# Reevaluation of Brush Border Motility: Calcium Induces Core Filament Solation and Microvillar Vesiculation

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ABSTRACT The report that microvillar cores of isolated, demembranated brush borders retract into the terminal web in the presence of $Ca^{++}$ and ATP has been widely cited as an example of $Ca^{++}$-regulated nonmuscle cell motility. Because of recent findings that microvillar core actin filaments are cross-linked by villin which, in the presence of micromolar $Ca^{++}$, fragments actin filaments, we used the techniques of video enhanced differential interference contrast, immunofluorescence, and phase contrast microscopy and thin-section electron microscopy (EM) to reexamine the question of contraction of isolated intestinal cell brush borders. Analysis of video enhanced light microscopic images of Triton-demembranated brush borders treated with a buffered $Ca^{++}$ solution shows the cores disintegrating with the terminal web remaining intact; membranated brush borders show the microvilli to vesiculate with $Ca^{++}$. Using $Ca^{++}$/EGTA buffers, it is found that micromolar free $Ca^{++}$ causes core filament dissolution in membranated or demembranated brush borders while the rootlets are insensitive to this $Ca^{++}$ effect. In the case of membranated brush borders, $Ca^{++}$ causes microvillar core solation followed by complete vesiculation of the microvillar membrane. The lengths of microvilli cores and rootlets were measured in thin sections of membranated and demembranated controls, in $Ca^{++}$-, $Ca^{++}$ + ATP-, and in ATP-treated brush borders. Results of these measurements show that $Ca^{++}$ alone causes the complete solation of the microvillar cores, yet the rootlets in the terminal web region remain of normal length. These results show that microvilli do not retract into the terminal web in response to $Ca^{++}$ and ATP but rather that the microvillar cores disintegrate.

NBD-phallicidin localization of actin and fluorescent antibodies to myosin reveal a circumferential band of actin and myosin in mildly permeabilized cells in the region of the junctional complex. The presence of these contractile proteins in this region, where other studies have shown a circumferential band of thin filaments, is consistent with the hypothesis that brush borders may be motile through the circumferential constriction of this "contractile ring," and is also consistent with the observations that ATP-treated brush borders become cup shaped as if there had been a circumferential constriction.

While the brush border is widely cited as an excellent model system for the study of nonmuscle cell motility, direct evidence of movement by intestinal epithelial cell microvilli or the brush border as a whole is equivocal. Reports of in vivo brush border or microvillar motility (26, 27) are not thoroughly documented and have yet to be confirmed. However, two lines of current reasoning support the contention that the brush border is a motile apparatus. These lines of thought incorporate the structural chemistry of the brush border and experiments reporting "reactivation" of brush border motility.

The structure and chemistry of the brush border cytoskeleton are somewhat analogous to that in muscle, and indeed models for brush border movement are based on muscle structure (6, 19, 25). Each microvillus contains a bundled core of unidirectionally polarized actin filaments which extend into the terminal web (1, 22). Within the microvillus are at least two actin filament bundling proteins, villin (3, 4, 16, 20) and fimbrin (10). The filaments are cross-linked to the plasma membrane by periodically spaced cross-filaments likely composed of a 110,000 $M_r$ polypeptide associated with calmodulin (9, 13, 15,

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17). The extent of detailed information regarding the chemistry of the structural elements in the terminal web, where the microvillar cores penetrate into the apical cytoplasm, is quite limited when compared with that known for the microvillar core. Both myosin (21) and tropomyosin (19) have been purified from brush borders, and immunolocalization studies have placed these proteins in the terminal web (2, 8, 21), probably associated with the core filament rootlets (6, 8, 11). In addition, $\alpha$-actinin is found in the region of the junctional complex (5, 8).

Perhaps the most exciting findings which suggest that the brush border is a motile organelle are two reports indicating that brush borders appear to contract in vitro (19, 25). Using isolated, demembranated brush borders, Mooseker (19) reported that, in a $Ca^{++}$- and ATP-requiring manner, core filaments can be induced to plunge into the terminal web region. On the other hand, Rodewald et al. (25) reported that membranated brush borders can be induced to contract radially in the terminal web region in the presence of divalent cations and high levels of ATP.

Very recently, a series of reports on the $Ca^{++}$-induced actin severing characteristics of the microvillar actin filament bundling protein villin (3, 4, 13, 16, 20) have raised questions regarding $Ca^{++}$-induced brush border movement. These questions include: (*a*) How can micromolar levels of $Ca^{++}$ induce core filament retraction into the terminal web when the same levels induce actin filament severing by villin?; (*b*) Are microvillar cores *in situ* somehow protected from villin by other associated proteins?

The present study was undertaken with these questions and the structural chemistry kept in mind. The question of in vitro brush border motility was reinvestigated by performing an ultrastructural and quantitative study of the lengths of microvillar cores and rootlets under various "reactivating" conditions. These qualitative and quantitative results, coupled with fluorescence microscopy and video enhanced images of brush borders in various "reactivation" conditions, seem to rule out brush border motility based on retraction of microvillar cores into the terminal web. The results are consistent with, but do not unequivocally prove, brush border motility based on contraction of the terminal web as mediated by a contractile ring.

## MATERIALS AND METHODS

### Isolation of Brush Borders

Epithelial cells were collected from chicken intestines as described earlier (15). Brush borders were isolated from these cells using the protocol outlined by Mooseker et al. (20), with the modification that the pH of the solutions was adjusted to 6.9, a pH that is optimal for the actin bundling abilities of villin (16). The proteolytic inhibitor phenylmethylsulfonyl fluoride (PMSF) in dimethyl sulfoxide (DMSO) was added to all preparations at a final concentration of 0.1 mM. For demembranation, purified brush borders were incubated in 1.0% Triton X-100 in 75 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM Imidazole, pH 6.9 at room temperature for 30 min. Demembranated brush borders were then washed twice in the above solution minus the Triton before further use.

### Reactivation Conditions

Small pellets of washed demembranated or membranated brush borders were resuspended in Microfuge tubes in various reactivation solutions for various lengths of time at room temperature. The zero $Ca^{++}$ solution contained 100 mM KCl, 3 mM $MgCl_2$, 3 mM EGTA, 10 mM Imidazole, (or $PO_4$) pH 6.9.

Brush borders were exposed to the above solution containing $CaCl_2$ at 0.8 mM, 2.1 mM, and 3.0 mM. The $Ca^{++}$ concentration of the stock $CaCl_2$ solution had been determined by atomic absorption. Free $Ca^{++}$ was calculated using a computer program written by Dr. Peter Chantler (Brandeis University, Waltham, MA) which takes into consideration the temperature, pH, $Mg^{++}$, EGTA, and ATP which all affect the level of free $Ca^{++}$ in solution. At 0.8 mM, 2.1 mM, and 3.0 mM $CaCl_2$ in the above solution containing 5 mM ATP, the free $Ca^{++}$ is $1.6 \times 10^{-7}$ M, $1.0 \times 10^{-6}$ M, and $1.0 \times 10^{-5}$ M, respectively. Without ATP the same three $CaCl_2$ concentrations give solutions whose free $Ca^{++}$ is $1.7 \times 10^{-7}$ M, $1.1 \times 10^{-6}$ M, and $3.7 \times 10^{-5}$ M, respectively. Incubation times varied from ~15 s (the amount of time necessary to fill the tube and place it in the Microfuge) to 30 min. Reactions were stopped by spinning for 30 s in a Beckman Microfuge (Beckmann Instrument Co., Palo Alto, CA), removing the supernatant, and fixing the pellet for electron microscopy.

### Electron Microscopy

Samples were fixed for 30 min at room temperature in 3% glutaraldehyde, 0.2% tannic acid, 0.1 M phosphate buffer (pH 7.0), rinsed for 10 min in 10% Sucrose, 0.1 M phosphate buffer (pH 7.0), and postfixed for 1 h on ice in 1% $OsO_4$ in 0.1 M phosphate, (pH 6.0). After embedding in EPON, silver sections were cut with a diamond knife, stained with lead citrate and uranyl acetate, and examined on a JEOL 100CX operated at 60 kV. All micrographs used for measurements were taken at 16,000 times magnification. Micrographs of brush borders were taken only when the plane of sectioning revealed the entire length of microvilli from their dense tip to rootlet extension in the terminal web. Between 23 and 43 individual brush borders were used for measurements and 5–30 microvilli (or rootlets) were measured per brush border. The data reported for the series of experiments performed on demembranated brush borders resulted from the brush borders of one animal although identical results were obtained using brush borders from many animals. A Student's *t* test was done on the aggregated data.

### Light Microscopy

Video enhanced differential interference contrast light microscopy was done using a Hamamatsu C-1000 camera and control unit coupled to a GYYR video timer, a SONY TVO-9000 videocassette recorder and a Panasonic Model WV5310 television monitor. The light microscope was a Leitz Diavert inverted microscope with Smith-T Interference Contrast optics. Images were recorded from the videomonitor using 35 mm Plus X film.

For fluorescence microscopy, a Leitz Diavert was used equipped with a 40X oil phase 1.3 N.A. objective and a 50 W mercury Ploem epiilluminator. The fluorescein I2 Leitz filter cube was used. NBD-phallicidin (Molecular Probes, Inc., Plano, TX) was used in a 1:100 dilution on isolated epithelial cells and fragments that had been mildly permeabilized in 0.1% Triton X-100, 75 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 10 mM Imidazole, pH 6.9. Excess phallicidin was removed before observation of the cells.

Antibodies to human platelet myosin were used to localize myosin in cells and in epithelial cell sheets (21). Epithelial cells and sheets were mildly permeabilized as above, washed and treated in a 60-fold dilution of immune or preimmune serum from the same rabbit for 30 min at room temperature. After two washes, tissues were incubated in a 50-fold dilution of fluorescein-conjugated goat anti-rabbit IgG (Cappel Labs, Cochranville, PA) for 30 min at room temperature. Tissues were washed several times and examined as above. All fluorescence micrographs were taken with Kodak Tri-X film and developed in Diafine.

## RESULTS

### Light Microscopy

Differential interference contrast images of isolated membranated brush borders reveal a clear view of the fingerlike microvilli separated from the underlying terminal web (Fig. 1*a*). Simple perfusion of a $Ca^{++}$ solution ($10^{-4}$ or $10^{-5}$ M) causes dramatic and rapid changes in the structure of the microvilli. After viewing the videotape several times one is left with the impression that the microvilli first become flaccid and tend to exhibit rapid Brownian movement. Then, almost immediately the microvilli vesiculate in the $Ca^{++}$; the apical tips of microvilli bud off first followed by complete vesiculation of the entire microvillar shaft (Fig. 1 *b* and *c*). Identical results are obtained with ATP included in the $Ca^{++}$ solution. No changes are noted in the terminal web region.

The reaction in demembranated brush borders to $Ca^{++}$ solution ($10^{-4}$ or $10^{-5}$ M) perfusion as revealed by video enhanced images is dramatically different from the response of

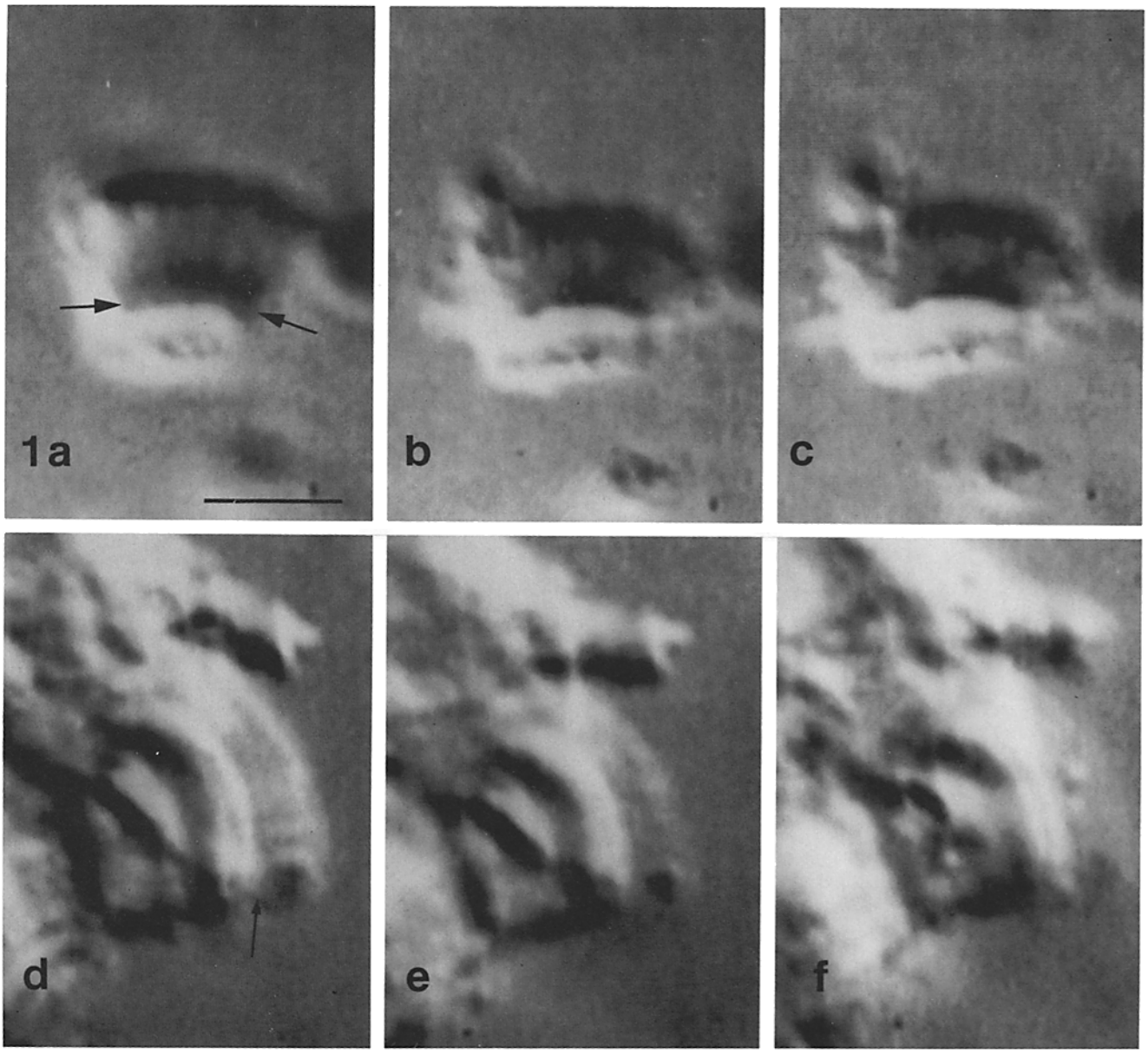



FIGURE 1 Video enhanced differential interference contrast images of a membranated brush border. The total elapsed time from *a* through *c* is ~8 s. (*a*) before perfusion with $10^{-5}$ M $Ca^{++}$ solution; (*b*) ~4 s after $Ca^{++}$ perfusion revealing changes in the apical region of the microvillar region; (*c*) 8 s after $Ca^{++}$ perfusion showing degradation of microvilli but little change in terminal web region. Video enhanced differential interference image of demembranated brush border. The total elapsed time from *d* through *f* is <2 s. (*d*) before $Ca^{++}$ perfusion; (*e*) first visual change after $Ca^{++}$ showing some degradation of the microvillar region; (*f*) ~2 s after $Ca^{++}$ perfusion showing degradation of microvillar region. Arrows: junction between microvilli and terminal web. Bar, 2 μm.

membranated brush borders. The microvillar core filament region appears to bunch together. This change appears in differential interference contrast microscopy as a change in the contrast or density of the core filament region (Fig. 1 *d* and *e*). In many instances the entire microvillar core filament region washes completely away from the terminal web which shows no obvious changes in structure.

The distribution of actin in these cells as revealed by NBD-phallicidin treatment is shown in Fig. 2. This pattern on mildly extracted cells is the same as has been reported in other studies using immunofluorescence, in terms of actin distribution in microvilli and the terminal web region (2, 6). In addition, careful optical sectioning through the epithelial cell fragments reveals a circumferential band of actin, at the level of the junctional complex, encircling the apical border of the cells (Fig. 2 *b*). A band of thin filaments has been recently described in intestinal epithelial cells at the ultrastructural level (14).

Fluorescence microscopy confirms the presence of myosin in the terminal web region of single epithelial cells as has been reported (2, 6, 21). However, when permeabilized epithelial sheets are optically sectioned such that the plane of sectioning passes across the terminal web at the level of the junctional complex, a striking pattern of myosin distribution is revealed (Fig. 3). Not only is there a rather even distribution of myosin across the terminal web, but there is, like that of actin distribution, an intensely staining circumferential band at the cell margins. Such staining is only observed in intact sheets of epithelial cells optically sectioned at the proper level.

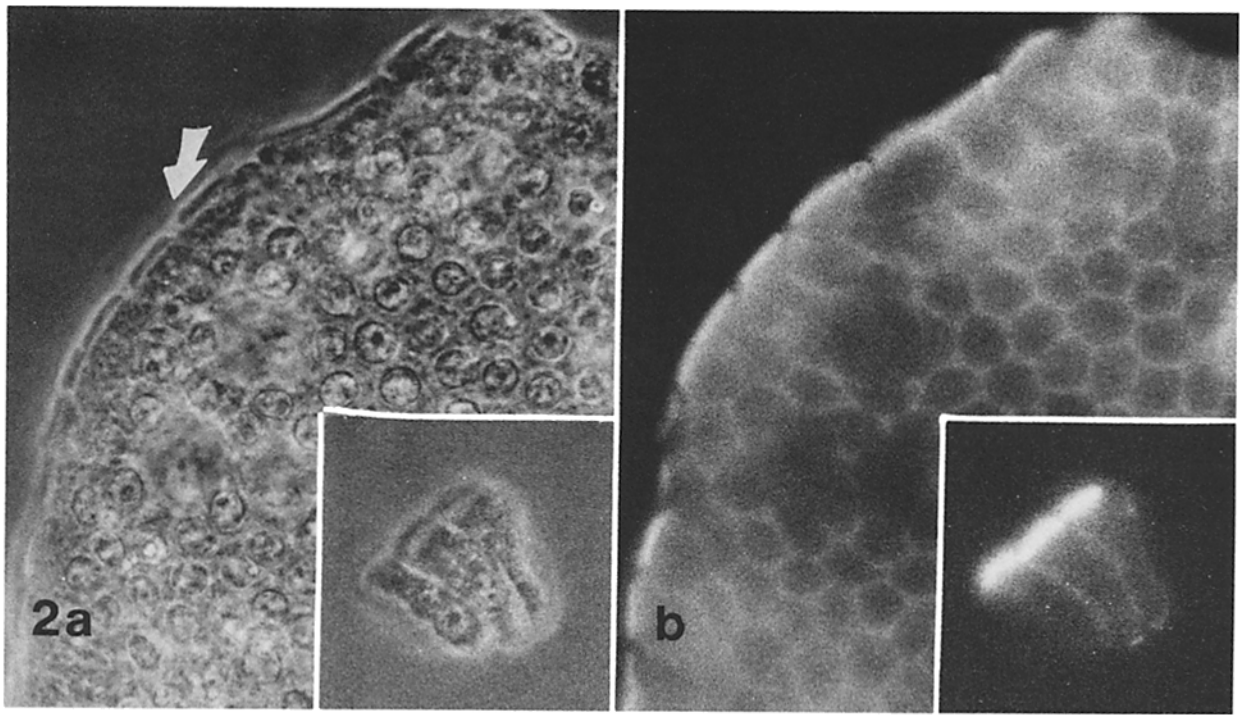



FIGURE 2 (*a*) Phase contrast image of a sheet of permeabilized epithelial cells treated with NBD-phallicidin revealing brush border region (arrow). *Inset*: phase contrast view of three permeabilized epithelial cells. (*b*) Pattern of actin distribution in the same sheet and group of cells as revealed by fluorescence of NBD-phallicidin. This micrograph was taken at a focal plane slightly above the corresponding phase micrograph. Actin is revealed in the brush border region and in circumferential rings at the periphery of the apical regions of the cells.

## $Ca^{++}$ Effects upon Membranated Brush Borders

Several ultrastructural landmarks of the brush border are of major relevance to the data presented here. Along the length of the microvillar shaft are found cross filaments which link the core filament bundle to the membrane. These cross filaments are not found in the rootlet (that part of the core filament bundle extending into the terminal web [Figs. 4 and 6]). The apical portion of the terminal web is relatively devoid of organized filaments, except for the penetrating rootlet bundle, when compared with the basal zone which possesses coarse skeins of 100 Å filaments. These structural features provide useful landmarks for length measurements of microvilli and rootlets.

Thin sections of $Ca^{++}$-treated brush borders show that in solutions containing $10^{-6}$ M or higher free $Ca^{++}$, the microvillar cores begin to disassemble immediately. At $10^{-5}$ M $Ca^{++}$, the effect is apparent at the shortest treatment, whereas at $10^{-6}$ M $Ca^{++}$, the effect occurs within 2 to 3 min. No matter what $Ca^{++}$ level is used, the loss of core filament structure with the concomitant vesiculation of the surrounding sleeve of membrane always occurs (Fig. 4). Usually just a small, short nub of a microvillar shaft, lacking most internal structures, remains. Membrane vesicles are present, sometimes as strings which mark the sites where intact microvilli were located. No intact microvilli are present. The vesicles appear either empty or contain electron dense material. Brush borders treated for the shortest time show that vesiculation begins at the tips of the microvilli and proceeds down their shaft. The same core dissolution followed by vesiculation occurs in brush borders treated with micromolar (or greater) $Ca^{++}$ and 5 mM ATP (Fig. 5*a* and *b*).

In contrast to the effects of $Ca^{++}$ and $Ca^{++}$/ATP on microvilli, these agents produce only slightly discernable effects on the morphology of the terminal web region. The rootlets, identified by their lack of cross-filaments, remain tightly bundled (Fig. 4). The only difference between $Ca^{++}$-treated and $Ca^{++}$-ATP-treated brush borders is that with ATP the terminal web region occasionally appears to have pinched inward (Fig. 5*a*). Such radial contractions are usually only apparent after long (5–15 min) exposure to ATP and always leave the terminal web region appearing cup-shaped.

## $Ca^{++}$ Effects upon Demembranated Brush Borders

Ultrastructural effects of micromolar or greater $Ca^{++}$ and micromolar or greater $Ca^{++}$ + 5 mM ATP upon demembranated brush borders are equally detrimental to the microvillar cytoskeleton. Neatly bundled microvillar cytoskeletons are not present after either treatment; in fact, no organized microfilamentous structures are apparent in what was the core filament region. Instead of organized core filaments, micromolar $Ca^{++}$ or $Ca^{++}$ + ATP treated demembranated brush borders have highly granular, electron dense material in this region (Figs. 5 and 6). Again, the terminal web rootlets appear to remain well bundled and intact although after 15 to 30 min some rootlets become somewhat splayed. The terminal web region in some $Ca^{++}$ + ATP treated demembranated brush borders also becomes cup-shaped after extended treatment.

## Quantitative Analysis of $Ca^{++}$ Effects

Measurements of microvillar shaft (or core filament) lengths and rootlet lengths were made for membranated and demembranated controls (0 $Ca^{++}$), $Ca^{++}$-treated ($10^{-4}$ M) or $Ca^{++}$

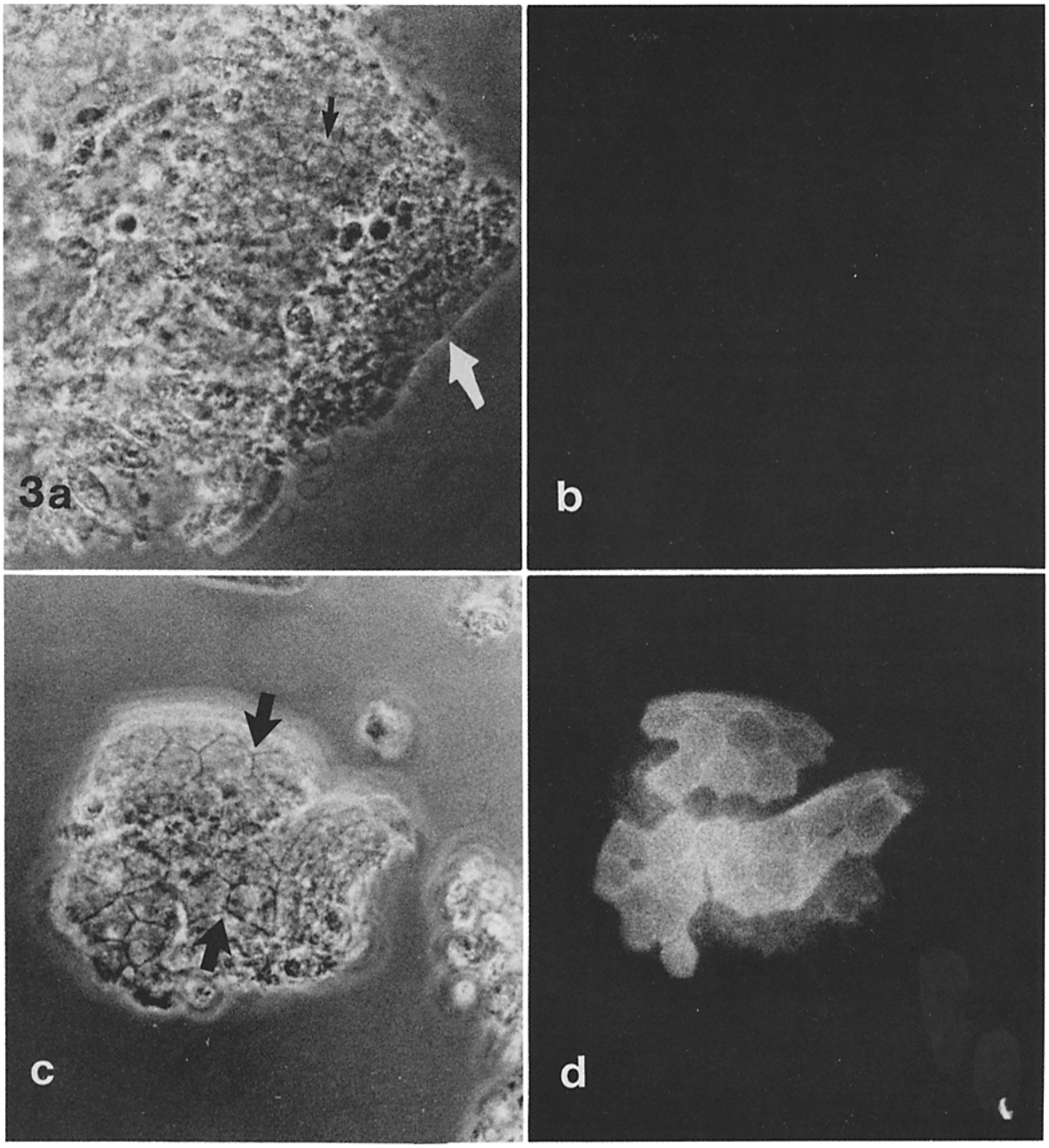
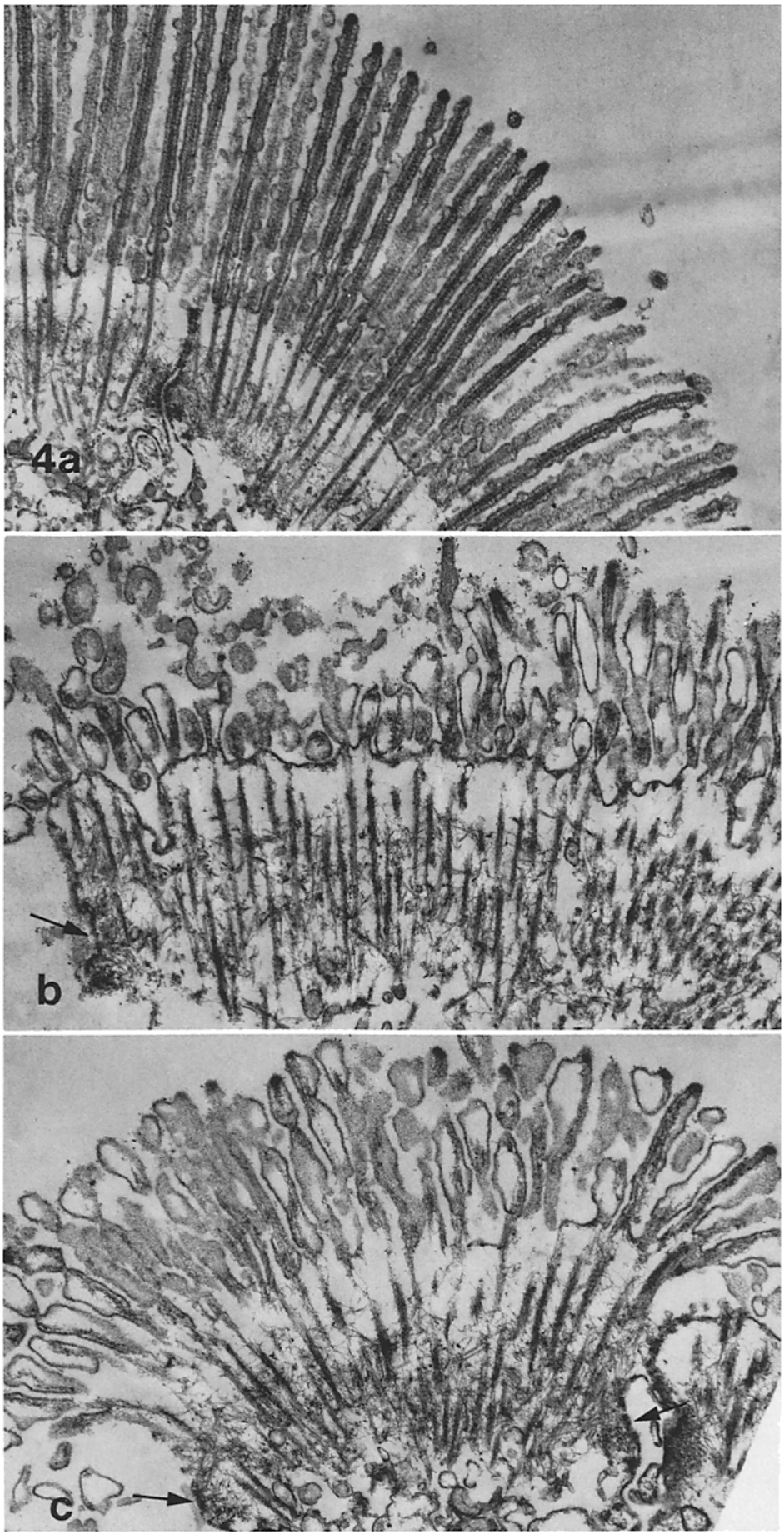



FIGURE 3 (*a*) Sheet of permeabilized epithelial cells treated with pre-immune serum showing brush border region (large arrow) and junctional complex region in optical section (small arrow). (*b*) Corresponding fluorescence micrograph of the same field in *a* taken and printed under the same conditions as *d*. (*c*) Phase contrast image of sheet of permeabiltreated with antimyosin antibody taken at the level of the junctional complex (arrows). (*d*) Corresponding fluorescence micrograph of same group of cells as in *c* taken at the same focal plane revealing myosin to be localized in a circumferential ring in the junctional complex region.

($10^{-4}$ M) + ATP (5 mM)-treated brush borders. High $Ca^{++}$ levels were used to achieve maximal effects upon microvilli and rootlets. While these measurements were easily accomplished in membranated brush borders under all conditions, they were difficult in the $Ca^{++}$ or $Ca^{++}$ + ATP-treated demembranated brush borders because there was no clear morphological marker for the apical cell surface. The presence of cross-filaments on the core filaments and the relatively filament-free apical zone of the terminal web provided the major morphological markers. Results are presented in Table I.

Although the lengths of microvilli and rootlets from membranated brush borders are significantly longer than those from demembranated brush borders these differences are most likely due to differences among animals. It was noted that microvillar and rootlet lengths were consistently very similar (with low standard deviations) in brush borders from the same animal while comparisons between animals yielded larger standard deviations. For this reason the measurements reported for experiments with demembranated brush borders came from brush borders prepared from one animal.

Results of measurements of the effects of $Ca^{++}$ or $Ca^{++}$ + ATP upon membranated brush borders revealed that microvilli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b
c

were reduced in length to 10% or less than the lengths of controls. The rootlets, however, did not change length after $Ca^{++}$ treatment but were significantly shorter (shorter by only 0.09 μm out of a length of 0.77 μm) after $Ca^{++}$ + ATP treatment.

Microvillar length after $Ca^{++}$ or $Ca^{++}$ + ATP exposure in demembranated brush borders was reduced to essentially zero. On the other hand rootlet length was ~16% longer after exposure to either $Ca^{++}$ or $Ca^{++}$ + ATP. While demonstrating that rootlets were significantly longer after $Ca^{++}$ or $Ca^{++}$ + ATP exposure, it should be noted that these measurements were extremely difficult ones to make because of the lack of definitive morphological landmarks and that the longest rootlet ever measured was 0.9 μm in length.

## DISCUSSION

### *$Ca^{++}$ Effects upon Microvilli*

The results presented in this study support a reevaluation of experiments on reactivation of brush border motility (19, 25). The first suggestion that these reports should be reevaluated came with the demonstration that one of the microvillar core filament bundling proteins, villin, is a $Ca^{++}$-activated actin filament severing protein (3, 4, 16, 20). The level of $Ca^{++}$ required to activate villin's actin severing capabilities ($1–2 \times 10^{-6}$ M) is the same amount reported to be required (with ATP) to activate motility in demembranated brush borders (19).

The light and electron microscopic analysis of $Ca^{++}$ + ATP treated brush borders as reported here reveals that what was viewed as core filament bundle retraction was instead core filament dissolution. Such microvillar core filament dissolution is likely the result of the action of villin, in response to micromolar levels of $Ca^{++}$, upon actin filaments to sever them.

The fact that after $Ca^{++}$ or $Ca^{++}$ + ATP treatment, rootlets of demembranated brush borders are slightly, but significantly, longer than those in controls is suggestive of contraction. However, a more likely explanation for slight increases in rootlet length under these conditions is that one is measuring not only just rootlet but part of the base of core filaments unaffected by $Ca^{++}$ or by $Ca^{++}$ + ATP. Definitive points where one can say core filaments end and rootlets begin are difficult to discern, especially since these are two regions of the same bundle. Note also, a much lower standard deviation in the experimentals when compared with the control which tends to support such an explanation.

Another reason for difficulty in interpreting $Ca^{++}$ or $Ca^{++}$ + ATP effects on demembranated brush borders, particularly those encountered in light microscopy, is the fact that the disrupted core filament material remains associated with the intact terminal web. Electron micrographs show that the core filaments are reduced into fine, dense, granular material in the

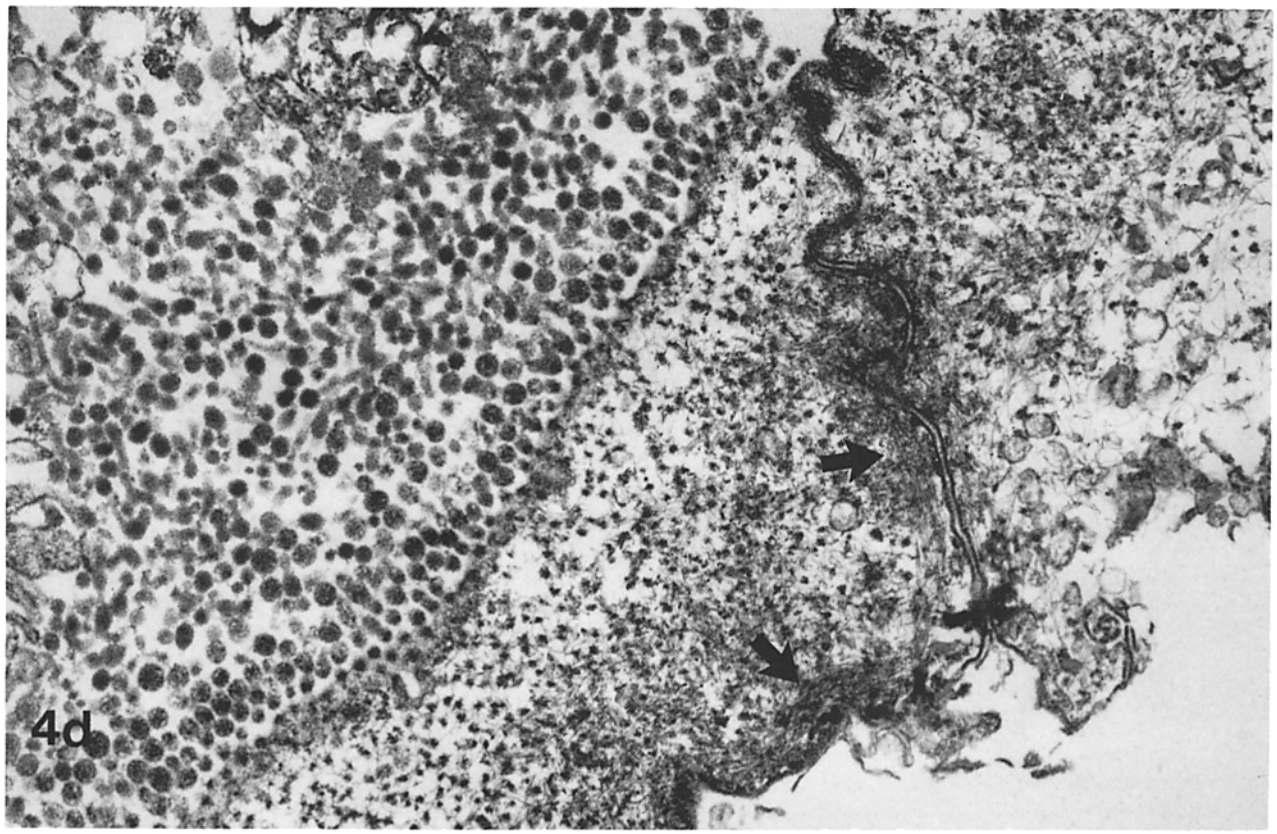


FIGURE 4 Thin sections of membranated brush borders. (*a*) Control brush borders in buffered EGTA (0 $Ca^{++}$) solution. (*b*) 2-min treatment with $10^{-5}$ M $Ca^{++}$ solution causes complete microvillar core solation and microvillar membrane vesiculation. Rootlets remain intact, associated with terminal web thick filaments and in proper relationship to junctions (arrow). (*c*) After 5-min treatment with $10^{-6}$ M $Ca^{++}$ solution membranated brush borders have lost microvillar core filaments and vesiculated their microvillar membrane. Rootlets are intact and penetrate to the level of the junctional complex (arrows). (*d*) Tangential section across brush border of $10^{-5}$ M $Ca^{++}$-treated membranated brush border showing microvillar core solation, membrane vesiculation, and normal appearing rootlets in cross section. In the region of the zonula adherens the circumferential band of fine filaments remains intact in spite of $Ca^{++}$ treatment (arrows). *a*, × 25,550; *b* and *c*, × 30,000; *d*, × 32,300.

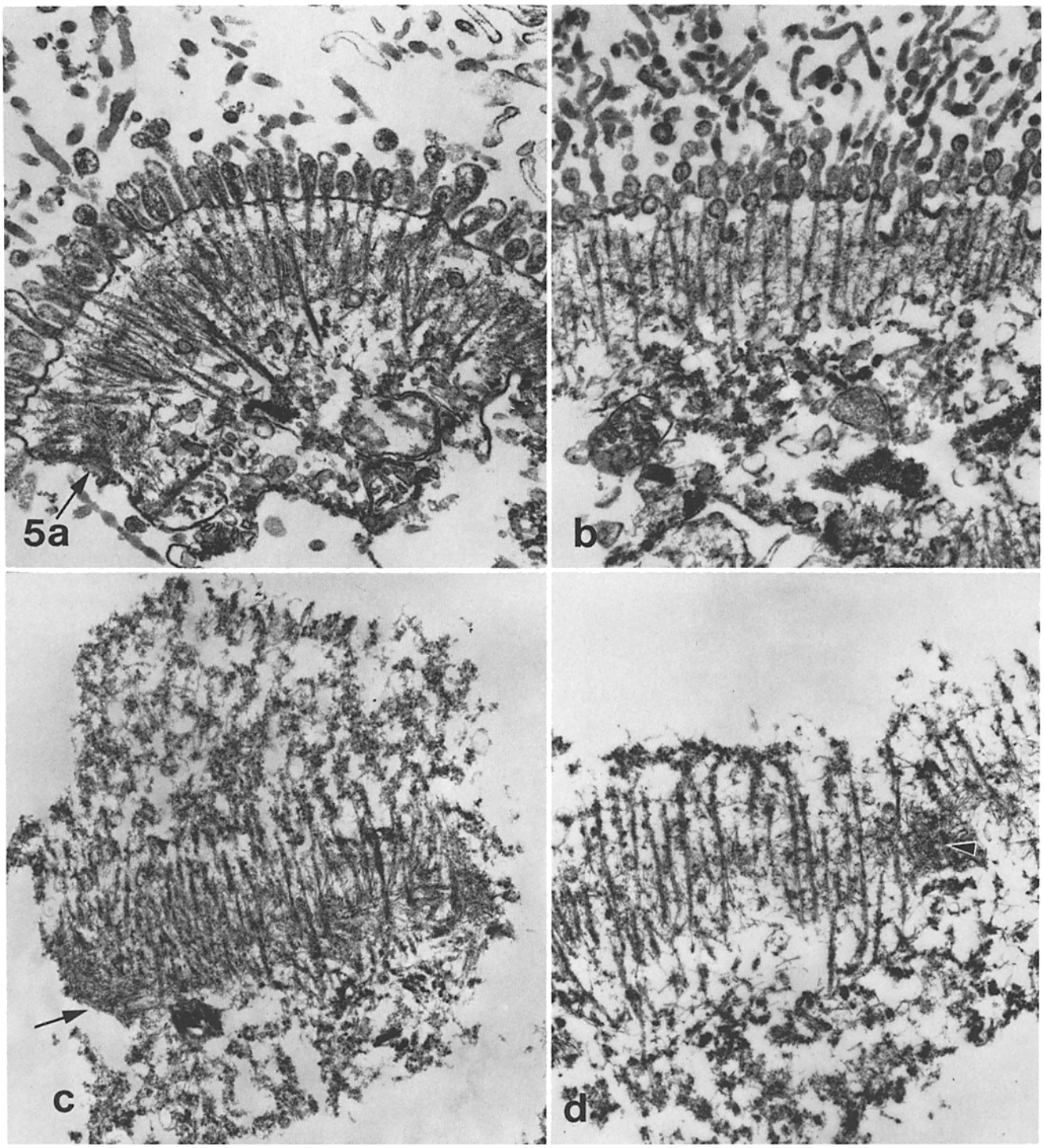


FIGURE 5 Thin sections of brush borders treated with $10^{-5}$ M $Ca^{++}$ + 5 mM ATP. (*a*) and (*b*) Membranated brush borders have shed their microvilli almost completely leaving behind short nubs possessing little or no internal structure. In *a* the brush border was treated for 30 min in $Ca^{++}$ + ATP which resulted in a cup-shaped brush border. Note rootlet penetration to level of junctional complex (arrow). The brush border in *b* was treated for 5 min with $Ca^{++}$ + ATP. (*c*) and (*d*) Demembranated brush borders after $Ca^{++}$ + ATP treatment showing rootlet stability. In *c* the core filaments have turned into finely granular, amorphous material, whereas the rootlets remain well bundled, associate properly with terminal web 100 Å filaments, and penetrate to the level of the junctions (arrow). In *d* little evidence of core filament material remains. Again, rootlets are intact and retain proper association with the junctional region (arrow). All × 25,550.

FIGURE 6 Thin sections of demembranated brush borders. Without the membrane, clear landmarks for the core filament vs. rootlet regions are difficult to discern. Arrow indicates region of juncture between cores and rootlets. Above the arrow the cores display cross-filaments clearly. (*a*) and (*b*) Control brush borders in 0 $Ca^{++}$ buffer. (*c*) and (*d*) Brush border treated with $10^{-5}$ M $Ca^{++}$ solution showing complete core filament disruption and rootlet stability. Core filaments are replaced by finely granular, dense aggregated material. (*e*) and (*f*) Brush borders treated with $10^{-6}$ M $Ca^{++}$ solution (0 ATP) demonstrating core filament solation yet rootlet stability. Note proper association with terminal web thick filaments and penetration to the level of the junctional complex (arrows). All × 25,550.

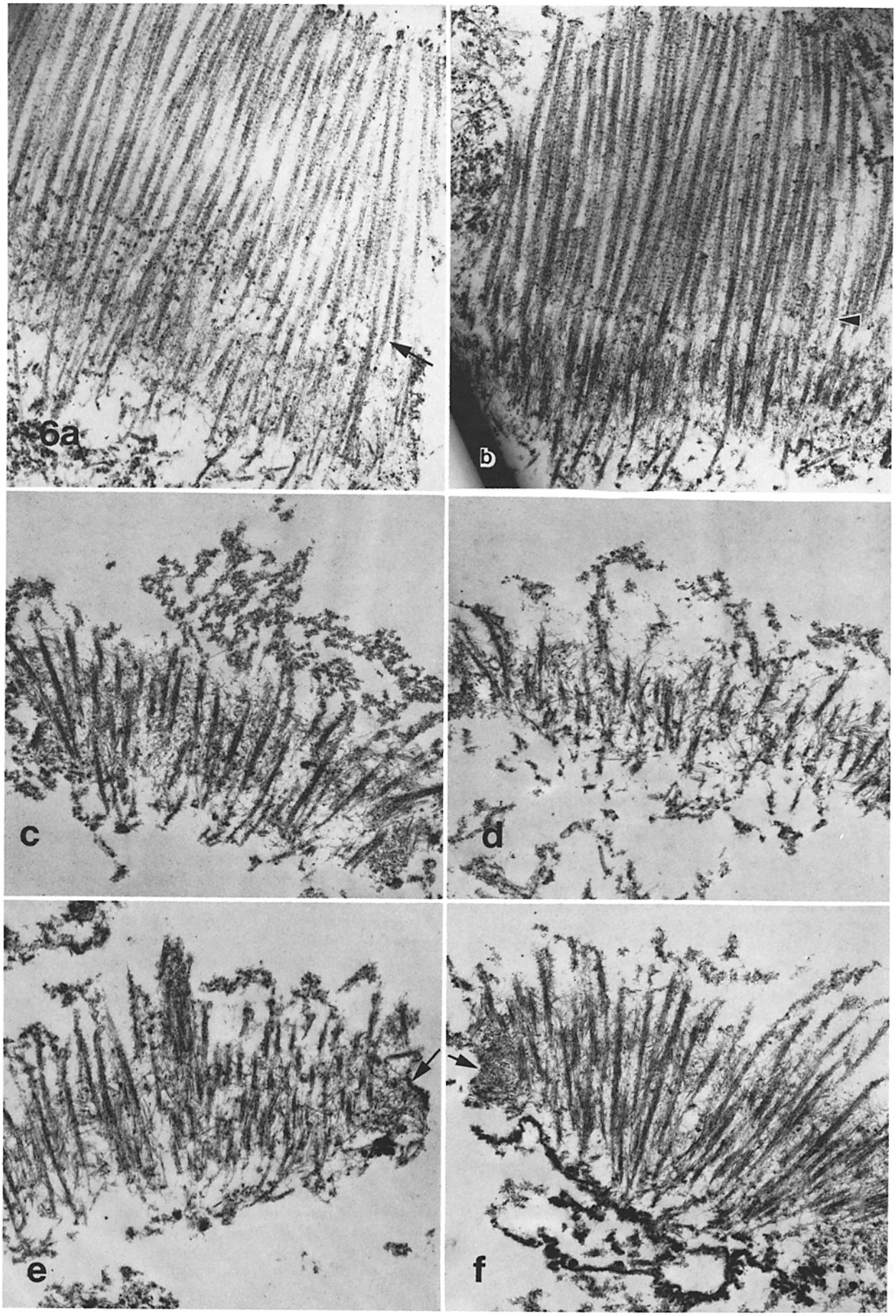
6a
b
c
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TABLE I

*Core Filament and Rootlet Lengths*

| | Core filament | Rootlet | n |
|---|---|---|---|
| | μm | μm | |
| Membranated | | | |
| control | 1.91 ± 0.34 | 0.77 ± 0.12 | 23 |
| + $Ca^{++}$ | 0.19 ± 0.11 | 0.75 ± 0.10 | 20 |
| + $Ca^{++}$ + ATP | 0.15 ± 0.10 | 0.68 ± 0.07 | 28* |
| Demembranated | | | |
| control | 1.74 ± 0.27 | 0.66 ± 0.21 | 40 |
| + $Ca^{++}$ | 0.04 ± 0.05 | 0.75 ± 0.11 | 43* |
| + $Ca^{++}$ + ATP | 0 | 0.75 ± 0.10 | 25* |

* Significantly different at the P = 0.05 level.

presence of micromolar $Ca^{++}$ or $Ca^{++}$ + ATP which usually sticks to, but is occasionally washed away from (Figs. 5 and 6), the terminal web. This association or stickiness is probably due to the cross-filament protein (110,000 $M_r$) which is very difficult to solubilize and tends to aggregate and clump with other brush border proteins upon attempted isolation.

The fact that membranated and demembranated brush borders react in the same way to either micromolar $Ca^{++}$ or micromolar $Ca^{++}$ + ATP also supports arguments against retracting microvilli. $Ca^{++}$ is free to diffuse into microvilli to induce solation of the cores because the brush borders are not closed vesicles. It appears that the core filaments are disrupted before vesiculation of the membrane. The sleeve of membrane left after core solation is unstable and immediately begins to pinch off.

## Rootlets are Stable in $Ca^{++}$

The contention that those bundles of filaments left after $Ca^{++}$ or $Ca^{++}$ + ATP treatment are rootlets and not core filaments is supported by several observations. First, their location, in terms of penetration to the tonofilament region in the terminal web and association with the junctional complex, is identical to that of rootlets in controls. Secondly, those bundles remaining lack cross filaments as do rootlets in controls. Finally, the lengths of these bundles are almost the same as rootlets. Rootlet stability in the presence of villin's $Ca^{++}$-activated actin severing capability may be due to accessory proteins: immunofluorescence studies suggest that tropomyosin is associated with the rootlets but not with the core filaments (6). Preliminary evidence (23) indicates that muscle tropomyosin protects actin filaments from villin's severing abilities.

## Models for Brush Border Motility

The data presented here and elsewhere show that: (*a*) Microvillar cores, but not rootlets, are solated under "reactivation" conditions (see also reference 13); (*b*) Micromolar free $Ca^{++}$ alone without ATP produces the same core solation; (*c*) Villin is an actin severing protein *in situ* at the same levels of free $Ca^{++}$ as that suggested for "motility"; (*d*) Thick section stereo, and serial thin sectioning of rootlets demonstrates a lack of splaying of rootlet filaments and documents fine filament attachment to the sides of rootlets (18); and (*e*) Quick-freeze, deep-etch freeze fracture studies demonstrate very little splaying of rootlet filaments (12). As a result, one must seriously question models for brush border motility which assume that filament bundles plunge through the terminal web as their rootlet filaments, splaying at their ends, slide past myosin in the terminal web (6, 19). The three published models of in vitro brush border motility (6, 19, 25) all envision some form of core filament retraction into the terminal web and some interaction of rootlet actin with myosin. None of these models predicts the whereabouts of an equal, but opposite force which would be necessary to return the microvilli to their original precontracted lengths.

Several lines of reasoning and new evidence argue for a reinterpretation of brush border motility with special attention focused on the terminal web region. Based on structural and chemical data we have recently suggested that the terminal web myosin and actin are in a state of isometric tension providing a structural scaffolding to keep microvilli erect (18). If this were not true then the reactivation conditions reported by Rodewald, et al. (25) and Mooseker (19) should cause the rootlets to come closer together through a massive radial contraction. However, under these conditions Rodewald et al. (25) and the present study always see brush borders rather cup-shaped in appearance with the rootlet bundles not more tightly packed.

Electron microscopy by Rodewald et al. (25) and Hull and Staehelin (14) clearly demonstrate the presence of a band of microfilaments encircling the cell at the level of the adherens junction just at or below the ends of the rootlets (also see Fig. 4*d*). This band is enriched in both actin and myosin (Figs. 2, 3, and reference 2) relative to the rest of the terminal web raising the interesting possibility that brush borders may be motile via the contraction of a contractile ring. Not only are actin, myosin, and tropomyosin localized in this region, but like the contractile ring of dividing cells (7) this region of intestinal epithelial cells possess α-actinin (5). This band of thin filaments is clearly stable to free $Ca^{++}$ (Fig. 4*d*) unlike the microvillar cores.

A contractile ring hypothesis is consistent with the observations that reactivation conditions result in cup-shaped brush borders (Fig. 5*a* and reference 25) with fanned out microvilli. Such a contractile ring obviates the necessity of explaining microvillar shortening under core filament solating conditions and still allows for gentle stirring of the water layer next to the microvilli. A contractile ring exerting a purse-string action also allows for the terminal web to be a firm structural framework for microvillar support. If such a contractile ring were activated by a local release of free calcium, such a flux of $Ca^{++}$ in this region would not endanger the integrity of microvilli because of its restricted locale. The likelihood that a contractile ring is operating in intestinal epithelial cells is heightened because of the recent report of such an active organelle in pigmented retinal epithelial cells (24). Owaribe, et al. (24) found that glycerinated epithelial sheets contracted to form cups in the presence of ATP and $Mg^{++}$ (being $Ca^{++}$-independent) and that these cells had circumferential bands of actin containing filaments in their adherens region. Such circumferential apical constrictions were also blocked by *N*-ethylmaleimide.

Only with further careful observations of epithelial sheets in vivo or in vitro will we be able to substantiate any model for brush border motility.

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