Mechanism of Brush Border Contractility Studied by the Quick-freeze, Deep-etch Method

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ABSTRACT   We have analyzed terminal web contraction in sheets of glycerinated chicken small intestine epithelium and in isolated intestinal brush borders using a quick-freeze, deep-etch, rotary shadow replication technique. In the presence of Mg-ATP at 37°C, the terminal web region of each cell in the glycerinated sheet and of each isolated brush border became severely constricted at the level of its zonula adherens (ZA). Consequently, the individual brush borders rounded up, splaying out their microvilli in fanlike patterns. The most prominent ultrastructural changes that occurred during terminal web contraction were a dramatic decrease in the diameter of the circumferential ring composed of a bundle of 8–9-nm filaments adjacent to the zonula adherens and a decrease in the number of cross-linkers between the microvillus rootlets. Microvilli were not retracted into the terminal web. We have used myosin S1 decoration to demonstrate that most of the circumferential bundle filaments are actin and that the actin filaments are arranged in the bundle with mixed polarity. Some filaments within the bundle did not decorate with myosin S1 and had tiny projections that appeared to be attached to adjacent actin filaments. Because of their morphology and immunofluorescent localization of myosin within this region of the terminal web, we propose that these undecorated filaments are myosin. From these results, we conclude that brush border contraction is caused primarily by an active sliding of actin and myosin filaments within the circumferential bundle of filaments associated with the ZA.

The brush border of epithelial cells in the small intestine is composed of highly differentiated cellular cortices. The relative ease with which brush borders can be isolated has facilitated correlating biochemical with ultrastructural studies in order to better understand the cytoskeletal structures of the brush border (28).

Previous studies have shown that contractile proteins, such as actin, myosin, and light chain kinase of myosin are all constituents of the brush border (3, 7, 23, 30). In addition, two in vitro phenomena suggested that brush borders might move in vivo. One was a shortening of microvilli after incubation with Ca^{++}-ATP (26) and the other was a "pinching in" of the terminal web at the level of the zonula adherens in the presence of ATP (37).

Recently, it has been proved that the former is due to solation of actin core bundles by the Ca^{++}-sensitive protein villin or MV-95K (6, 11, 25, 27). Also, the quick freeze-deep etch (QF-

DE) studies have shown that the terminal web does not have a suitable structural basis for retracting microvilli into the terminal webs (17, 20).

On the other hand, Hirokawa et al. (20), and Burgess and Prum (5) have hypothesized that a "pinching-in" of the terminal web could be caused by a constriction of the actin bundle inside the zonula adherens. A similar kind of constriction of a circumferential actin bundle inside the zonula adherens has been observed in the glycerinated pigmented epithelium of the retina after adding ATP (32, 33). Furthermore, Keller and Mooseker (23) have recently found that severe constriction of the terminal web occurs at the level of the zonula adherens in the isolated chicken brush borders most prominently in the presence of Ca^{++} (1 μM) and ATP, and that at the same time there is a Ca^{++}- and calmodulin-dependent phosphorylation of the light chain of brush border myosin. (After this manuscript was submitted Burgess [4] also reported, independently, that

contraction occurs in glycerinated intestinal epithelial cells.)

Nonetheless, it is still not clear how the contractile proteins interact with each other to generate forces for this contraction. In this study, we have attempted to find the structural bases for understanding the mechanism of this contraction in brush borders by analyzing both glycerinated whole epithelium and isolated brush borders with the QF-DE method. We have found that the circumferential ring inside the zonula adherens is composed of actin of mixed polarity and small myosin filaments. Our results strongly suggest that contraction of the brush border in the presence of ATP is primarily due to contraction of this circumferential filament bundle by an actomyosin-sliding mechanism.

Results similar to those reported here have appeared in preliminary form (18).

MATERIALS AND METHODS
Glycerination of Sheets of Epithelial Cells

Small intestines were dissected out from chicks (1–2 wk) and the lumens were washed with Ca^{++}-free physiological saline (155 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 3 mM EGTA, and 5 mM HEPES, pH 7.4). The luminal surface was scraped with a cover glass to obtain large sheets of epithelial cells. They were incubated with 50% glycerol, 100 mM KCl, 5 mM EDTA, 10 mM phosphate buffer, pH 7.0 for 24 h at 4°C (32).

Brush Border Isolation

Brush borders were isolated from chicken epithelial cells as described by Keller and Mooseker (23). Instead of rigorously homogenizing the fragments of epithelial sheets to get small clusters of isolated brush borders, we gently homogenized the fragments to obtain larger sheets of 10–25 brush borders that were still connected by their intercellular junctions.

Contraction Experiments

GLYCERINATED WHOLE EPITHELIAL CELLS: Glycerinated whole epithelial cells were washed with 100 mM KCl, 5 mM EDTA, 10 mM phosphate buffer for 30 min on ice. They were incubated with 3 mM ATP, 5 mM $MgCl_2$, 100 mM KCl, 10 mM phosphate buffer or 3 mM ATP, 5 mM $MgCl_2$, 2 mM EGTA, 100 mM KCl, 10 mM phosphate buffer pH 7.0 at 37°C for 5–10 min.

Controls were incubated with solutions without ATP. Some of the glycerinated epithelia were incubated with 10 mM NEM, 100 mM KCl, 10 mM phosphate buffer for 30 min on ice after washing. They were then transferred to the solution that contained 10 mM NEM, 5 mM $MgCl_2$, 3 mM ATP, 100 mM KCl, 10 mM phosphate buffer, pH 7.0, for 5–10 min at 37°C. After incubation, the epithelia were fixed by adding 70% glutaraldehyde to get a final concentration of 1% glutaraldehyde for 1 h on ice. Some of them were quick frozen and the others were observed with a phase-contrast light microscope or were postfixed with 1% OsO_4 in 10 mM phosphate buffer, pH 6.8, for 45 min, dehydrated, embedded, and processed for thin sectioning.

ISOLATED BRUSH BORDERS: The brush borders were incubated at 37°C in a buffer containing 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM DTT, 25 mM piperazine-N,N'-bis [2-ethane sulfonic acid] (PIPES) pH 6.9. This solution also contained Ca^{++} buffers consisting of ratios of $CaCl_2$ to EGTA of either 0.1 mM ($-Ca^{++}$, $<10^{-8}$ M free Ca^{++}) or 0.9 mM:1 mM ($+Ca^{++}$, 1×10^{-6} M free Ca^{++}) and either no added ATP ($-$ATP) or 2 mM ATP ($+$ATP).

From the ratios of Ca^{++} and EGTA added and the pH of the solution, the final concentrations of free Ca^{++} were calculated by the method of Portzehl et al. (36). Contraction was assayed by resuspending brush borders in ice-cold buffer and immediately transferring them to a 37°C water bath. The brush borders were fixed by adding 8% glutaraldehyde to get a final concentration of 1% glutaraldehyde.

Decoration of Brush Borders by S1 Fragments of Myosin

Large sheets of brush borders were incubated with S1 of myosin (1 mg/ml) in stabilization buffer (70 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole, 0.25 mM PMSF) for 10 min at 20°C, washed and fixed with 1% glutaraldehyde plus 0.2% tannic acid in stabilization buffer for 1 h at 20°C.

Quick Freeze, Deep Etch, Rotary Shadowing

Samples were washed with distilled water after fixation and were quick frozen with a liquid-helium-cooled machine and freeze-fractured in a Balzers apparatus (Balzers, Hudson, NH) as previously described (15, 17). Deep etching was carried out at $-95°C$ for 5 min. Replicas were made by rotary shadowing with a mixture of platinum and carbon. The replicas were cleaned in chrome sulfuric acid for several hours and then picked up on Formvar-carbon-coated 75-mesh grids. They were examined with a JEOL 100CX or 200CX electron microscope operated at 100 kV and photographed in stereo at $\pm10°$ tilt.

Immunocytochemistry of Myosin

Isolated large sheets of brush borders or glycerinated sheets of epithelial cells were prefixed with 1% formaldehyde in stabilization buffer for 1 h on ice. Some of the samples were incubated with 3 mM ATP and 5 mM $MgCl_2$, for 5–10 min at 37°C and were fixed with the same fixative. The samples were washed with stabilization buffer and were incubated with 50-fold diluted anti-human platelet myosin rabbit serum or preimmune serum kindly provided by K. Fujiwara (Dept. of Anatomy, Harvard Medical School) (9) for 1 h at 20°C. They were washed and incubated with 50-fold-diluted fluorescein-labeled anti-rabbit IgG goat IgG for 1 h at 20°C. Then they were mounted on a glass slide and observed with a Leitz fluorescence microscope.

Solubilization of Proteins During Contraction

Protein solubilization in glycerinated cells was assayed by resuspending the cells in 100 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM phosphate buffer, pH 7.0 containing 3 mM ATP and either 0.9 mM $CaCl_2$ or no added $CaCl_2$ and incubating the cells at 37°C. After 3, 5, and 10 min of incubation, the samples were centrifuged at 15,000 g for 1 min and aliquots of the pellets and supernatants were mixed with Laemmli SDS-sample buffer (24) containing 1 mM EGTA and immediately boiled for 1 min. The samples were then electrophoresed on SDS (linear 5–15%) polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue by the method of Fairbanks et al. (8).

RESULTS
Light Microscopic Observation of Brush Border Contraction After Incubation with ATP

After glycerination of large sheets of epithelial cells, the cells remained attached to each other by the junctional complexes. With phase-contrast microscopy, the microvilli and terminal web regions of these epithelial cell sheets looked like a continuous belt (see Fig. 1 A). This morphology dramatically changed after incubation with 3 mM Mg^{++} ATP for 5–10 min at 37°C. Severe constriction of individual brush borders within the sheets occurred at the level of their zonula adherens, resulting in an extensive decrease of cell diameters at this level (Fig. 1 B, F, and G). The junctional complexes detached from each other and the brush border regions of individual cells rounded up, splaying out their microvilli in fanlike patterns (Fig. 1 B, F, and G). Since the contracted cells remained attached below the level of their apical junctional complexes, they still formed large sheets of cells (Fig. 1 B and F).

Incubation of glycerinated cell sheets with a solution lacking ATP did not induce any morphological changes (Fig. 1 A, D, and E). We can appreciate this by comparing cross sections of sheets of epithelial cells after incubation with or without ATP (Fig. 1 E and G). The morphological changes that occur during incubation in ATP are equivalent to those observed in large fragments of isolated brush borders (23), but in the sheets of glycerinated epithelia, brush borders contracted equally well in the presence or absence of Ca^{++}.

On electron micrographs, we measured the largest diameter of 50 cells at the zonula adherens level after incubation with or without ATP. The diameter of each brush border was decreased by an average of ~49% after incubation with ATP. This contraction was inhibited by preincubating samples with 10 mM N-ethylmaleimide (Fig. 1 C).
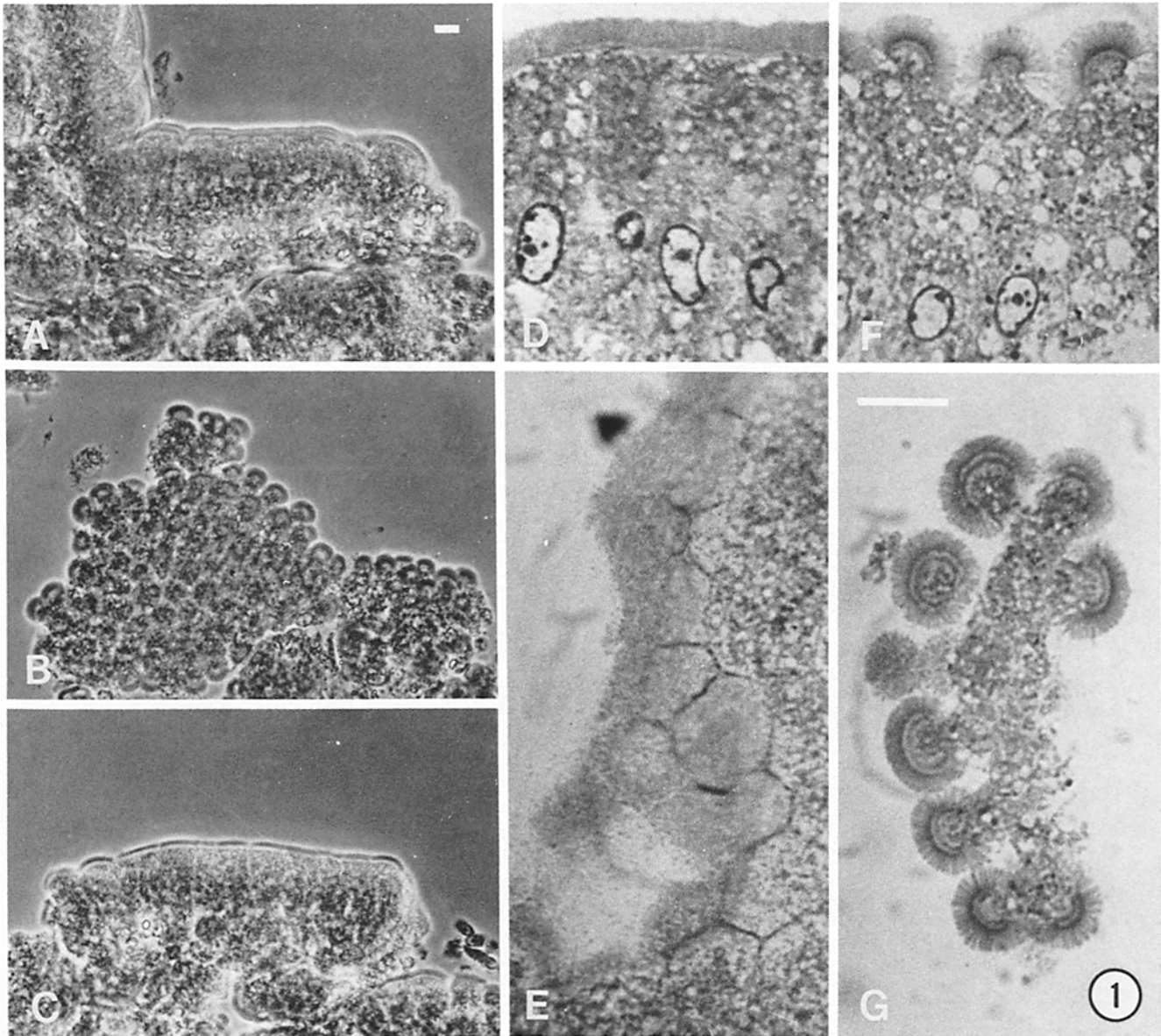
FIGURE 1 Light micrographs of sheets of glycerinated epithelial cells. (A) A phase-contrast light micrograph. Cells were incubated with no ATP at 37°C for 10 min. The epithelial cells are attached to each other by junctional complexes to form flat sheets. (B) A phase-contrast light micrograph. Cells were incubated with 3 mM Mg^{++}-ATP at 37°C for 10 min. Remarkable contraction occurs within the brush borders. The apical portions of the cells detach from each other and round up although the cells remain associated with each other basally. (C) A phase-contrast light micrograph. Cells were preincubated with 10 mM NEM and then incubated with 3 mM Mg^{++}-ATP plus 10 mM NEM at 37°C for 10 min. No contraction occurs. (A–C) Bar, 10 μm, × 400. (D–G) Light micrographs of 1-μm sections embedded in araldite. Bar, 10 μm; × 1,400. (D and E) Cells are incubated with no ATP at 37°C for 10 min. The epithelial cells are hexagonally packed and attached with each other by junctional complexes. (D) Longitudinal section. (E) Cross section. (F and G) Cells are incubated with 3 mM Mg^{++} ATP at 37°C for 10 min. Remarkable constriction occurs at the level of zonula adherens. The brush borders round up and junctional complexes are fallen apart. The cell diameter at the zonula adherens level is reduced prominently. (F) Longitudinal section. (G) Cross section.

Fluorescence Microscopy of Antimyosin Antibodies Before and After ATP Treatment

To determine whether myosin is present in the glycerinated cell sheets and, if so, where it is localized in the brush borders before and after ATP treatment, we used indirect immunofluorescence microscopy. In both glycerinated epithelial cells and isolated brush borders myosin was detected throughout the terminal web, but was concentrated near the junctions between adjacent cells before incubation with ATP. Fig. 2a shows the localization of myosin in a typical sheet of 50–100 cells hexagonally or pentagonally connected to each other before ATP treatment. As seen in this *en face* view, each cell displayed bright staining at its apical boundary and in terminal web regions (Fig. 2a).

After incubation with ATP the staining pattern with antimyosin was drastically changed. As shown in Fig. 2b antimyosin stained the terminal web region as small separated rings even though the cells were still attached to each other at a more basal level. The rest of the terminal web region was
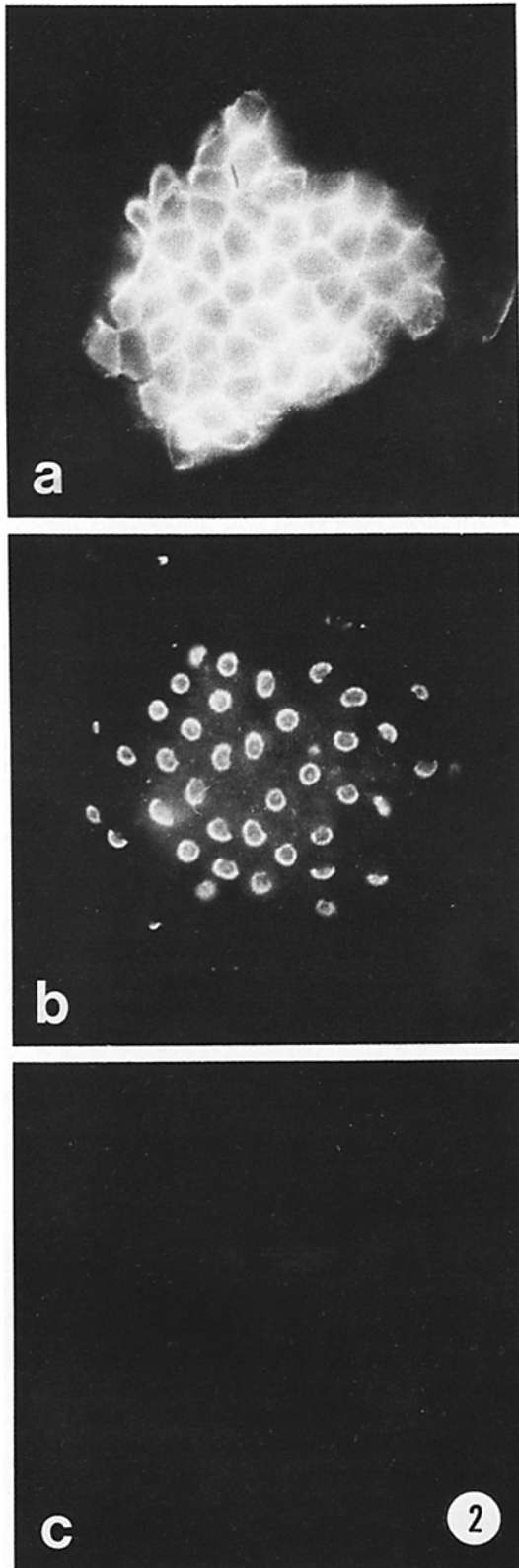
also stained as can be seen in the side views of some of the brush borders in Fig. 2b, but there appears to be an overall loss of fluorescence staining from the terminal web after ATP treatment. This figure also demonstrates clearly how drastic the constriction is at the level of the zonula adherens (Fig. 2b). Fluorescence was not detectable before (Fig. 2c) and after ATP treatment (data not shown) in the brush borders incubated with preimmune serum.

Quick-Freeze, Deep-Etch Study of the Glycerinated Epithelial Cells and Isolated Brush Borders after ATP Treatment

The cytoplasm of the terminal webs in whole nontreated epithelial cells is filled with granula material, probably soluble proteins, making it difficult to observe the cytoskeletal architecture clearly (17). Fortunately, glycerination removes all of the granular material in the cytoplasm allowing a clear visualization of the cytoskeletal elements (Fig. 3). Although the microvillus rootlets are much longer and the system of cross-linking elements between rootlets is denser, the basic structure of the terminal web of the chicken intestinal epithelial cells appears to be similar to that of the mouse brush borders previously described (17, 20). The cross-linkers have various diameters and form a strikingly dense and complicated network between the straight microvillus rootlets (Fig. 3). Inside the zonula adherens there is a circumferential bundle of 8–9 nm filaments tightly packed together (22) (Fig. 4A) and connected by thin (~5 nm), short (~20 nm) strands (Fig. 4B). Some of the circumferential filaments are anchored into a dense meshwork of nonetchable structures inside the zonula adherens membrane (Fig. 4A and B).

To analyze the components of this circumferential bundle, we treated isolated brush borders with myosin S1 in order to determine which components were composed of actin. Although the cross-linkers between rootlets were removed by S1 decoration, the rootlet actin filaments were decorated showing a characteristic double helical structure. Moreover, a careful examination of the surface of the helices shows that the gyres are somewhat angular and the asymmetry of each gyre, which appears fatter on one side than on the other, allows us to determine the polarity of the filaments (see reference 19). As shown in Fig. 5, many of the filaments in the circumferential bundle inside the zonula adherens were decorated by S1, proving that they are actin. Careful observation of these decorated samples reveals that the actin filaments within the bundle have a mixed polarity (as indicated by the arrows in Fig. 5A and B).

Another view of the circumferential bundles of the cross-fractured epithelial cells displayed that some of the actin filaments in the circumferential bundles are anchored at the plasma membrane, although the dense meshwork of substances underneath the plasma membrane disappeared after S1 decoration (data not shown). In most of these actin filaments the S1 arrowheads point away from the plasma membrane, indicating

FIGURE 2 Indirect immunofluorescence microscopy with anti-myosin serum and preimmune serum. (a) *En face* view of a sheet of glycerinated epithelial cells stained with antimyosin serum before treatment with ATP. Polygonal epithelial cells are attached to each other by junctional complexes. Antimyosin stains inside the zonula adherens as well as in the terminal web. (b) *En face* view of a sheet of glycerinated epithelial cells stained with antimyosin serum after treatment with ATP. Antimyosin stains constricted regions inside the zonula adherens as rings. The terminal web region is also stained, but the reaction is weaker than before treatment with ATP. Although cells are detached at the apical region, they are attached at a lower level so that they still form a sheet of cells. Note the diameter of cells at the zonula adherens level decreases to less than half after ATP treatment. (c) *En face* view of a sheet of glycerinated epithelial cells stained with preimmune serum before treatment with ATP. No fluorescence is detectable.
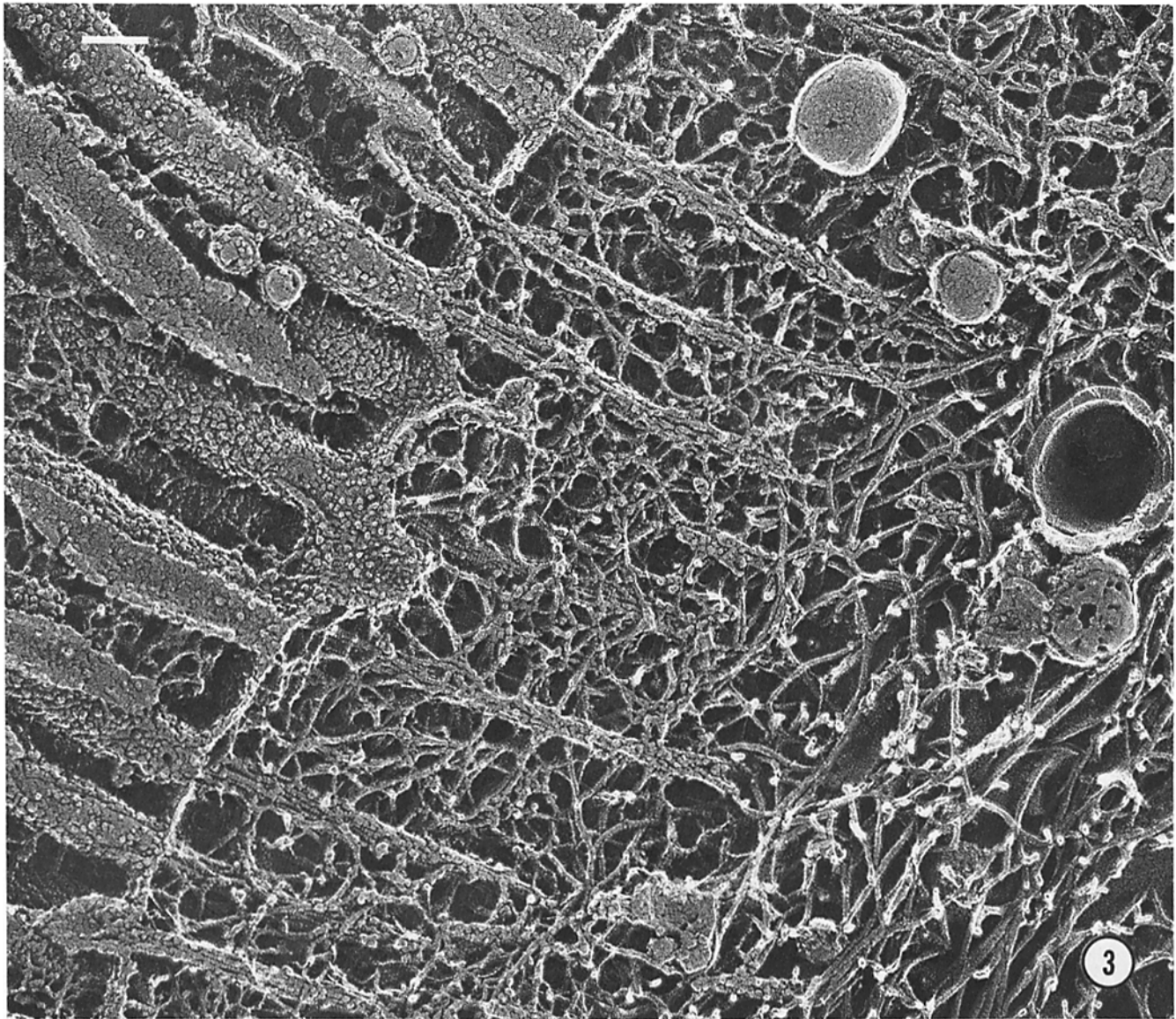
FIGURE 3 Glycerinated epithelial cell incubated with no ATP at 37°C for 10 min. Terminal web region. Long straight rootlets are cross-linked by a dense and complicated network of cross-linkers. Intermediate filaments make a foundation for the rootlets. Bar, 0.1 μm. × 94,000.

that the circumferential actin bundle is anchored to the plasma membrane at many points.

Another important thing about the S1-decorated circumferential bundle is that there are some undecorated filaments (~9 nm in diameter) interposed between decorated actin filaments (Fig. 5 B, C, and D). Many of these undecorated filaments have tiny projections or heads that appear to be attached to adjacent actin filaments (Fig. 5 C), in a manner similar to what we would expect of small myosin molecules. Fig. 5 D shows clearly undecorated filaments interposed between actin filaments of mixed polarity.

After incubation with ATP, the most prominent morphological change was constriction of the terminal web at the level of the zonula adherens. Fig. 6 provides an *en face* view of the circumferential bundle after ATP treatment by the QF-DE method, which demonstrates the pinched-in zonula adherens and associated circumferential filament bundle. Fig. 7 A displays an epithelial cell that has contracted slightly. Although it is evident that the circumferential filament bundle has con-

tracted to decrease the cell diameter at the zonula adherens level, it is difficult to find remarkable changes in the terminal web (Fig. 7 B). However, after severe constriction further morphological changes were detectable in the terminal webs (Figs. 8 and 9). As shown in Fig. 8 the zonula adherens region has extensively "pinched in" and at the same time the distance between rootlets has decreased remarkably at the zonula adherens level, so that the brush border looks like a fan. In the terminal webs of extensively contracted cells, cross-linkers were still present, but there were fewer of them and they appeared to be shorter in length than in the control (Fig. 9). These same morphological changes were also observed in the isolated brush borders incubated with Ca^{++} and ATP.

In direct correlation with terminal web contraction in glycerinated cells, some proteins become soluble and no longer associate with the glycerinated cell structure. This is shown in Fig. 10, which is a Coomassie-Blue-stained SDS polyacrylamide gel of glycerinated epithelial incubated at 37°C in the presence of ATP and in the presence (+Ca^{++}) and absence
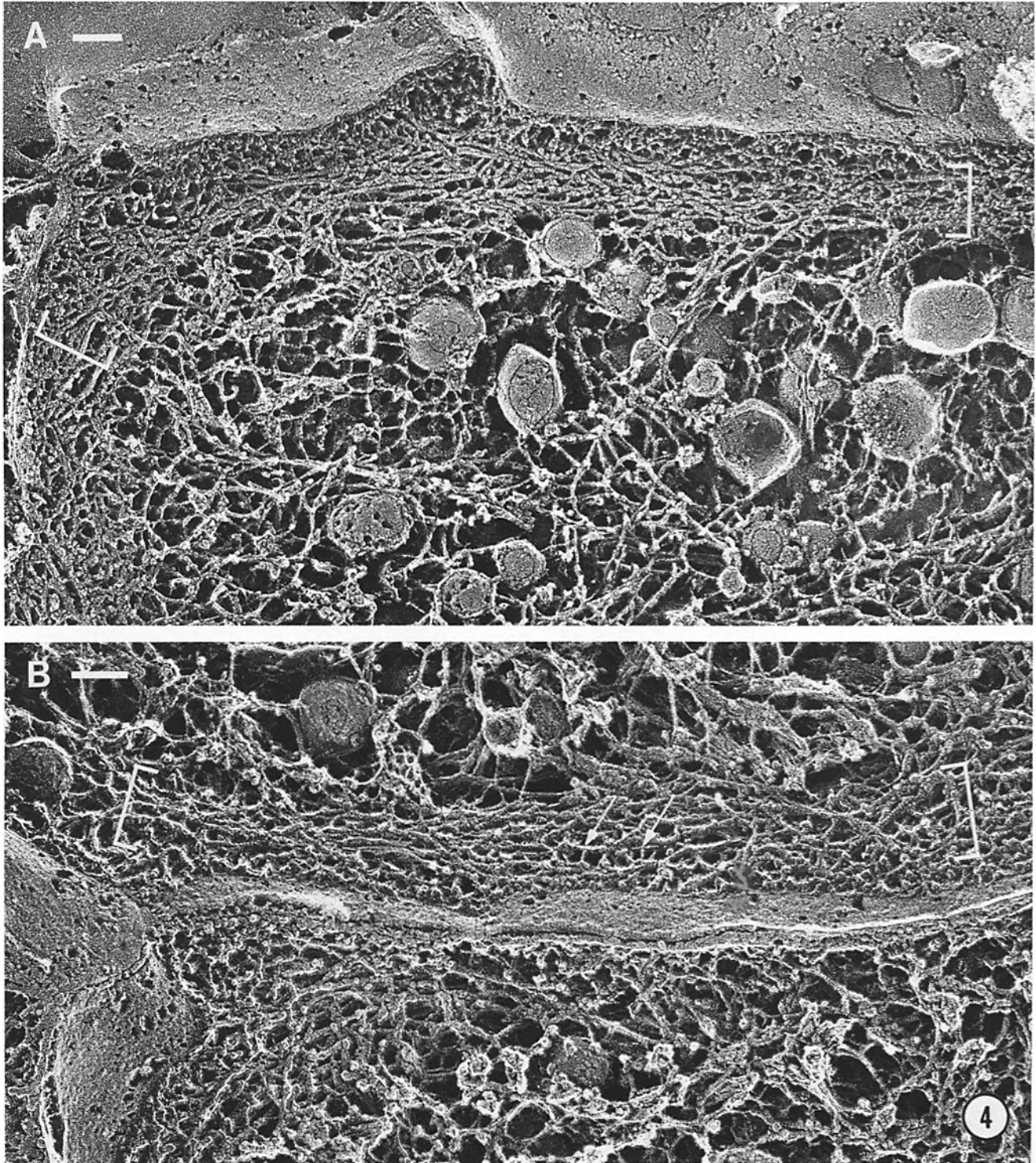
FIGURE 4   Glycerinated epithelial cells incubated with no ATP at 37°C for 10 min. (A) Cross cut of an epithelial cell to show the circumferential filament ring inside the zonula adherens ([   ]). It is composed of 8-9-nm filaments mostly parallel to the plasma membrane. Just underneath the plasma membrane observed is a meshwork of unetchable substances. Some of the circumferential filaments are anchored in this meshwork. Bar, 0.1 μm. × 79,000. (b) Oblique section of epithelial cells to expose inside the tight junction (lower cell) and inside the intermediate junction (upper cell). This micrograph clearly display short thin strands (arrows) between adjacent filaments in the circumferential filaments bundle inside the zonula adherens ([   ]). Bar, 0.1 μm. × 97,000.
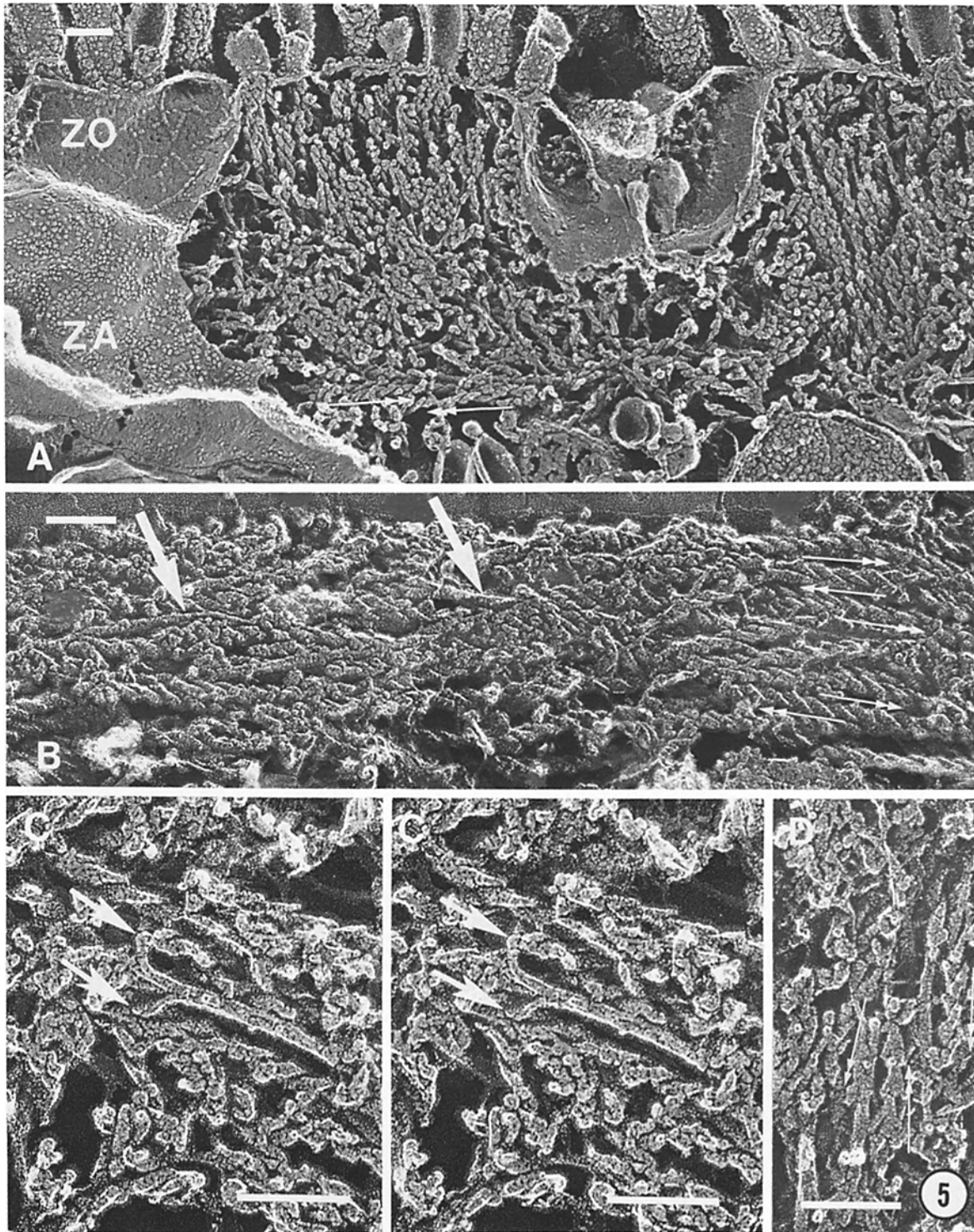
FIGURE 5   Circumferential bundle inside the zonula adherens (ZA) of the S1-decorated brush borders. Bars, 0.1 μm. (A) Circumferential bundle contains many actin filaments that are decorated with S1 and display double helical structure. The actin filaments have mixed polarity. Some of the actin filaments are anchored at the plasma membrane. ZO, Zonula occludens. (B) Higher magnification view to show the mixed polarity of the decorated filaments. The circumferential filament bundle also contain nonactin filaments (thick arrows) that sometimes remain after S1 decoration. (C) A stereo pair showing an undecorated filament projects two tiny heads (arrows) that attach to adjacent actin filaments. (D) Undecorated ~9 nm filaments are interposed between decorated actin filaments having mixed polarity.
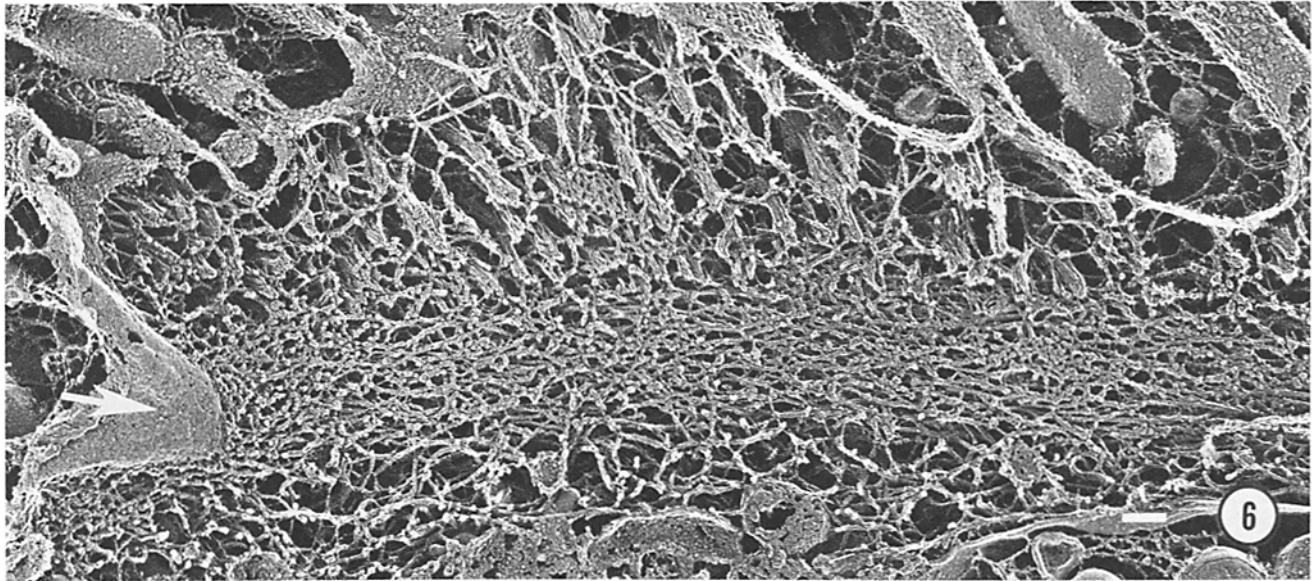
FIGURE 6 A glycerinated cell after Mg^{++}-ATP incubation. The intercellular junctions are split and the zonula adherens region is pinched in. The circumferential filaments bundle inside the zonula adherens is contracted. Bar, 0.1 μm. × 58,000.

($-$Ca^{++}) of 1 μM Ca^{++}. After 3, 5, and 10 min, the epithelial were centrifuged and the pellets (P) and supernatants (S) were electrophoresed. After 10 min of incubation in parallel samples, the extent of terminal web contraction both in the presence and absence of Ca^{++} was similar to that shown in Fig. 1 *B*.

During contraction, some of the myosin is solubilized although most of the TW 260/240, the spectrinlike complex found in the terminal web (12), remains associated with the contracted epithelial cell. This loss of as much as 60% of the myosin after incubation most likely corresponds to the loss of cross-linkers between microvillus rootlets observed under these conditions. There is also a loss of some of the actin and other microvillus proteins, probably indicating loss of some whole microvilli because of the incubation at 37°C.

DISCUSSION

What is the Force-generating Mechanism for Contraction of Brush Borders?

We have observed a severe contraction of terminal webs in both glycerinated sheets of intestinal epithelial cells and in isolated brush borders after incubation with ATP at 37°C. A similar contraction phenomenon was reported by Rodewald et al. (37) in isolated rat brush borders incubated with ATP. Our structural study reveals that contraction remarkably decreases the diameter of the cell at the level of zonula adherens. This terminal web contraction appears to be due to a circumferential bundle of filaments inside the zonula adherens (22). We demonstrate clearly that this bundle is composed of actin filaments of mixed polarity and undecorated ~9-nm filaments interposing between actin filaments. Because it has been shown immunocytochemically by others (3, 5) and by us that myosin exists within this region and because we observe that these undecorated filaments project heads that attach to adjacent actin filaments, it is reasonable to suppose that these undecorated filaments are small bipolar myosin filaments. Since we found severe contraction of this circumferential filament bundle after incubation with ATP, the contraction of this bundle via an actomyosin-sliding mechanism could be the main force-

generating system for the brush border contraction observed here. A similar kind of constriction of the circumferential actin bundle has been reported in the glycerinated pigmented epithelial cells of retina after incubation with ATP (32, 33). Because Hirokawa and Tilney (19) found a circumferential ring of actin filaments of mixed polarity inside the zonula adherens in sensory hair cells of the inner ear and the same kind of structure exists in ciliated epithelial cells of trachea (Hirokawa, unpublished observation), this actomyosin circumferential ring appears to be a general structure for many kinds of epithelial cells.

Although small bipolar filaments have been formed in vitro from platelet myosin (31) and from brush border myosin (29), myosin filaments of comparable dimensions to those in skeletal muscle are not usually found in nonmuscle cells except protozoa (35) and the slime mold Physarum (1). In fact, in many cells, e.g., fibroblasts, macrophages, neurons, leucocytes, the existence of myosin has been unequivocally proven immunocytochemically (14) and biochemically, but no one has shown clearly whether the myosin in those cells forms filaments or is in a globular form. From our findings and a recent study about myosin II by Pollard (34), we assume this is partly because myosin could exist as small bipolar filaments, in many cases having about the same diameter as actin. Thus, it may be impossible to detect myosin in many systems with conventional thin section electron microscopy. Even by the QF-DE method it is difficult to discriminate myosin from actin in undecorated circumferential filament bundles. However, in chicken intestinal epithelial circumferential bundles we found some short thin strands cross-linking filaments. These strands could be either heads of myosin or other actin-binding proteins such as α-actinin, which has been shown with immunocytochemical localization to occur in this region (10). Only after mild S1 decoration, can we unequivocally identify undecorated filaments that possibly are myosin cross-linking actin filaments within the bundle. Thus, we hope that this method can further contribute to the analysis of the structural basis for motility of other nonmuscle cells in future.

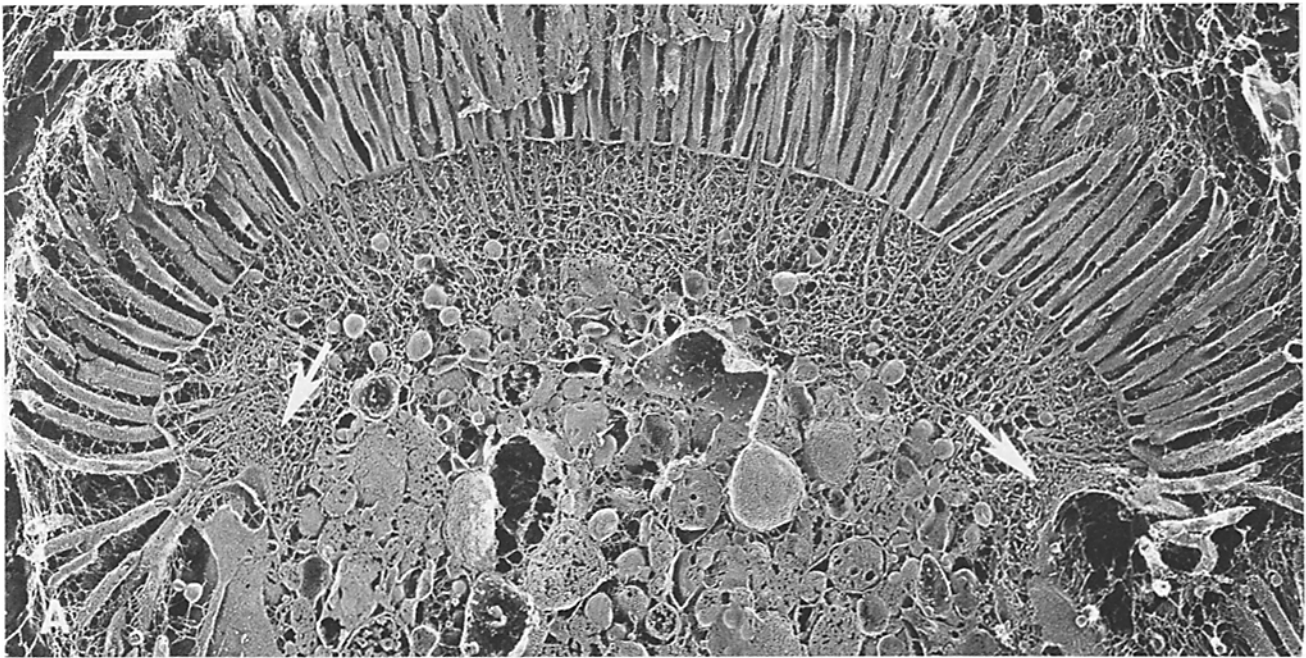The present study revealed another morphological change

FIGURE 7   (A) A glycerinated cell that shows slight contraction after Mg^{++}-ATP incubation. In this cell the zonula adherens region (arrows) is prominently pinched in, but in the terminal web it is difficult to detect remarkable morphological changes. Bar, 0.1 μm. × 16,000. (B) A higher magnification view of a glycerinated cell which shows slight contraction after Mg^{++}-ATP incubation. Although the distance between rootlets has become a little bit narrower at the basal part, the overall density of the cross-linkers is not changed. Bar, 0.1 μm. × 70,000.
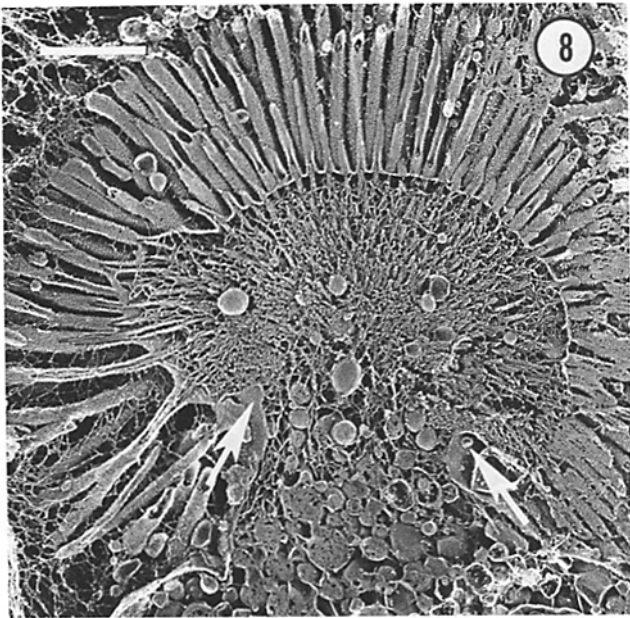
FIGURES 8 and 9

Fig. 8:   A low magnification picture of a severely contracted glycerinated epithelial cell. The zonula adherens (*arrows*) is extensively pinched in and the rootlets are packed very densely in the terminal web. Bar, 0.1 μm. × 14,000. Fig. 9:   The terminal web of a glycerinated cell which has extensively contracted after ATP treatment. The basal ends of the rootlets are closer than in uncontracted brush borders. Although some cross-linkers between adjacent rootlets remain, the cross-linkers are reduced in number. Bar, 0.1 μm. × 104,000.
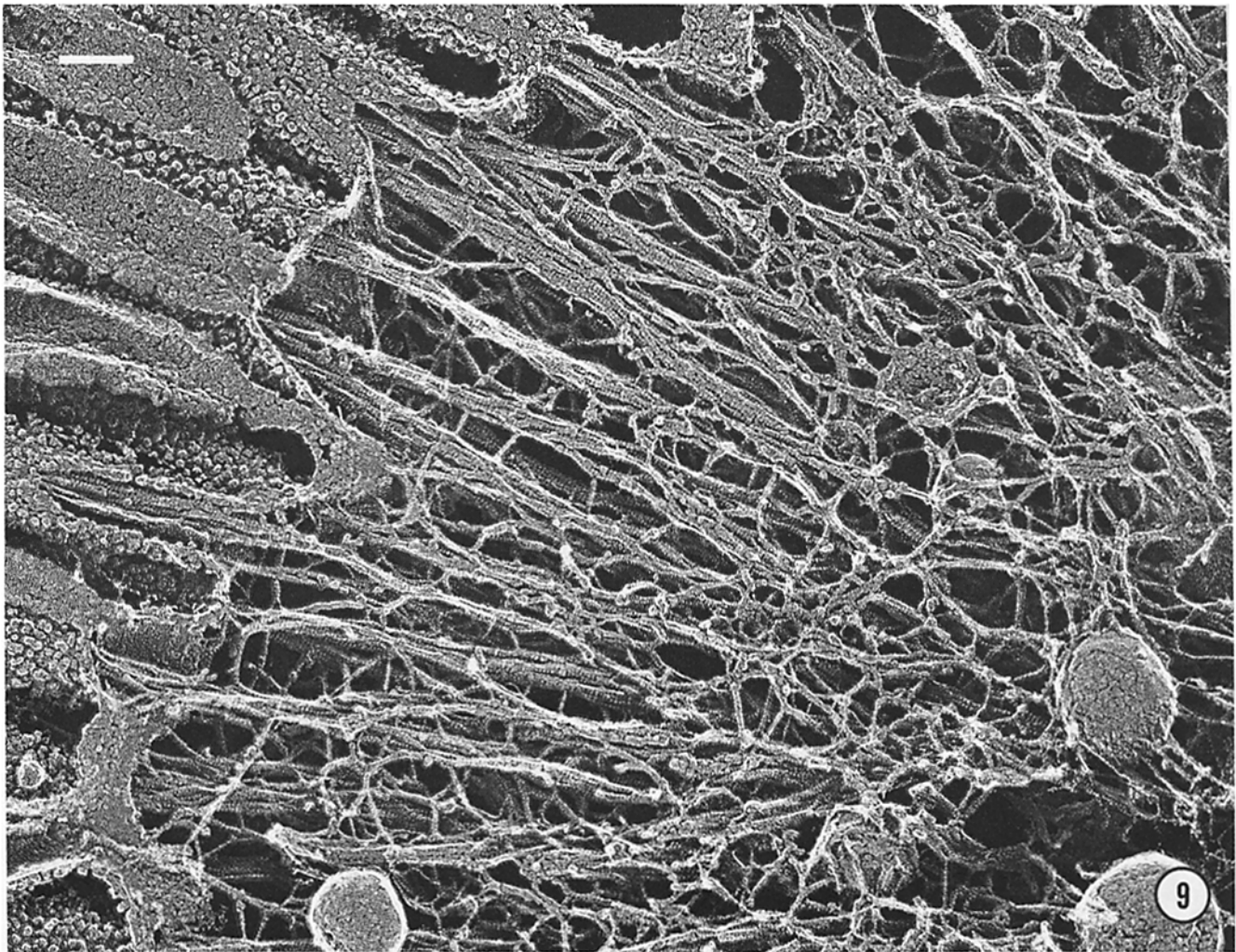
in the terminal web after ATP treatment. In the severely contracted brush borders, we found that the rootlets had gotten closer at the zonula adherens level. Although some short cross-linkers remained between the rootlets, the number of cross-

linkers was decreased. It has been postulated that the cross-linking structures between rootlets are composed mainly of myosin and a spectrinlike, high molecular weight actin-binding protein (12, 16, 20). In the present study, although myosin still
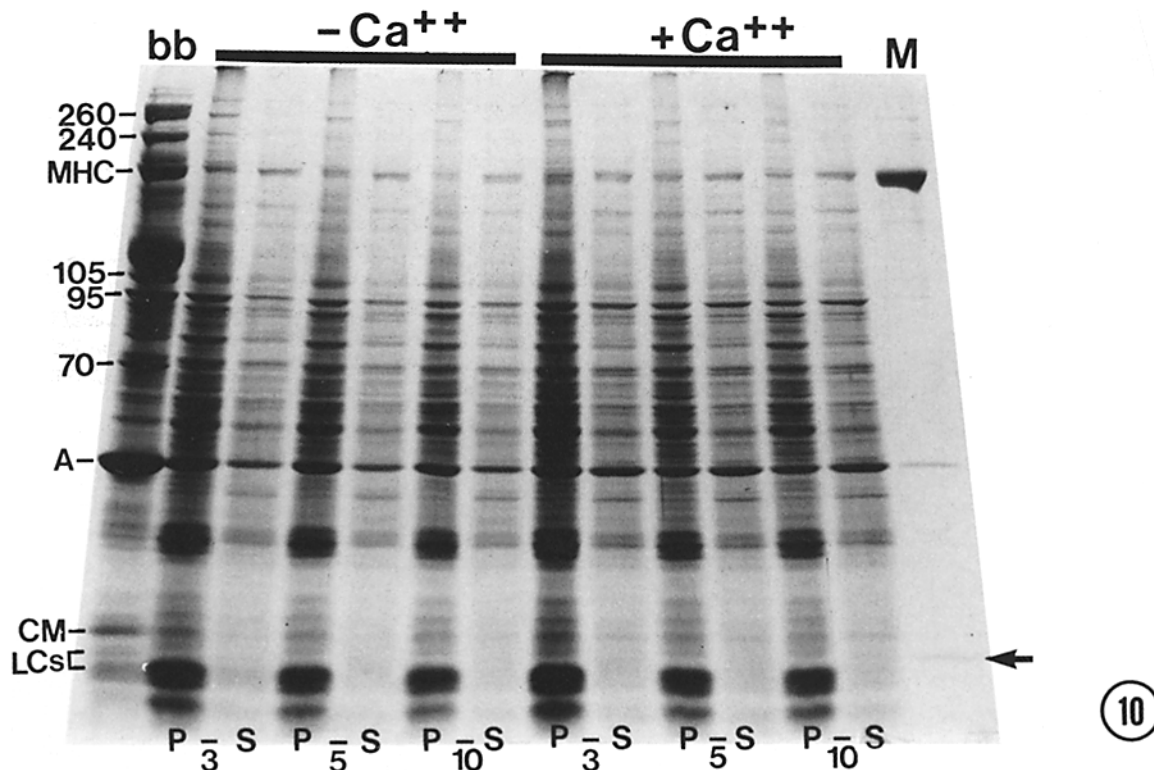
FIGURE 10 Analysis of protein solubilization during terminal web contraction in glycerinated epithelial cells. Sheets of glycerinated epithelia were incubated at 37°C in buffers containing 3 mM ATP and either 1 mM EGTA ($<10^{-8}$ M Ca^{++}, $-Ca^{++}$) or approximately 1 μM free Ca^{++} ($+Ca^{++}$). After 3, 5, and 10 min of incubation, (*3, 5,* and *10*) samples of the glycerinated cells were centrifuged at 15,000 g for 1 min. Samples of the pellets (*P*) and supernatants (*S*) were boiled in SDS, electrophoresed on a SDS (5–15% linear gradient) polyacrylamide gel, and stained with Coomassie Blue. The migration positions of previously identified brush border cytoskeletal proteins—the 260/240-kdalton spectrinlike terminal web protein (*260, 240*), myosin heavy chain (*MHC*), the 105-kdalton (*105*), 95-kdalton (*95*), and 70-kdalton (*70*) microvillus proteins, actin (*A*), calmodulin (*CM*), and the myosin light chains (*LCs*)—are indicated. The first lane is an isolated brush border standard (*bb*) and the last lane (*m*) is a purified brush border myosin standard with the migration position of the 20-kdalton light chain indicated by the arrow. The extent of terminal web contraction monitored in parallel samples both the presence and absence of Ca^{++} was equivalent to that in the glycerinated cells in Fig. 1 B.

existed in the circumferential ring and the terminal web regions after ATP treatment, we found that >50% of the myosin was released into the supernatant after incubation with Mg^{++}-ATP at 37°C. Hence, a possible explanation of the contraction in terminal web is that some of bipolar myosin filaments between rootlets must first be released by incubation with ATP at 37°C before the basal region of the rootlets can be squeezed by the circumferential bundle. Thus, although the circumferential bundle may be solely responsible for brush border contractility (as we have seen in slightly contracted cells), there may be the additional possibility that some reorganization of myosin filaments between rootlets must occur to facilitate terminal web contraction.

Is Ca $^{++}$ Necessary for Brush Border Contraction?

We used both glycerinated epithelial cells and isolated brush borders for these contraction experiments. Each system has advantages and disadvantages. Isolated brush borders, which bear an inherent possiblity that some mechanical distortion of the cytoskeletal elements could be induced during sample preparation, are superior for biochemical analysis. On the other hand, glycerinated epithelial cells are relatively free from mechanical distortion during preparation and therefore may give a more realistic representation of contraction as it might occur in a sheet of epithelial cells.

In the present study, the structural data from both systems coincide well with each other. However, there was a prominent difference between two systems. In the isolated brush border the contraction was Ca^{++}-dependent and phosphorylation of the light chain of myosin has been shown to be Ca^{++}- and calmodulin-dependent (23), but in the glycerinated epithelium contraction occurred even in Ca^{++}-free medium. Preliminary results (not shown) indicate that the lack of Ca^{++} sensitivity for contraction in glycerinated cells may be due to a lack of Ca^{++} dependence for myosin light chain phosphorylation. It has been shown previously that when myosin light chain kinase is partially proteolyzed, it maintains its kinase activity, but it loses its dependence on Ca^{++} and calmodulin (13, 38). Therefore, terminal web contraction in epithelial cells, which in vitro does not depend on Ca^{++}, may be regulated by Ca^{++} in vivo.

Possible Function of the Contractile Ring In Vivo

Herman et al. (14) have used immunocytochemistry to study the relationship between motility and the distribution of actin and myosin in many kinds of cells. They suggested that large actomyosin filament bundles such as stress fibers in the fibroblasts are potentially motile, but are associated with nonmotile cytoplasm and that actively motile cytoplasm has a more diffuse distribution of these proteins. From their data about actomyosin filament bundles in other cell systems and our

structural data, we suggest that the circumferential actomyosin bundle inside the zonula adherens could be motile, but it may also be primarily a structural element. More specifically, the circumferential bundle inside the zonula adherens could work primarily to maintain tension between adjacent epithelial cells within sheets of cells. For example, this bundle could generate tension towards the cytoplasm causing adjacent cells to pull on each other through extracellular bridges at the zonula adherens (17), thus generating tension within the sheet as a whole. This possible role is supported by the finding that a circumferential bundle generally exists in epithelial cells. This ring might be actively contractile in some cases such as formation of tubular and vesicular structures from epithelia during organogenesis, as suggested by Baker and Schroeder (2) and Owaribe et al. (32). Alternatively, in the intestinal epithelial cells, it might generate some movement of cell surfaces to mix the fluid surrounding the microvilli or it might regulate the state of the junctions between the cells.

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