*) of colonic crypts incubated with [3 H]glucosamine for 30 min, maintained on cold chase medium for 3 h, and then treated as indicated. PAS-iron hematoxylin. \times 1,350. (Fig. 2) Control. Goblet cells are full of intact mucous granules. (Fig. 3) ACh- eserine, 30 min. (*a*) Mucus released from the apical granules is streaming into the lumen. (*b*) The labeled goblet cell mucus seen in the lower and central portions of the theca has not yet been secreted. Radioactivity in the crypt lumen is derived predominantly from immature columnar cells. (Fig. 4) ACh- eserine with atropine, 60 min. Atropine prevents the secretory response to ACh. The cells are identical to untreated controls (see Fig. 2). (Fig. 5) ACh- eserine, 60 min. (*a*) Goblet cells are nearly depleted of mucous granules. (*b*) Most of the labeled mucus is now present in the crypt lumen.

basal portion of the mass of stored granules. At later times (between 4 and 8 h), there is great variation in the location of the radiolabeled mucous granules among adjacent crypt goblet cells. Even within a single cell, labeled granules are often widely separated, suggesting that newly formed granules do not necessarily move together toward the cell surface (Fig. 3*b*). Because the position of the radiolabeled mucous granules is fairly uniform up to 3 or 4 h, the release of labeled mucus in response to ACh was investigated in goblet cells that had been pulse-labeled with [3 H]glucosamine 3 h previously. Columnar absorptive cells also incorporated [3 H]glucosamine into nascent macromolecules and rapidly transported the labeled product (within 1-2 h) to their apical microvillus borders.

The secretory response was documented first by light microscopy and autoradiography, then by SEM and TEM, and finally by freeze-fracture. By light microscopy, the goblet cells of colonic crypts exposed to ACh- eserine for 30 min show cavitated, deeply concave apical surfaces from which streams of PAS-stained mucus emerge to join a thick mucus stream in the crypt lumen (Fig. 3*a*). Surface goblet cells, in contrast, show no secretory response and are indistinguishable from their counterparts in normal, uncultured mucosa and in control tissue cultured without ACh or eserine. Autoradiographs of mucosa exposed to ACh- eserine for 30 min show that the majority of the labeled goblet cell mucus in crypt cells has not yet been released, whereas the predominantly unlabeled granules located more apically have been secreted (Fig. 3*b*).

Much of the labeled material present in the lumen at this time is apparently derived from the immature columnar cells, whose apical surfaces are heavily labeled 3 h after the 30-min pulse. After a 60-min exposure to ACh- eserine (Fig. 5 *a*), light microscopy reveals that most crypt goblet cells are nearly depleted of intracellular mucous granules, whereas surface cells are still unaffected. Autoradiographs of these tissues show that labeled mucus has by this time been released from most crypt goblet cells, resulting in heavy labeling of the mucus stream in the crypt lumen (Fig. 5 *b*). Goblet cells in biopsies exposed to ACh- eserine for either 30 or 60 min with the addition of 10^{-6} M atropine are indistinguishable from control cells either by light microscopy (Fig. 4) or by autoradiography. Goblet cells in biopsies exposed to 10^{-6} M atropine alone (without ACh or eserine) for periods ranging from 30 min to 4 h show the same morphology and the same slow baseline transport of labeled mucous granules as do those in untreated tissues. Concentrations of ACh up to 10^{-4} M fail to elicit a secretory response in surface goblet cells.

By SEM, the velvety, microvillus-covered sur-

face of normal, unstimulated rabbit colonic mucosa is punctuated at regular intervals by the small, circular openings of colonic crypts, and a narrow stream of mucus emerges from each crypt opening (Fig. 6 *a*). Although most surface goblet cells are hidden by epithelial folds, some are recognizable as small, convex smooth areas (arrows, Fig. 6 *a*). After a 30-min exposure to ACh- eserine in organ culture (Fig. 6 *b*), thick streams of mucus appear to erupt from widened crypt openings (Fig. 6 *b*). The SEM appearance of goblet cells on the mucosal surface is unchanged by the ACh treatment (arrows, Fig. 6 *b*).

The ultrastructural features of goblet cells in normal, uncultured rabbit mucosa and in mucosa maintained for 4 h in organ culture are identical. The individual mucous granules crowded in the apical cytoplasm are limited by intact unit membranes. After standard glutaraldehyde-paraformaldehyde fixation, with OsO_4 and uranyl acetate postfixation (Figs. 9–12), after simultaneous glutaraldehyde- OsO_4 fixation (Fig. 7 *a*), and after fixation by rapid freezing and subsequent substitution with OsO_4 in acetone (Fig. 7 *c*), the mem-

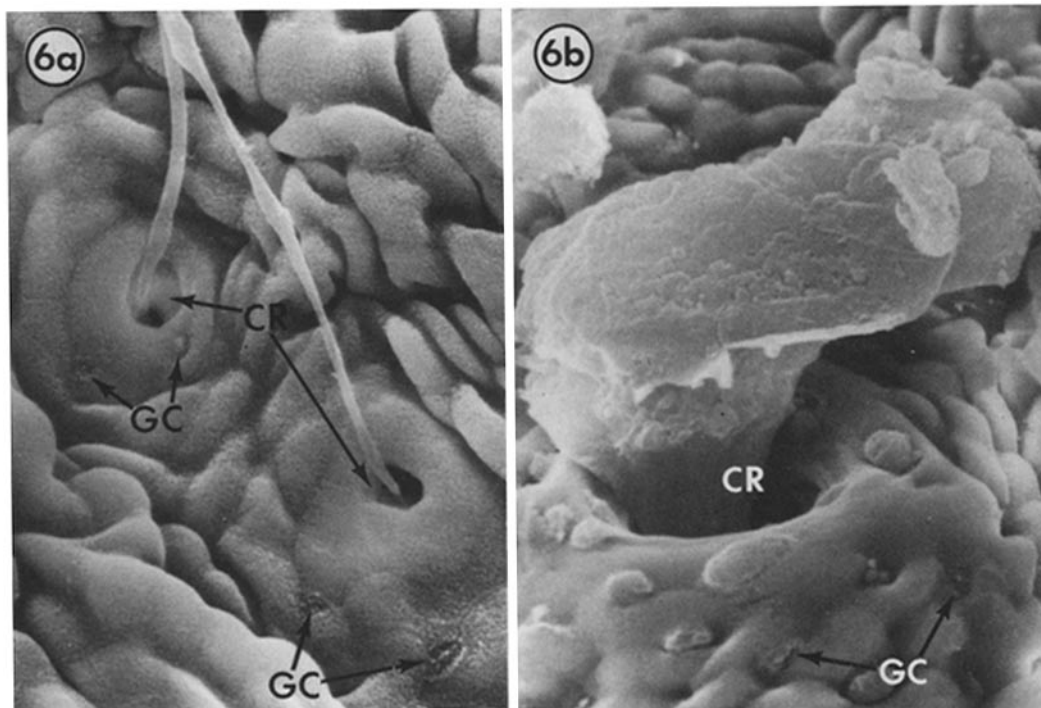


FIGURE 6 SEM views of the mucosal surface of rabbit colonic biopsies. (*a*) Control. Thin streams of mucus emerge from the openings of two crypts (CR). Most surface goblet cells (GC) are hidden in mucosal folds. $\times 1,360$. (*b*) ACh- eserine, 30 min. Mucus derived from crypt goblet cells flows from a dilated crypt opening (CR). Surface goblet cells (GC) have not secreted. $\times 1,070$.

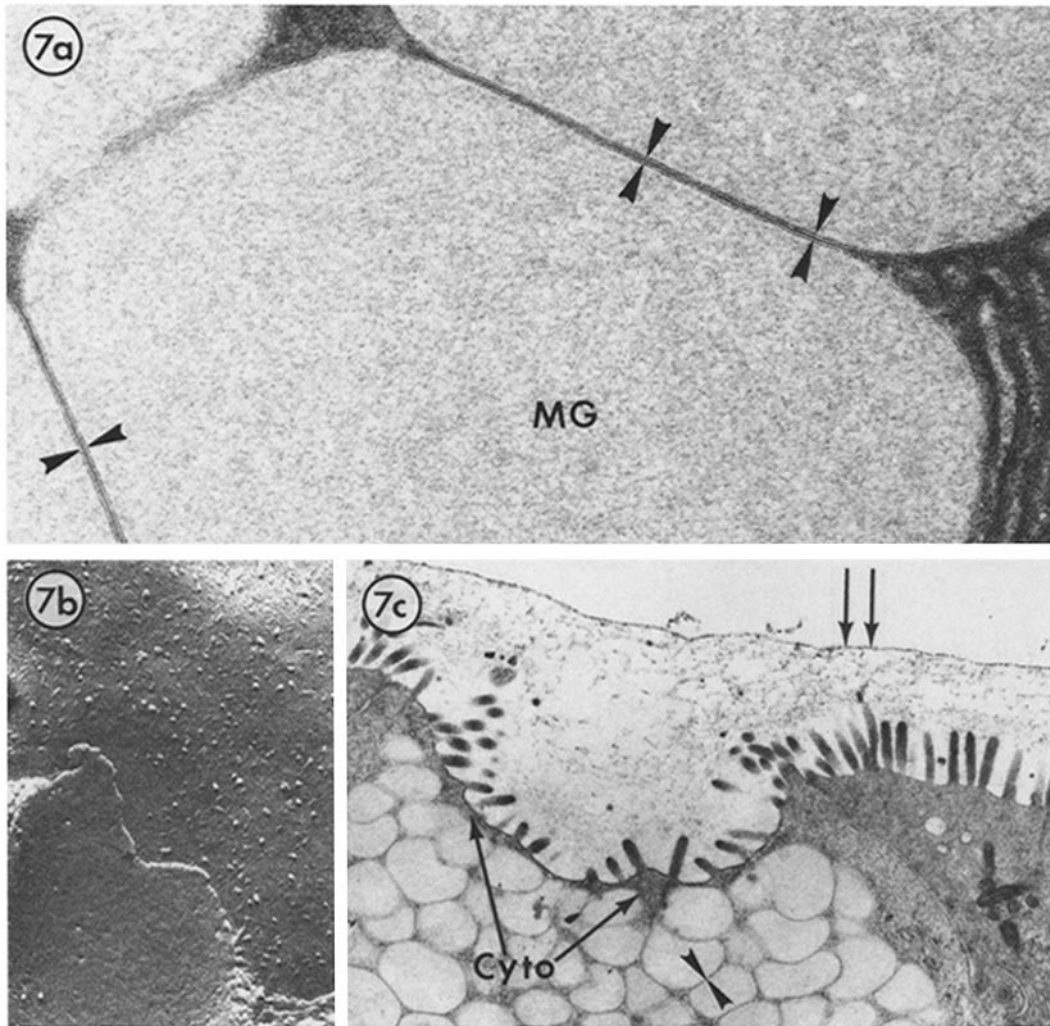


FIGURE 7 (a) Electron micrograph of a goblet cell fixed simultaneously with glutaraldehyde and OsO_4 . Arrowheads indicate pentalaminar fusion sites between the membranes of adjacent mucous granules (MG). $\times 91,800$. (b) A freeze-fracture replica of mucous granules in a rapid-frozen surface goblet cell. In areas of close apposition, mucous granule membranes are devoid of intramembrane particles. $\times 63,100$. (c) Rapid-frozen, freeze-substituted surface epithelium of rabbit colon. The upper portion of a surface goblet cell lies $\sim 2\text{--}5\ \mu\text{m}$ below the upper edge of the hydrated mucous cushion that had contacted the helium-cooled copper block (double arrows). A thin layer of cytoplasm separates the apical plasma membrane from underlying mucous granules. The membranes of adjacent granules are as closely apposed as they are in aldehyde-fixed cells (arrowheads). $\times 10,200$.

branes of adjacent mucous granules are closely apposed to each other, with little intervening cytoplasm, and show areas of direct contact, which appear as fused, pentalaminar structures in thin sections. In freeze-fracture replicas of both aldehyde-fixed and rapid-frozen rabbit goblet cells, the contact areas are devoid of intramembrane P- and E-face particles (Figs. 7b and 13 inset). Mu-

cous granules immediately underlying the apical cell surface are closely apposed to each other but are generally separated from the plasma membrane by a layer of cytoplasm of variable thickness (Fig. 7c). Occasionally, however, sites of direct contact or fusion of apical granules and plasma membranes are observed, and images suggesting the recent exocytosis of a single mucous granule

are commonly seen in both crypt and surface goblet cells of untreated and atropine-treated control tissues (Fig. 8). Because the goblet cells of control tissues are uniformly filled with intracellular granules, these exocytotic events are presumably limited to one or a few granules at a time and are balanced with the continual production of new mucous granules in the Golgi region of the cell.

After a 2-min exposure to ACh- eserine, we observed a marked increase in the number of images of recent exocytosis in the central apical region of crypt goblet cells (Fig. 9). Continued exposure to ACh- eserine results in an orderly series of exocytotic events, apparently involving the sequential breakdown of pentalaminar contact regions between adjacent mucous granule membranes (Figs. 10–13). Sequential fission of granule membranes is propagated first toward the center of the cell and later includes more peripheral mucous granules (Figs. 9 and 10). After 30 min of ACh- eserine

exposure, the upper portion of the theca is empty, but sequential exocytosis continues into the center of the lower theca (Figs. 10 and 13). During this process, fragments of intergranule cytoplasmic matrix rich in microfilaments and containing an occasional small vesicle appear on the caviated cell apex. They are bounded by the remaining portions of the membranes of secreted mucous granules, which may include some intact pentalaminar regions (Fig. 12*a*). Although in thin sections they appear most frequently as long tags (Figs. 11*a* and 12*b*), in three dimensions they probably represent thin sheets or empty “cages” of membrane-enclosed cytoplasm (Fig. 12*a* and *b*). These thin extensions of the newly amplified apical membrane rapidly disappear, leaving a deeply concave but smooth and intact apical plasma membrane (Figs. 10, 11*b*, and 12*b*). Although normal cell height and width are maintained throughout the 30 min of ACh stimulation, they are drastically altered at later times. By 60 min (Fig. 11*b*), crypt goblet cells contain a variable but sparse population of mucous granules and are markedly shortened. At the same time, crypt lumina are widely dilated. Goblet cell apical plasma membranes are no longer deeply concave but still are ultrastructurally intact.

In freeze-fracture replicas of untreated, aldehyde-fixed or rapid-frozen cells and of cells treated for 30 min with ACh- eserine before aldehyde fixation, the exposed membrane faces of intracellular mucous granules display flattened, particle-poor areas (Figs. 7*b* and 13 *inset*) similar to those previously reported in primate goblet cells (44). During the secretory response, smooth membrane areas also appear in regions of contact between the newly amplified plasma membrane and the membrane of the subjacent mucous granule, which presumably is the next to be secreted (Fig. 14*b*). Thin sections of the same tissue show that these particle-poor contact areas appear as pentalaminar membrane profiles (Fig. 14*a*); they presumably correspond to those that had formed between adjacent intracellular granules before secretion.

DISCUSSION

Evidence for the factors that control and modulate intestinal goblet cell secretion is fragmentary and circumstantial (15, 16, 19). Mucus release is known to occur in response to mechanical insult, chemical irritation, and certain bacterial toxins (13, 16, 19, 41, 55, 59, 60). The response to intraluminal mustard oil has been reported to be independent of

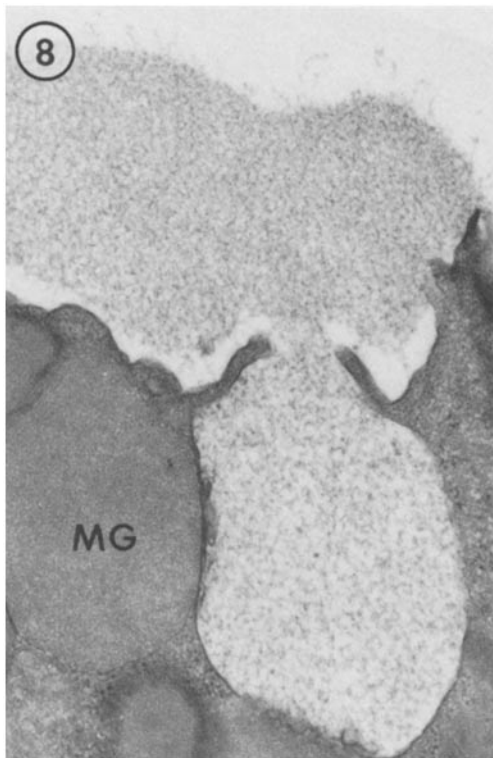


FIGURE 8 The release of mucus from unstimulated cells involves the intermittent fusion of a mucous granule membrane and the apical plasma membrane. Atropine does not inhibit this process. In this cell, exocytosis is occurring despite the fact that the ACh-mediated secretory response was blocked by atropine. $\times 39,300$.

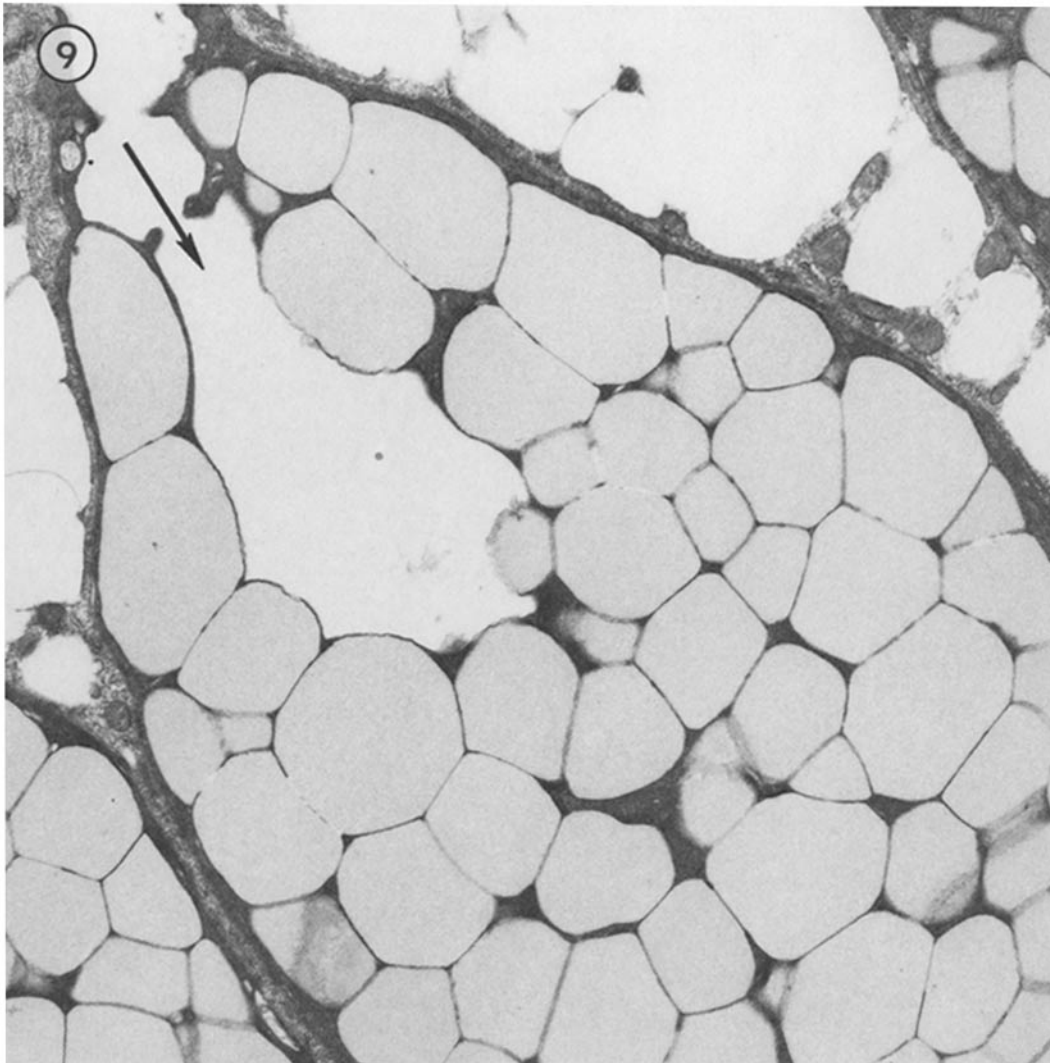


FIGURE 9 ACh- eserine, 2 min. In response to ACh- eserine, the centrally located apical mucous granules have emptied through a series of membrane fission events (arrow), whereas more peripheral granules are spared. $\times 12,700$.

autonomic nerves (9, 16), but whether individual goblet cells respond directly to luminal signals is not known, and whether there are receptors on goblet cell apical membranes has not been investigated. With regard to neurotransmitters, cholinergic drugs induce the appearance of visible mucus in the lumen of dog and cat intestine (15, 16) and stimulate glycoprotein release from rabbit colonic mucosa in organ culture (39); in both cases, goblet cells were assumed, but not proved, to be the source. Adrenergic agents and cyclic AMP do not affect intestinal glycoprotein secretion (17, 19, 36).

In the respiratory passages where surface goblet cells are relatively long-lived, the cells do not respond to cholinergic or adrenergic agents (4, 54, 56). Our results show that in the intestine, where the epithelial cell population is renewed every few days (6), goblet cells are responsive to ACh only as long as they are within the intestinal crypts. As they move onto the surface of the mucosa, they no longer respond to ACh. This loss of sensitivity, which occurs at a precise site at the crypt mouth, is abrupt and complete since large doses of ACh (up to 1×10^{-4} M) failed to elicit a secretory

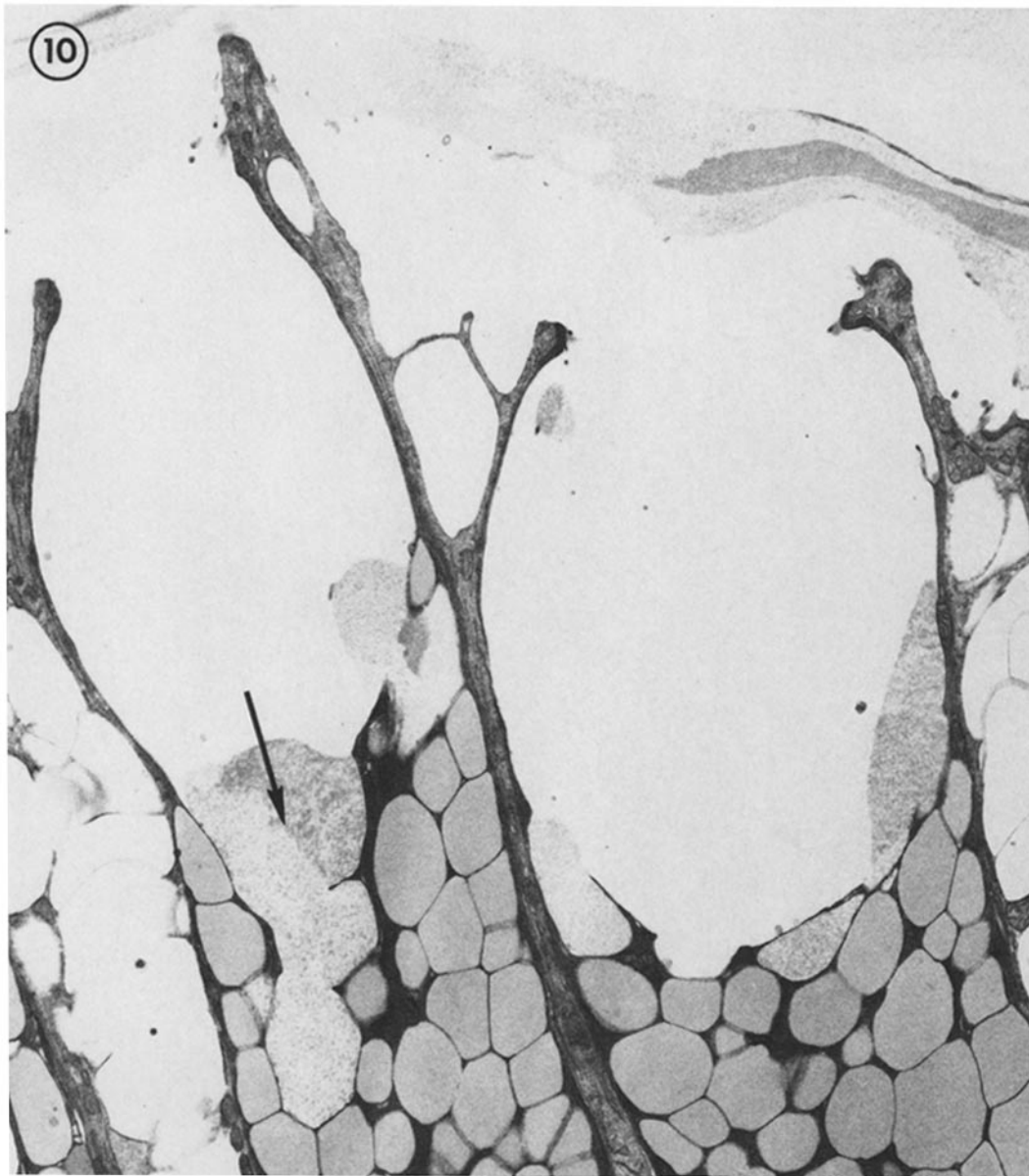


FIGURE 10 ACh-esterine, 30 min. The apical surfaces of crypt goblet cells are deeply cavitated. Although the sequential release of both central and peripheral mucous granules has greatly amplified the apical plasma membrane, the limiting membranes of intracellular granules remain intact. As the secretory response continues into deeper levels of the cell, secretion of central granules precedes secretion of peripheral granules (arrow). $\times 8,425$.

response in colonic surface cells. On the other hand, when we instilled intraluminal mustard oil into ligated rabbit colonic loops, the surface goblet cells rapidly secreted mucus, whereas crypt cells did not. It is possible that the irritant may not

have been able to enter the mucus and fluid-filled crypts. In any case, the secretory response of surface cells to mustard oil involved a sequential fission of mucous granule membranes comparable to that seen in crypt cells stimulated by ACh.

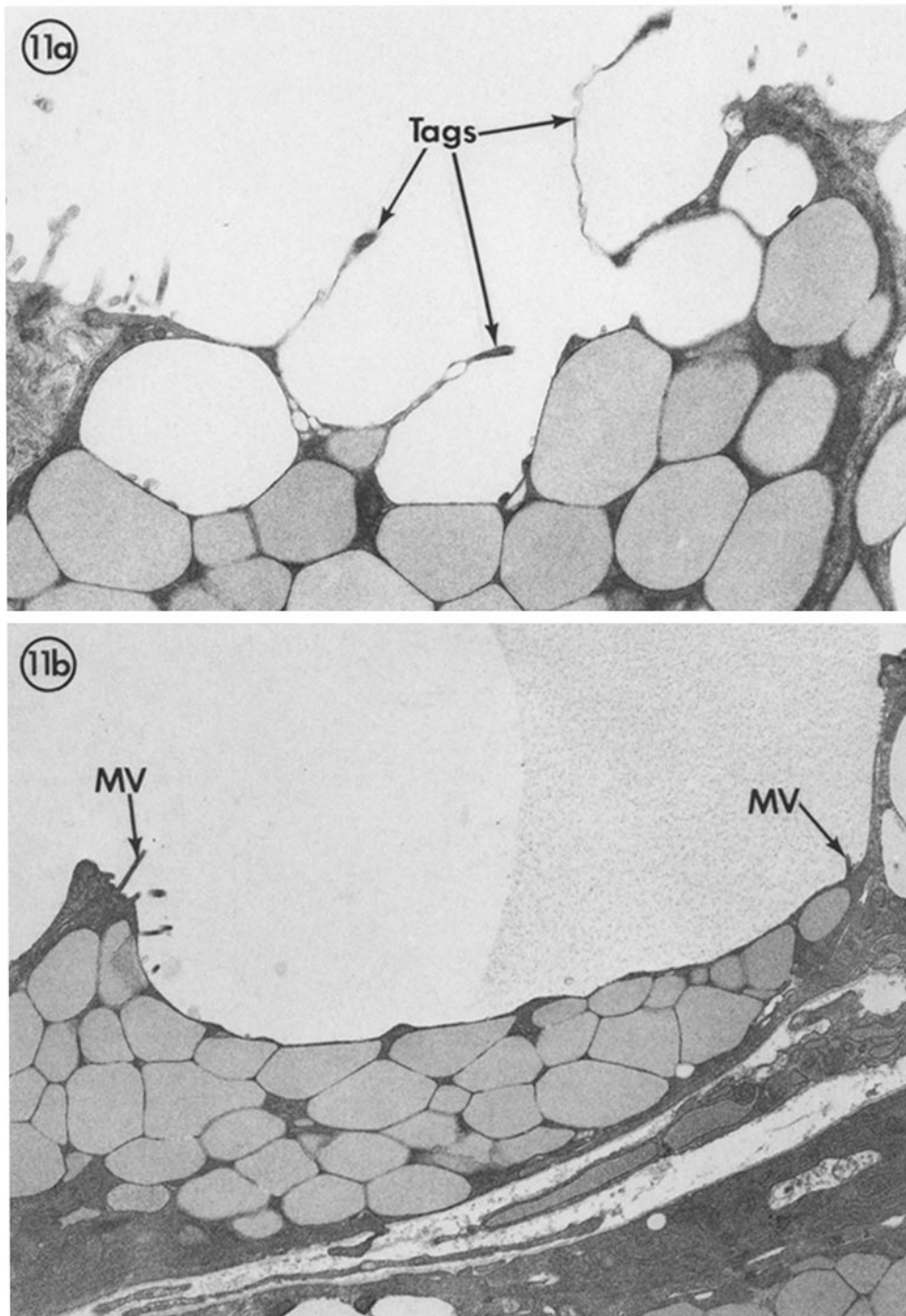


FIGURE 11 (a) ACh-esterine, 30 min. Following accelerated mucus secretion, tags of cytoplasm bounded by granule membranes are isolated on the cavitated surface of goblet cells. $\times 14,100$. (b) ACh-esterine, 60 min. After prolonged secretion, goblet cells are greatly shortened, and the apical membrane-bound tags have disappeared. Microvilli (MV) are restricted to the rim of the apical plasma membrane. $\times 8,000$.

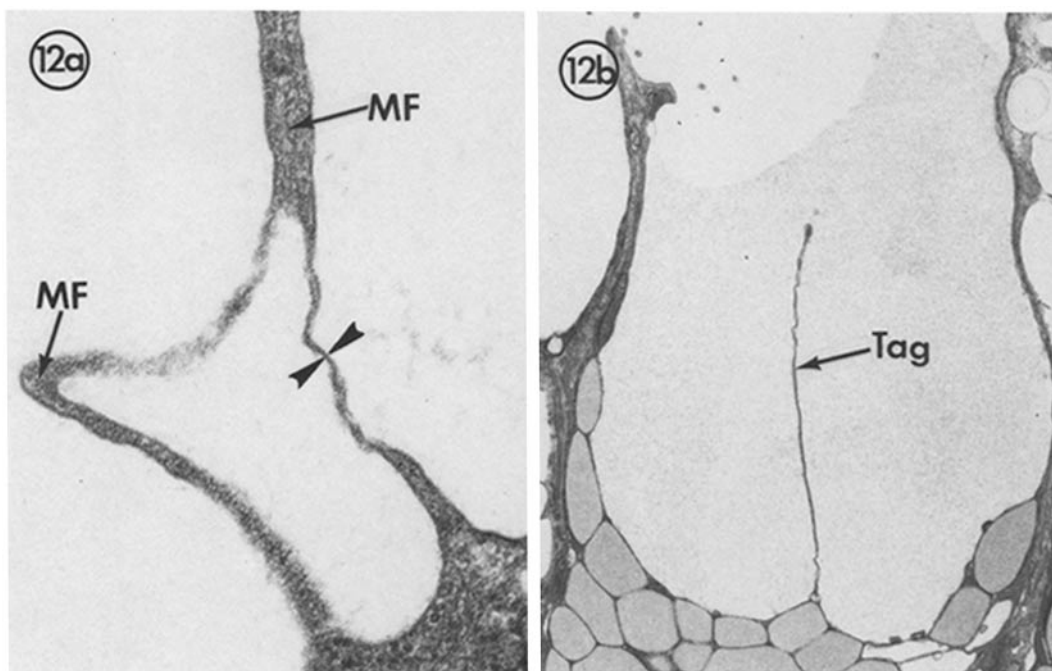


FIGURE 12 (a) ACh-esterine, 60 min. The membrane-bounded remnants of intergranular cytoplasm are thin interconnected sheets in three dimensions, which may appear as empty "cages" after the contents of several adjacent granules have been released. Their cytoplasmic matrix is rich in microfilaments (MF), and their membranes may include intact pentalaminar sites (arrowheads). $\times 67,640$. (b) ACh-esterine, 30 min. A thin sheet, appearing in section as a long, narrow tag of cytoplasm limited by redundant apical plasma membrane, is tenuously attached to the surface of a half-empty goblet cell. It will rapidly disappear, apparently by sloughing into the crypt lumen. $\times 8,830$.

In the absence of irritants or neurotransmitters, goblet cells are known to secrete mucus slowly and continuously (42, 43), intermittently releasing the contents of a single mucous granule by conventional exocytosis (34, 47, 58). Although short-term transport and secretion rates vary widely among individual goblet cells, average rates within a given species, *in vivo* or in organ culture, are remarkably constant (15, 39, 42, 43). We have shown that this "baseline" secretory activity is not inhibited by atropine in organ culture; thus, it is presumably not due to the release of ACh from nerve endings in the lamina propria. Although the factors that control baseline secretion are unknown, maintenance of a slow, continuous secretory rate in the normal mucosa may ensure the constant renewal of the mucus coat, which cushions against particulate matter (16, 19), adsorbs microorganisms (22, 23), and binds toxins and antigenic macromolecules (59). An acute threat to the lumen (chemicals, trauma) would directly induce a local, rapid release of a limited amount of fresh mucus from

surface goblet cells. Further local or systemic challenge could evoke the massive release of the mucus reserves from the intestinal crypts by inducing ACh release from parasympathetic nerve endings.

Although baseline mucus secretion is clearly merocrine, the exact mode of rapid mucus release from stimulated goblet cells has long eluded discovery, largely due to the lability of mucous granule membranes during fixation and tissue processing. Several ultrastructure studies describe massive apocrine mucus secretion involving rupture of the apical plasma membrane and outpouring of whole mucous granules, membranes, and cytoplasmic fragments (20, 29, 33). The cell is said to recover from this catastrophe by rapid coalescence of reserve intracellular membrane vesicles to form a new apical plasma membrane (20, 33). In the light of our recent understanding of rapid secretion in other cell types, however, true apocrine secretion seems a needlessly radical and costly solution.

In thin sections of aldehyde-fixed, normal goblet cells (44), as in other mucous cells (33, 57), exten-

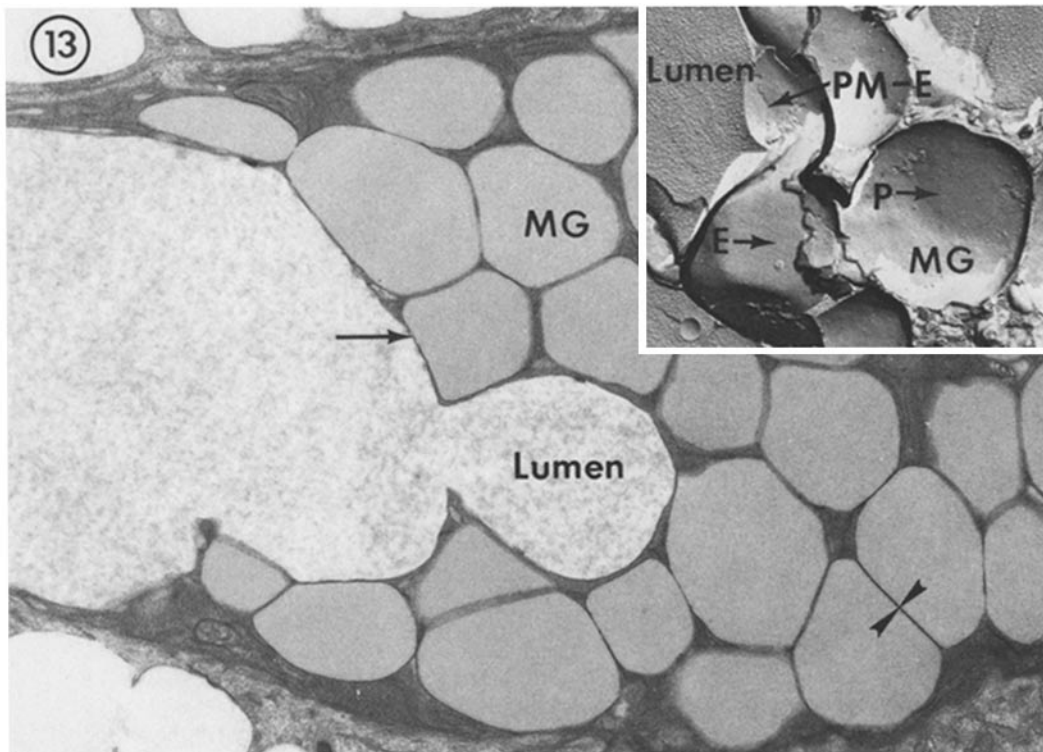


FIGURE 13 ACh-esterase, 30 min. The crypt lumen extends deep into a secreting goblet cell. Areas of close membrane contact representing potential fusion sites are seen between the plasma membrane and a mucous granule (arrow), and broad pentalaminar fusion sites are seen between adjacent intracellular mucous granules (arrowheads). $\times 14,900$. (Inset) ACh-esterase, 30 min. A freeze-fracture replica of a comparable goblet cell reveals the internal membrane faces of several intracellular mucous granules (MG). Particle-poor areas on both the P and E faces correspond to pentalaminar fusion sites (arrows). A small particle-poor area of plasma membrane E face (PM-E) is closely apposed to a mucous granule membrane. $\times 19,200$.

sive pentalaminar contact or fusion sites are seen between adjacent mucous granule membranes. In freeze-fracture replicas these contact sites are largely devoid of intramembrane particles (44); thus, they resemble the transient fusion sites seen during stimulated exocytosis in other cell types. In goblet cells, however, these contact areas appear to be relatively stable. Inasmuch as autoradiographs have shown that mucous granules within a single cell migrate upward at different rates (Fig. 3b, Reference 42), the membrane contact must be reversible and would not invariably lead to membrane fission and secretion in the unstimulated cell. In any case, the presence of membrane contact sites prompted us to speculate that this cell may be "primed" for rapid secretion, and that the appropriate secretagogue would trigger multiple, perhaps simultaneous, membrane fission, releasing

large quantities of mucus without rupture of the apical plasma membrane. The fact that the contents of adjacent mucous granules may be separated only by relatively protein-poor lipid bilayers, without a stabilizing layer of cytoplasmic matrix, would explain the tendency of mucous granule membranes to rupture during fixation, leading to the artifactual intracellular coalescence commonly observed.

We have shown in this study that during ACh stimulation, intestinal goblet cells efficiently release their stored secretion product neither by rupture of the apical membrane nor by indiscriminate breakdown of intergranular fusion sites but by an orderly series of membrane fission events, which begin at the apical plasma membrane, proceed first to the most central mucous granules, are then propagated to include peripheral mucous

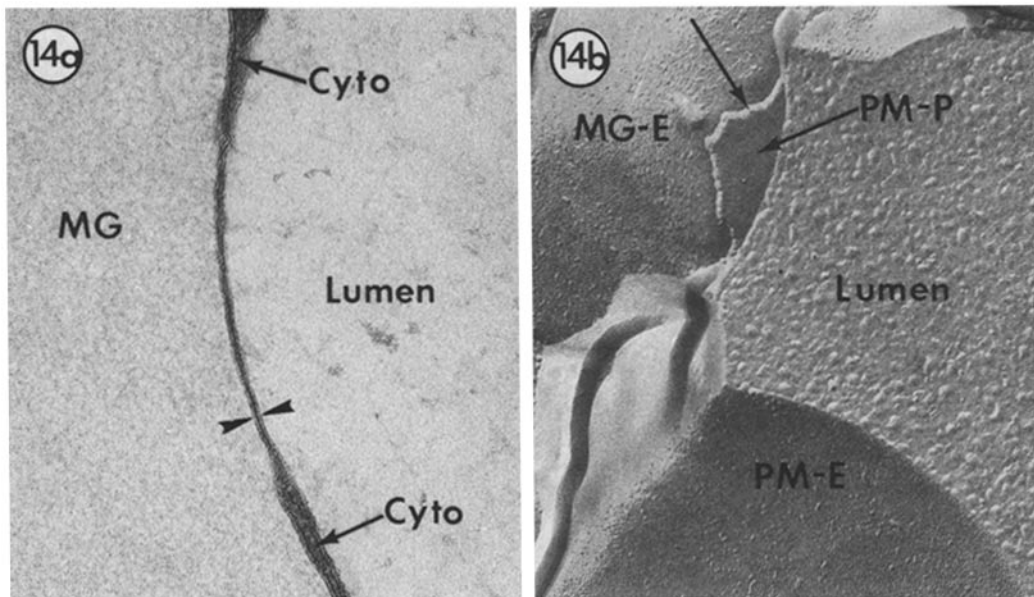


FIGURE 14 (a) Thin section of a deeply cavitated goblet cell apical membrane. The membrane of a mucous granule (*MG*) and the amplified plasma membrane form a pentalaminar fusion site (arrowheads), from which cytoplasm (*Cyto*) is excluded. This fusion site may originally have formed between adjacent intracellular mucous granules. $\times 64,300$. (b) Freeze-fracture replica of an area comparable to the area shown in *a*. The E face of an intracellular mucous granule (*MG-E*) is in close contact with a fragment of plasma membrane P face (*PM-P*). Although the "step" from *PM-P* to *MG-E* (arrow) is too large to assume direct membrane contact, the lack of intramembrane particles on *PM-P* suggests that an interaction may have occurred. *PM-E*, plasma membrane E face. $\times 62,000$.

granules, and finally spread to the most basal of the stored mucous granules. The response may be halted at any point by withdrawal of ACh and is completely inhibited by atropine. When secretion is arrested by fixation, the newly incorporated granule membranes in the highly redundant surface are seen to carry particle-poor, pentalaminar fusion sites, which may mark the location of the next membrane fission with the nearest subjacent mucous granule.

The presence of pentalaminar, particle-poor sites of apparent membrane fusion in fixed cells has been interpreted by us (44) and by others (1, 3, 5, 8, 11, 21, 25, 35, 37, 38, 40, 45-48, 50, 52, 57) as a sign of the real interaction of two lipid bilayers and the lateral movement of intramembrane particles that accompanies the fusion-fission process in the living cell. More recently, however, the application of rapid-freeze techniques to secreting cells has shown that such broad pentalaminar profiles may be artifacts that are formed during the relatively slow chemical fixation process (7). The stimulated goblet cells in this study were located in intestinal crypts and, thus, could not be

rapidly frozen. Nevertheless, when unstimulated, intact surface goblet cells of mouse and rabbit large intestinal mucosa were rapid-frozen, freeze-substituted, and examined in thin sections, broad pentalaminar membrane contact sites were observed between adjacent mucous granules. Freeze-fracture replicas of rapid-frozen rabbit colonic surface goblet cells showed that these contact sites are generally particle free. Pentalaminar profiles were also observed in crypt and surface goblet cells fixed in glutaraldehyde-OsO₄, a technique thought to minimize artifactual membrane fusion and vesicle formation (24, 49). Thus, unlike the secretion granules of other cell types, the mucous granule membranes in the living goblet cell may be capable of undergoing some initial step in the fusion process, even in the absence of stimulation by secretagogues. In any case, it is clear that these membranes are highly "fusible" and that very little, if any, intergranular cytoplasmic matrix is present *in vivo* to prevent intergranular membrane fusion.

Nonstimulated mast cells, serous exocrine cells, and endocrine cells differ from mucous cells in

that their secretion granules are generally separated by intervening cytoplasmic matrix. In these cells, the transient intergranular membrane fusion sites, which appear as pentalaminar profiles in thin sections, have been observed only during rapid, stimulated secretion. In mast cells, when these sites break down, the remaining tandemly fused membranes form long channels, which appear as deep, complex invaginations in the plasma membrane (5, 8, 35, 37). Similarly, stimulated parotid acinar cells incorporate multiple serous granule membranes into the apical plasma membrane during rapid secretion (2). A freeze-fracture study of parotid cells showed that those portions of the expanded apical membrane donated by secretion granules, recognizable by a paucity of intramembrane particles, may be selectively endocytosed and recovered as the cell resumes its baseline state (10, 40). Evidence of membrane retrieval was seen in thin sections as an abundance of small, smooth, membrane-bounded vesicles. More recently, elegant demonstrations of membrane retrieval after secretion in endocrine and in other cell types have established that many secretory cells efficiently recycle the limiting membranes of their secretion granules directly back into the secretory or lysosomal pathway (14, 26; for a review, see reference 40). These studies imply that little or no membrane is lost during secretion or recovery.

Although the goblet cell apical membrane is also dramatically amplified by incorporation of mucous granule membranes, the increase in surface area is not nearly sufficient to account for the original collective membrane surface area of the secreted granules. Small cytoplasmic vesicles are present (Figs. 11a and 12b) but are not numerous in secreting goblet cells. Because macromolecular tracers do not readily enter the mucus- and fluid-filled crypts, we have thus far been unable to determine the extent of endocytosis from goblet cell apical surfaces, and the possibility remains that some portion of the redundant apical membrane may be retrieved. On the other hand, a preliminary stereological analysis of these cells showed that during a 60-min period of ACh-induced secretion, large amounts of the original mucous granule membranes are lost (53) along with a significant volume of intergranular cytoplasmic matrix (unpublished observations).

These losses could most readily be explained by a rapid shedding into the lumen of the membrane-limited tags of intergranular cytoplasm from the

apical surface of the secreting cell. It would follow that the apical plasma membrane of a recently emptied goblet cell is derived largely from portions of peripheral, and not central, mucous granules. Thus, although rapid goblet cell secretion is accomplished by an extension of conventional exocytosis, it seems to be accompanied by a selective loss of mucous granule membranes and intergranular cytoplasm.

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