

Ca⁺⁺-Calmodulin-dependent Phosphorylation of Myosin, and Its Role in Brush Border Contraction In Vitro

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ABSTRACT We have reinvestigated the effects of Ca⁺⁺ and ATP on brush borders isolated from intestinal epithelial cells. At 37°C, Ca⁺⁺ (1 μM) and ATP cause a dramatic contraction of brush border terminal webs, not a retraction of microvilli as previously reported (M. S. Mooseker, 1976, *J. Cell Biol.* 71:417-433). Terminal web contraction, which occurs over the course of 1-5 min at 37°C, actively constricts brush borders at the level of their zonula adherens. Contraction requires ATP, is stimulated by Ca⁺⁺ (1 μM), and occurs in both membrane-intact and demembrated brush borders. Ca⁺⁺-dependent-solation of microvillus cores requires a concentration of Ca⁺⁺ slightly greater (10 μM) than that required for contraction.

Under conditions in which brush borders contract, many proteins in the isolated brush borders become phosphorylated. However, the phosphorylation of only one of the brush border proteins, the 20,000 dalton (20-kdalton) light chain of brush border myosin (BBMLC₂₀), is stimulated by Ca⁺⁺. At 37°C, BBMLC₂₀ phosphorylation correlates directly with brush border contraction. Furthermore, both BBMLC₂₀ phosphorylation and brush border contraction are inhibited by trifluoperazine, an anti-psychotic phenothiazine that inhibits calmodulin activity. These results indicate that Ca⁺⁺ regulates brush border contractility in vitro by stimulating cytoskeleton-associated, Ca⁺⁺- and calmodulin-dependent brush border myosin light chain kinase.

Ca⁺⁺ has been implicated as a regulator of both motility and cytoplasmic structure in nonmuscle cells. For example, changes in free Ca⁺⁺ concentration may regulate motility in vertebrate nonmuscle and smooth muscle cells by controlling phosphorylation of the regulatory light chain of myosin by a Ca⁺⁺-calmodulin-dependent myosin light chain kinase. Phosphorylation of the regulatory light chain of smooth and nonmuscle myosin increases its actin-activated Mg⁺⁺-ATPase activity, presumably increasing force production and causing motility (for review, see reference 1).

Ca⁺⁺ may also regulate cytoplasmic structure by disrupting the bundles or isotropic networks of cross-linked actin filaments that have been postulated to contribute to the gel-like properties of cytoplasm (see reference 12, for review). Raising the concentration of free Ca⁺⁺ in cytoplasmic extracts disrupts supramolecular actin filament structures. This indicates that Ca⁺⁺ may regulate the rapid changes in cytoplasmic viscosity that can occur in vivo. Recently, two major classes of Ca⁺⁺-sensitive actin-binding proteins which could modulate the reversible breakdown of actin filament structures have been identified. These include proteins which cross-link actin filaments in the absence, but not in the presence, of Ca⁺⁺ (9, 50,

58, 60, 79) and proteins which reversibly cut or shorten filaments in the presence of Ca⁺⁺ (6, 13, 26, 30, 46, 53). The mechanism by which changes in structure are coordinated with motility in vivo is still a matter of speculation (32).

One of the most extensively ordered arrays of contractile proteins in nonmuscle cells is found in the brush border of intestinal epithelial cells (for review of brush border structure, see reference 54). Brush borders are uniquely suited for the study of both motility and cytostructural regulation, because they can be isolated from cells with their cytostructures intact. Several years ago, we reported that, when Ca⁺⁺ and ATP were added to isolated, demembrated brush borders, their microvilli retracted into and through the terminal web (51). This report was made on the basis of light microscope observations (including a film shown at the 14th Annual Meeting, American Society for Cell Biology, San Diego, CA, November, 1974) in which Ca⁺⁺ and ATP caused a progressive loss of microvilli and an increase in density of terminal web. Electron micrographs of these contracted brush borders showed that the apical ends of the microvilli were at the level of the intercellular junctions, supporting further the interpretation of microvillar retraction (50). At about the same time, Rodewald et al. (63)

reported that the terminal webs of brush borders that were not demembrated with detergent "pinched in" at the level of the zonula adherens in the presence of ATP. These observations of microvillar movement *in vitro* lent credence to the observations of brush border motility *in vivo* (64, 74).

Since then, our lab has discovered that Ca^{++} disrupts microvillus structure (38), and several labs have independently demonstrated that concentrations of free Ca^{++} greater than micromolar cause the 95,000 dalton (95-kdalton) microvillus protein (variously referred to as villin or MV-95k) to sever the microfilaments in the core bundle (6, 13, 26, 27, 46, 53). These results have forced us and others to develop serious doubts about the validity of microvillus retraction in the presence of Ca^{++} . In reinvestigating this phenomenon with enhanced video microscopy at higher magnifications, we find it obvious, in hindsight, that what was originally interpreted as microvillar retraction is really a combination of a tip-to-base loss of microvillus cores due to Ca^{++} -dependent solation (while this work was in progress, similar results were independently obtained by Burgess and Prum, 7) and a contraction of the terminal web similar to that described by Rodewald et al. (63).

In reinvestigating motility in isolated brush borders, we have been able to separate the effects of Ca^{++} -dependent microvillus core solation from Ca^{++} - and -ATP-dependent contractility. Here, we demonstrate that Ca^{++} and ATP cause a dramatic contraction in brush border terminal webs but not a retraction of microvilli as previously reported (51). Furthermore, our results indicate that Ca^{++} regulates this brush border contraction by stimulating phosphorylation of the 20-kdalton light chain of brush border myosin by a calmodulin-dependent kinase.

Results similar to those reported here have appeared in preliminary form (52).

MATERIALS AND METHODS

Brush Border Isolation

Brush borders were isolated from chicken epithelial cells by a modification of the method of Mooseker et al. (56). Instead of vigorously 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. These larger sheets, which occasionally folded back on themselves during isolation, were more suitable for scoring contraction than the smaller groups of brush borders.

Other modifications of the isolation procedure included purifying the brush borders by loading them onto the sucrose step gradient in 50% sucrose (wt/wt in Solution A: 75 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 10 mM Imidazole-HCl pH 7.2) and floating them to the 40–50% sucrose interface, as suggested by Glenney and Weber (29), instead of pelleting them from 30% sucrose through 40% sucrose to the 40–50% interface, as was previously done (56). This modification significantly reduced contamination by nuclei which pellet through the 50% sucrose but which previously were trapped in the pad of brush borders at the 40–50% interface. To inhibit proteolysis, all solutions were made 0.1% (vol/vol) 10–20 trypsin inhibitor units [TIUs] per liter) in Aprotinin (Sigma Chemical Co., St. Louis, MO) and 0.1 mM in phenylmethylsulfonyl fluoride (PMSF: α -toluenesulfonyl fluoride, Eastman Kodak Co., Rochester, NY) which was diluted from a 100 mM stock made in 100% ethanol into aqueous solution immediately before use. Other protease inhibitors were tested for effectiveness in inhibiting proteolysis during brush border isolation including benzamide, diisopropylfluorophosphate, leupeptin, pepstatin A, and soybean trypsin inhibitor, but none of these inhibitors proved effective in completely inhibiting proteolysis in this system.

Isolated brush borders were demembrated (58) in Solution A containing 0.1 mM PMSF, 4% (vol/vol) Aprotinin, and 1–2% Triton-X-100 (Sigma Chemical Co.) or Nonidet P-40 (NP-40: Particle Data Laboratories, LTD., Elmhurst, IL). Demembrated brush borders were washed free of detergent either in Solution A or in Solution A with reduced concentrations of MgCl_2 (1 mM) and EGTA (0.1 mM), and pelleted at 2,000 g before use.

Contraction Studies

In all of the contraction studies presented in this paper the isolated brush borders were incubated at 37°C in a buffer containing 50 mM KCl, 5 mM MgCl_2 , 0.2 mM DTT, 25 mM PIPES, pH 6.9. This solution also contained Ca^{++} buffers consisting of ratios of CaCl_2 to EGTA of either 0:1 mM ($-\text{Ca}^{++}$, $<10^{-8}$ M free Ca^{++}) or 0.9:1 mM ($+\text{Ca}^{++}$, 1×10^{-6} M free Ca^{++}) and either no added ATP ($-\text{ATP}$) or 2–4 mM ATP ($+\text{ATP}$). The pH of the final solution was always readjusted to 6.9 after addition of Ca^{++} and EGTA to compensate for the hydrogen ions released when Ca^{++} binds to EGTA. 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. (62).

Contraction was assayed by resuspending brush borders in ice-cold buffer and immediately transferring them to a 37°C water bath. At specified times, the brush borders were fixed by adding 8% glutaraldehyde to a final concentration of 1%. They were then photographed in a light microscope with either phase contrast or differential interference contrast (Nomarski) optics. Alternatively, brush borders were resuspended in ice-cold buffer and immediately placed on nitric-acid-cleaned slides and warmed to 37°C on a temperature-controlled microscope stage. Contraction was then recorded on videotape and "stop-action" views were photographed directly from the monitor screen with a tripod-mounted 35 mm camera.

For ultrastructure studies, the brush borders were incubated in suspension as indicated above. They were then fixed and processed for electron microscopy by the method of Begg et al. (5). Micrographs of thin sections were taken with a Phillips 201 Electron Microscope at an operating voltage of 80 kV.

The effects of different compounds on contraction were determined either by adding them to the assay solutions instead of ATP, as in the cases of 2 mM AMP-PNP (Sigma Chemical Co.) and 2 mM ATP- γ -S (Boehringer Mannheim, Indianapolis, IN), or by adding them in addition to ATP, as in the cases of 0.2 mg/ml phalloidin (Boehringer Mannheim) and trifluoperazine hydrochloride (TFP—a gift from Smith, Kline, and French Laboratories, Philadelphia, PA). When TFP was tested for effect, all of the brush borders in the experiment were incubated in the assay solutions in the absence of ATP for 1 min on ice, to allow time for TFP binding to calmodulin before addition of ATP. The results of the contraction studies were determined in single-blind experiments in which one investigator scored qualitatively for extent of contraction without knowing the specific condition of the samples.

Phosphorylation Assay

In situ phosphorylation of brush border proteins was assayed either in Solution A at pH 7.0 with 1 mM MgCl_2 or in the PIPES solutions described above. When different concentrations of free Ca^{++} were required to determine the Ca^{++} -sensitivity, CaCl_2 was added to final concentrations from 0 mM to 1.1 mM. In all cases, the pH of the solution was readjusted after addition of CaCl_2 .

The stock of [γ - ^{32}P]ATP was used in the phosphorylation experiments made by adding [γ - ^{32}P]ATP ($<2,000$ Ci/mmol, Amersham, Arlington Heights, IL) to a stock of unlabeled ATP (Sigma Chemical Co.). This was then diluted into the reaction mixtures to give final concentrations of either 0.1 mM ATP (~ 0.1 Ci/mmol) or 2 mM ATP (0.005 Ci/mmol).

Phosphorylation of brush border proteins was determined by resuspending brush borders in the assay solutions and incubating them either at room temperature (21°–23°C) or at 37°C in a water bath. When trifluoperazine was tested for activity, all of the brush borders in the experiment were resuspended without ATP, either with or without TFP, and incubated on ice for 1 min before ATP was added and the brush borders were warmed. In all other experiments, ATP was included in the original suspension solution. The phosphorylation reaction was stopped at specified times by mixing an aliquot of the reaction mixture with an equal volume of 2x-Laemmli SDS- Sample Buffer (41) containing 2 mM EGTA and immediately boiling it for 1 min. Including EGTA in the sample buffer prevented calmodulin from migrating to a position that interfered with identification of the 20-kdalton light chain of myosin in SDS PAGE (see reference 8). The samples were electrophoresed on SDS (linear 5–15%) polyacrylamide gradient gels made with the buffers described by Laemmli (41). The gels were stained with Coomassie Blue by the method of Fairbanks, et al. (22) and dried for autoradiography. In some cases, the region of the gel containing the 20-kdalton light chain of brush border myosin (BBMLC₂₀) was excised with a razor blade either before or after drying, dissolved in 0.3 ml of 30% H_2O_2 at 60°C overnight, mixed with 3.0 ml of Aquasol (New England Nuclear, Boston, MA), and the radioactivity was counted in a scintillation counter. The amount of BBMLC₂₀ in each brush border sample was estimated by assuming a 1:1 molar ratio of BBMLC₂₀ to BB myosin heavy chain (56). The amount of brush border myosin heavy chain was then determined by comparing, by densitometry, its staining with the staining of the heavy chain of known amounts of a highly purified chicken skeletal muscle myosin standard that were electrophoresed in

the same gel. The protein concentration of the skeletal muscle myosin standard was quantitated by the method of Lowry et al. (45).

Analysis of the Relationship between Phosphorylation and Contractility

To compare levels of BBMLC₂₀ phosphorylation with extent of brush border contraction, equivalent samples of brush borders isolated at the same time were incubated at 37°C in 2 mM ATP (to score for contraction) or in 2 mM [γ -³²P]ATP (to determine BBMLC₂₀ phosphorylation). After incubation for specified times (1, 3, 5, and 10 min) aliquots of brush borders either were mixed with SDS, boiled, and electrophoresed as described above for determination of BBMLC₂₀ phosphorylation, or were fixed for light microscopy and scored for contraction. Contraction in this experiment was scored within the categories (–), no detectable contraction; (–/+), only a slight amount of contraction in a small percentage of the brush borders; (+/–), most brush borders contracted to variable extents; (+) contraction detectable in all brush borders, and (++) extensive contraction in all brush borders.

Isolation of Brush Border Myosin with ³²P-labeled, 20-kdalton Light Chains

Demembrated isolated brush borders were incubated with Ca⁺⁺ and [γ -³²P]ATP as described above. To stop the reaction and extract the ³²P-labeled myosin, the brush border sample was then mixed with an equal volume of 2× KI extraction solution containing 1.8 M KI, 0.2 M NaF, 10 mM MgCl₂, 2 mM EDTA, 2 mM EGTA, 20 mM NaH₂PO₄, 1 mM DTT, 10 mM ATP, 40 mM Imidazole-HCl, pH 7.0, cooled to 0°C, homogenized with a Dounce homogenizer, and spun at 100,000 g for 60 min. The supernatant was fractionated at 4°C on a 2.5 × 90-cm column of Bio-Gel A-15m (Bio-Rad Laboratories, Richmond, CA) equilibrated with 0.6 M KCl, 2 mM MgCl₂, 1 mM EDTA, 0.1 M NaF, 1 mM NaH₂PO₄, 1 mM DTT, 0.5 mM ATP, 20 mM Imidazole-Cl, pH 7.0. Before loading the brush border sample onto the column, a 50 ml front of 1× KI extraction buffer was run into the column (61). After loading the brush border sample, the column was run at 20 ml per h and 5 ml fractions were collected.

RESULTS

Ca⁺⁺- and ATP-dependent Contraction of the Terminal Webs in Brush Borders—Observations with Light Microscopy

In the presence of Ca⁺⁺ (1 μ M) and ATP (1–4 mM), the terminal webs of isolated brush borders dramatically contract. This contraction is temperature-dependent, occurring best at 37°C and only slowly at room temperature (20°–25°C). At the light microscope level of resolution, contraction is most easily visualized in fragments of epithelial cell sheets consisting of 10–25 brush borders still attached to one another by their intercellular junctions. Within these sheets, Ca⁺⁺- and ATP-dependent contraction of their terminal webs causes an extensive rounding of individual brush borders (Fig. 1*d* and *e*). This active rounding of each brush border changes the morphology of the sheet as a whole by fanning-out each brush border's microvillar array, thereby giving the apical surface of the sheet a scalloped appearance (Fig. 1*d* and *e*).

Brush borders incubated in Ca⁺⁺ (1 μ M) in the absence of ATP do not contract (Fig. 1*b*). Moreover, brush borders incubated at 37°C in the presence of 1 μ M Ca⁺⁺ (Fig. 1*b*) are indistinguishable, with light microscopy, from brush borders incubated in the absence of both Ca⁺⁺ (<10⁻⁸ M) and ATP (Fig. 1*a*).

Although Ca⁺⁺ alone had no significant effect, incubation of brush borders in ATP (with the free Ca⁺⁺ concentration buffered below 10⁻⁸ M) had a variable effect. ATP alone usually caused either no contraction or only a slight contraction (Fig. 1*c*) that was reminiscent of Ca⁺⁺- and ATP-dependent

rounding of brush borders. However, in some preparations, the effect of ATP alone was almost as great as the effect of Ca⁺⁺ and ATP (not shown), in both the number of brush borders that contracted and the degree to which individual brush borders contracted.

Although the dependence of brush border contraction on Ca⁺⁺ was somewhat variable, ATP was an absolute requirement. Brush borders resuspended in the absence of ATP (Fig. 1*a* and *b*) or in the presence of the nonhydrolysable ATP-analogues AMP-PNP (Fig. 1*f*) or ATP- γ -S (not shown) did not contract. Moreover, neither ATP-analogue had any effect on brush border morphology detectable at the light microscope level of resolution.

Phalloidin and trifluoperazine (TFP) were also tested for their effects on Ca⁺⁺- and ATP-dependent brush border contraction. Phalloidin, a dicyclic peptide that stabilizes actin filaments (77) and blocks Ca⁺⁺-dependent solation of microvillus cores (38), did not inhibit contraction at a concentration of 0.25 mM. This result indicates that neither microvillus solation nor depolymerization of actin within the terminal web is required for contraction.

In preparations where Ca⁺⁺ (1 μ M) was necessary for contraction, contraction was inhibited by 0.1 mM TFP, an antipsychotic phenothiazine that inhibits calmodulin activity (44, 48). However, TFP, even at concentrations as high as 0.5 mM, did not inhibit the amount of contraction that occurred in the presence of ATP, but in the absence of Ca⁺⁺. Moreover, brush borders incubated with TFP, Ca⁺⁺ (1 μ M), and ATP were typically indistinguishable from brush borders incubated with ATP in the absence of Ca⁺⁺.

The effects of all of the conditions for brush border contraction that were tested are summarized in Table I, where (++) indicates extensive contraction, (–) indicates no detectable contraction, and (+/–) indicates a variable amount of contraction—variable both in the number of brush borders contracted and the extent to which individual brush borders within a single preparation contracted.

Kinetics of Ca⁺⁺- and ATP-dependent Contractility

The time course of brush border contraction was examined in two ways. Contraction in specific clusters of brush borders warmed to 37°C on a temperature-controlled microscope stage was recorded continuously with videomicroscopy. In addition, contraction of brush borders warmed to 37°C in suspension was monitored by fixing aliquots of the brush border preparations at specified times. Both techniques, which yielded similar results, demonstrated that, in the presence of Ca⁺⁺ and ATP at 37°C, isolated brush borders do not contract immediately. Contraction occurs slowly over the course of 1–5 min.

Photographs of three videotape sequences of sheets of isolated brush borders that were resuspended in Ca⁺⁺ (1 μ M) and 2 mM ATP at 0°C, then immediately transferred to a temperature-controlled slide and warmed to 37°C, are shown in Fig. 2. The first sequence (Fig. 2*a*) is of a sheet of brush borders that had folded back on itself during isolation. In the sheet, there are ~10 brush borders that are still connected by their junctions. When the temperature reached 37°C (00:09:43), there was no immediate change. However, over the course of 3 min at 37°C (to 00:12:44), individual brush borders within the sheet progressively rounded, contorting the sheet so that it

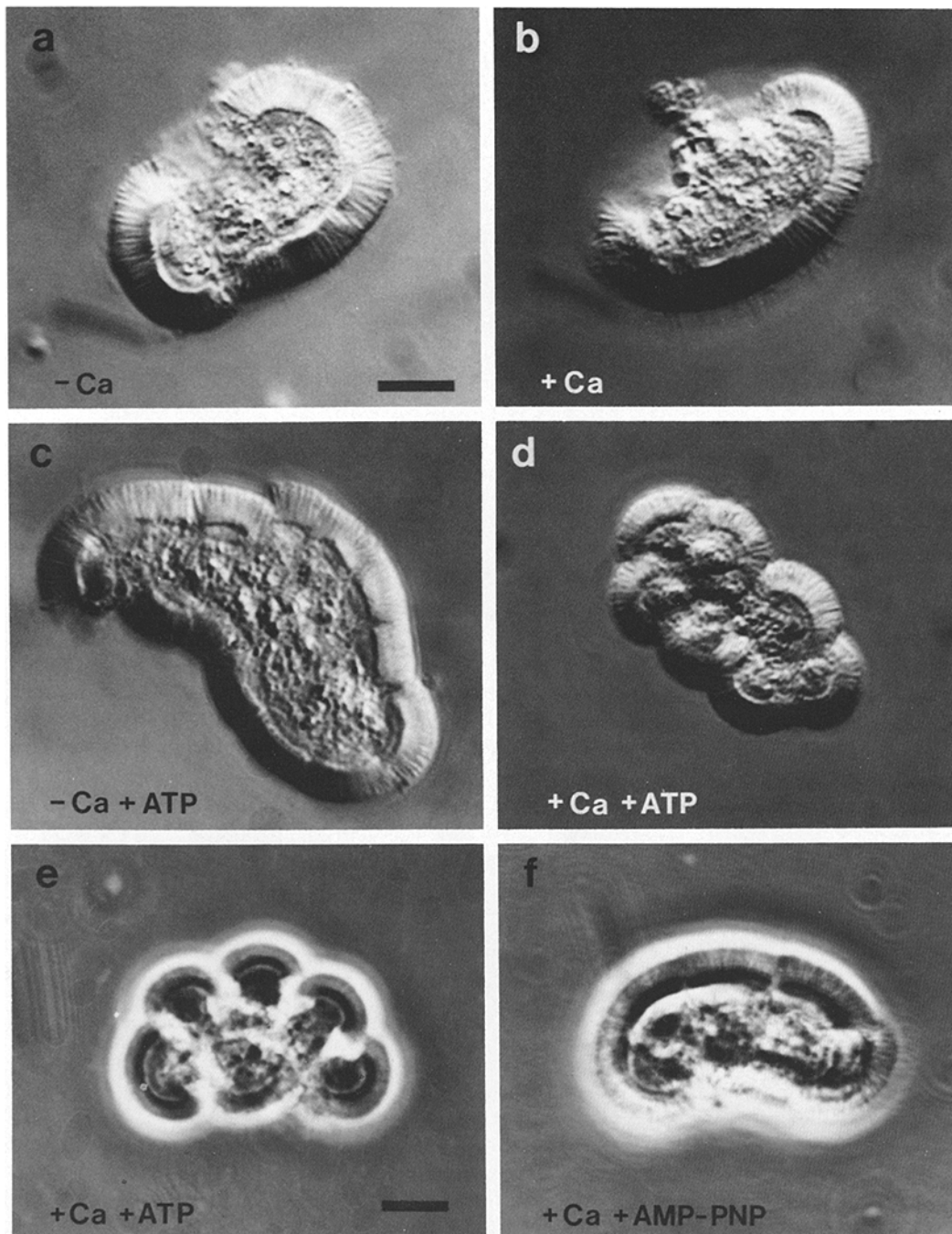


FIGURE 1 Light microscopy of contraction in isolated brush borders. Sheets of 5-10 brush borders interconnected by junctional complexes were suspended and incubated for 10 min at 37°C in solutions containing (a) <10 nM (free) Ca^{++} , no ATP (-Ca); (b) 1 μM Ca^{++} , no ATP (+Ca); (c) <10 nM Ca^{++} , 2 mM ATP (-Ca + ATP); (d and e) 1 μM Ca^{++} , 2 mM ATP (+Ca + ATP); (f) 1 μM Ca^{++} , 2 mM AMP-PNP (+Ca + AMP-PNP). In the presence of Ca^{++} and ATP (d and e), contraction of the terminal web region causes extensive "rounding" of the individual brush borders within the sheets. Slight contraction in the terminal web region occurs in the presence of ATP and absence of Ca (c); no contraction is observed in the absence of ATP or in the presence of the analog AMP-PNP. (a-d) Differential interference contrast optics; Bar, 5 μm ; \times 2,200. (e and f) Phase contrast optics; Bar, 5 μm ; \times 1,900.

was no longer in a single plane of focus. After 6 min (00:15:44), each individual brush border in the sheet was extensively rounded and the sheet itself was so contorted that half of it was completely out of focus.

In the second sequence, the extensive rounding of the indi-

vidual brush borders after 2 min at 37°C (17:37:45) had presumably disrupted the junctions between many of the brush borders. In this case, contraction within the brush borders caused an overall expansion and spreading of the sheet, creating the illusion of a deepening cleft where the brush border

TABLE I
Contraction of Brush Borders In Vitro

Conditions	Contraction
	37°C
-Ca	-
+Ca	-
-Ca+ ATP	+/-
+Ca + ATP	++
+Ca + AMP-PNP	-
+Ca + ATP- γ -S	-
+Ca + ATP + Phalloidin	++
+Ca + ATP + Trifluoperazine	+/-

Conditions tested for contraction of membrane-intact brush borders at 37°C. Brush borders were resuspended in 50 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.2 mM DTT, 25 mM PIPES, 0.1 mM PMSF, 10 μ g/ml Aprotinin, pH 6.9 with the following additions: 0.9 mM CaCl₂, for a final concentration of free Ca⁺⁺ of 10⁻⁶ M (+ Ca); 2 mM ATP (+ ATP), 2 mM AMP-PNP (+ AMP-PNP); 2 mM ATP- γ -S (+ ATP- γ -S); 0.25 mM Phalloidin (+ Phalloidin); and 0.1 mM Trifluoperazine hydrochloride (+ TFP). The brush borders were then incubated at 37°C for 10 min and scored for contraction by an investigator not aware of the solutions being tested. (++) represents extensive contraction, (-) represents no contraction, and (+/-) represents variable amounts of contraction, both in terms of extent and number of brush borders contracted.

had folded back on itself. Likewise, in the last example (Fig. 2c), contraction of the individual brush borders caused an overall expansion of the sheet. In all three cases, significant contraction of the brush borders required minutes in Ca⁺⁺ and ATP at 37°C, and contraction continued to progress for up to 5 to 6 min.

Although continuous monitoring of brush borders on a slide allowed visualization of contraction as it progressed in individual brush borders, contraction was inhibited under these conditions by a number of factors, including the continuous illumination required for recording and adhesion of the brush borders to the slides. Therefore, to make a more accurate assessment of the time necessary for contraction, the second method, in which brush borders were warmed in suspension to 37°C and then fixed at specified times, was routinely used to score contraction in large numbers of brush borders. Under these conditions in the presence of Ca⁺⁺, contraction was detectable in brush borders after 1 min of incubation and usually reached its maximum extent in <5 min. Results of a comparison of this time course of contraction with phosphorylation are presented below (see Fig. 9b).

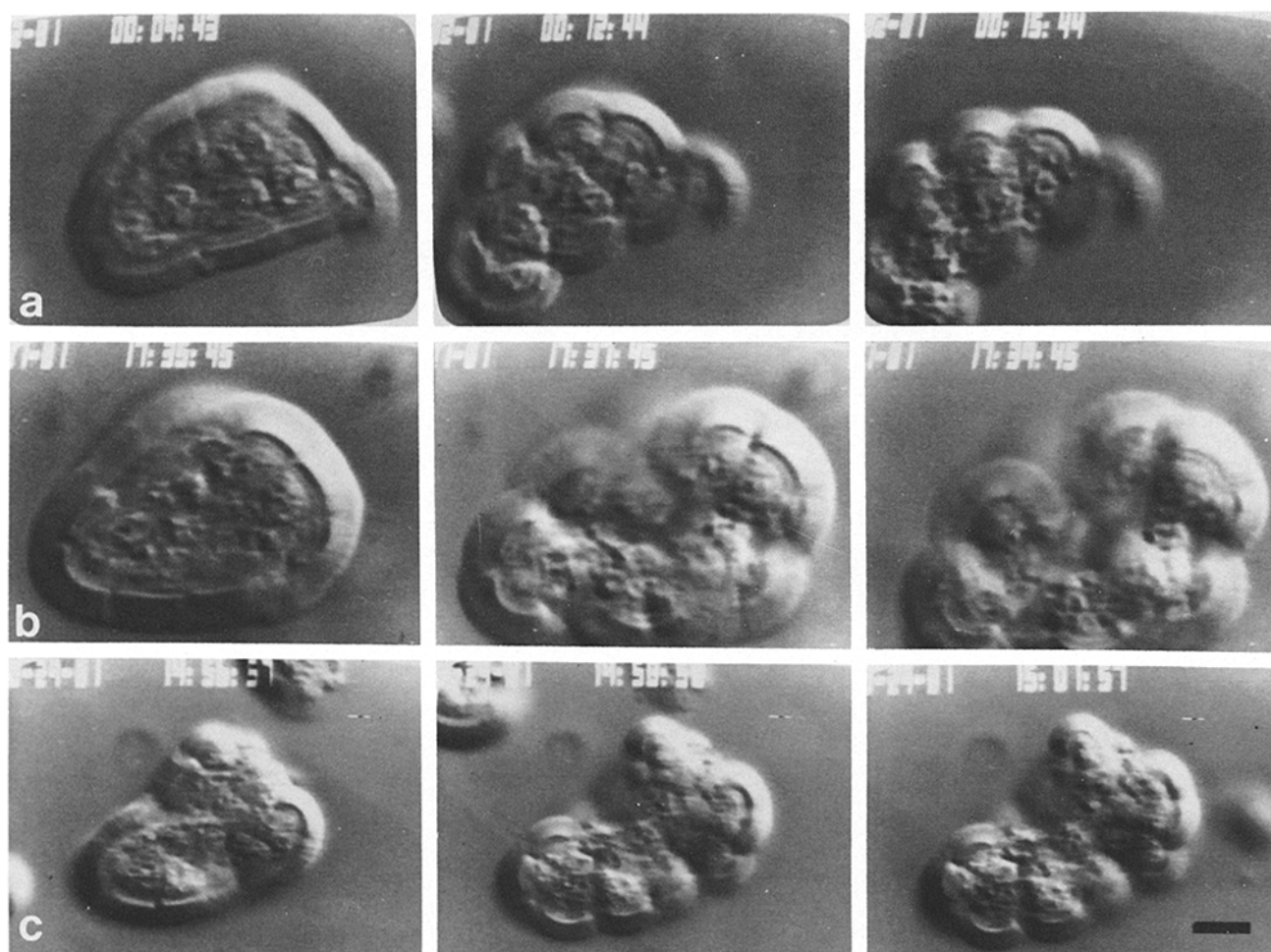


FIGURE 2 Kinetics of brush border contraction at 37°C. (a-c) Photographs of three video-taped sequences of brush border sheets that were suspended in contraction solution containing 1 μ M free Ca⁺⁺ and 2 mM ATP, transferred to a slide on a temperature-controlled stage and warmed to 37°C. The time elapsed is indicated at the top of each frame in h:min:s. The first frame in each sequence was recorded at the time the slide reached 37°C which usually required 10-15 s of warming. Bar, 5 μ m; \times 1,500.

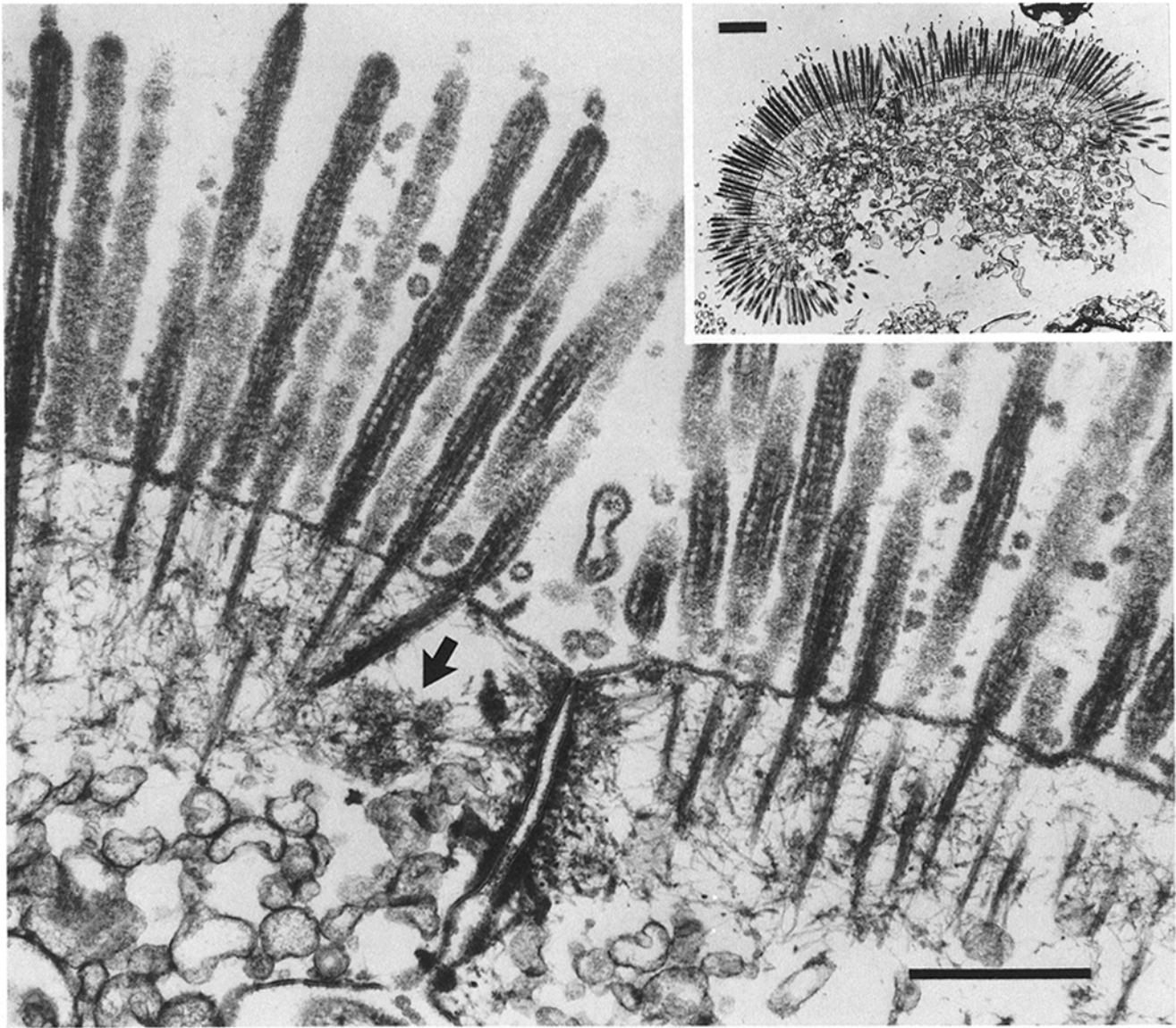
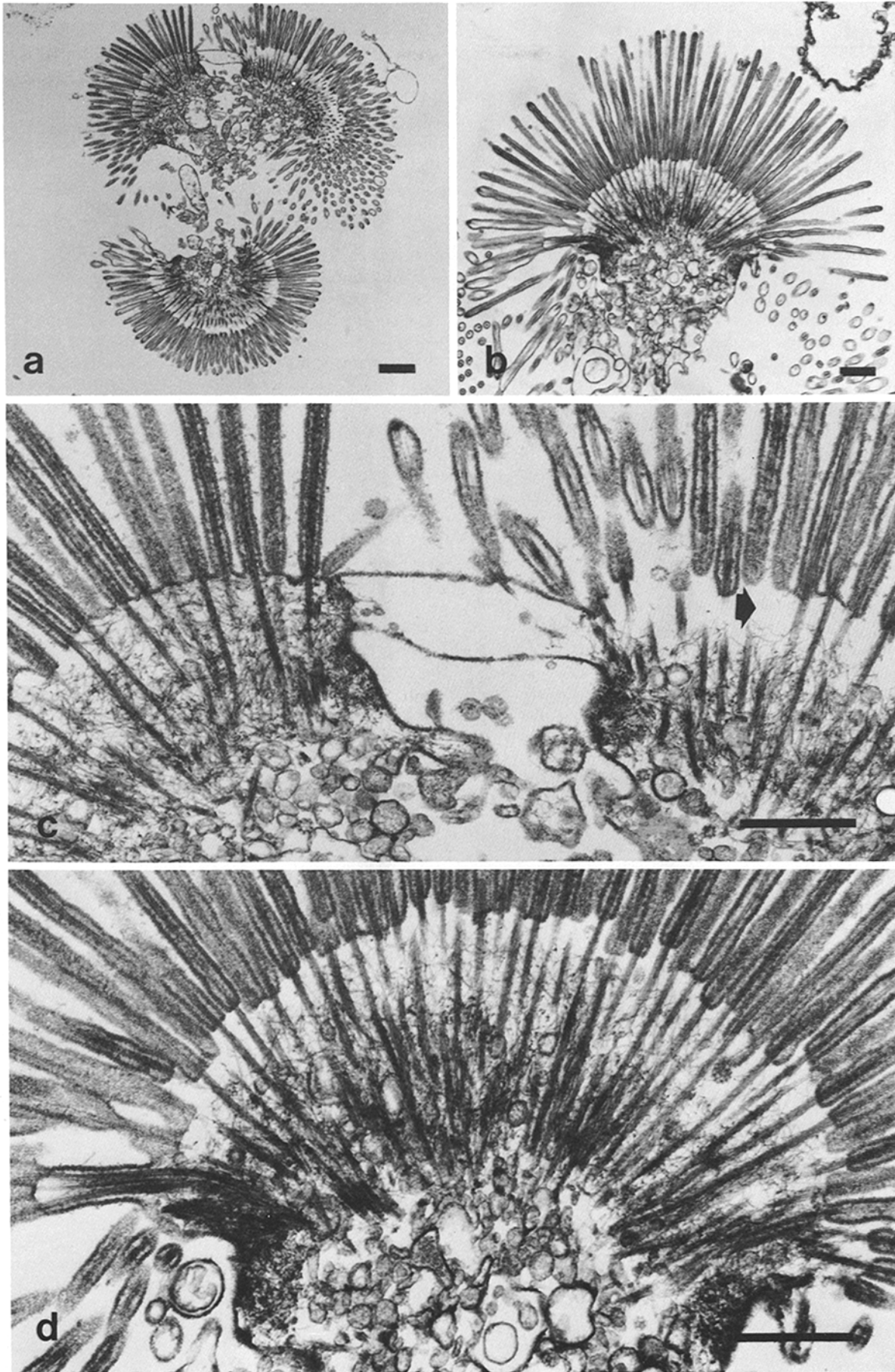


FIGURE 3 Electron microscopy of brush borders incubated at 37°C in the presence of Ca^{++} ($1 \mu\text{M}$) and absence of A1P. (Inset) Low magnification micrograph of a small sheet consisting of two brush borders joined by their junctional complex. The higher magnification micrograph enlarges the junctional complex. Note that at this concentration of Ca^{++} ($1 \mu\text{M}$) no solation of microvillus cores occurs, although some vesiculation of the microvillar membrane is apparent. In one of the brush borders the circumferential ring of filaments (arrow) has become detached from the zonula adherens. In these uncontracted brush borders, note the uniform distribution of filaments interdigitating with the rootlet portions of the microvillar cores along their full length in the terminal web. Bar, $0.5 \mu\text{m}$; $\times 54,000$. (inset) Bar, $1 \mu\text{m}$; $\times 6,700$.

FIGURE 4 Electron microscopy of brush border contraction. Isolated brush borders were incubated at 37°C for 10 min in the presence of $1 \mu\text{M}$ Ca^{++} and 2 mM ATP. (a) A cluster of three contracted brush borders that were most likely tightly connected to one another by junctions (as in Fig. 3) before contraction. Although the brush borders are still associated, the extensive contraction of their terminal webs has greatly disrupted the junctional connections between them. Bar, $10 \mu\text{m}$. $\times 6,300$. (b) Terminal web contraction in a single, isolated brush border. Bar, $0.5 \mu\text{m}$. $\times 12,000$. (c) Higher magnification of the junctional region between two of the brush borders in (a). The junctional region is torn apart, presumably as a result of the contraction in the terminal web regions of the brush border. In the brush border on the right, note the "clear zone" (arrow) in the terminal web directly below the plasma membrane which is free of interdigitating filaments between the core rootlets. Bar, $0.5 \mu\text{m}$. $\times 39,000$. (d) Higher magnification of the terminal web region of the brush border in (b). The lateral margins of the brush border appear to be "pinched in" at the level of the zonula adherens. Note the "clear zone" similar to that described in (c). Bar, $0.5 \mu\text{m}$. $\times 34,000$.



Electron Microscopy of Brush Border Contractility

Visualization of isolated brush borders with electron microscopy dramatically confirmed at the ultrastructural level the overall differences in morphology between contracted and noncontracted brush borders. At 37°C, incubation of brush borders in Ca^{++} ($1 \mu\text{M}$) with no ATP had no visible effect on terminal web ultrastructure (Fig. 3) and, except for some vesiculation of microvilli, brush borders incubated in $1 \mu\text{M}$ free Ca^{++} were indistinguishable from brush borders incubated with no added Ca^{++} (not shown). The lower magnification of the isolated sheet (*inset*) shows that after incubation in $1 \mu\text{M}$ Ca^{++} the brush borders were slightly curved, as they usually are after isolation. However, their junctions, although probably partially extracted during brush border isolation, still form close connections between the brush borders. The tight junctions remain connected and there is only a small intercellular space at the zonula adherens. In addition, numerous very thin filaments (5), probably composed of myosin (33, 36) and the 260/240 spectrin-like protein recently described (28, 29), are evenly distributed throughout the terminal web, with only slightly increased concentrations near the zonula adherens. At concentrations of free $\text{Ca}^{++} > 1 \mu\text{M}$, microvilli vesiculate and are lost, but their rootlets remain intact as does the overall morphology of the junctions and the terminal web (not shown).

As was seen with light microscopy, contraction in the presence of Ca^{++} ($1 \mu\text{M}$) and ATP causes extensive changes in brush border morphology. The major change that occurs in the brush borders during contraction is a constriction of the terminal web at the level of the zonula adherens (Fig. 4). This contraction pulls together the basal ends of the microvillus rootlets which constricts material in the terminal web and fans out the array of microvilli. Contraction also disrupts the junctions between brush borders by widening the intercellular spaces at the level of the zonula adherens. In some cases, contraction even rips tight junctions apart (Fig. 4*c*).

In addition to "pinching-in" the terminal web, contraction clears material from an area around the bases of the microvilli at the apical end of the terminal web (Fig. 4, arrow). This clearing, which is evident in most contracted brush borders, could result either from a redistribution of terminal web filaments that are already anchored primarily at the basal ends of the rootlets, or from an active recruitment of unanchored terminal web filaments to create an organized contractile apparatus which can then constrict the terminal web at that level. Alternatively, the clearing could be simply a sliding of the microvillus membrane sleeve up the microvillus. Clearing of material from the apical end of the terminal web is most evident in brush borders in which there is a loss of membrane

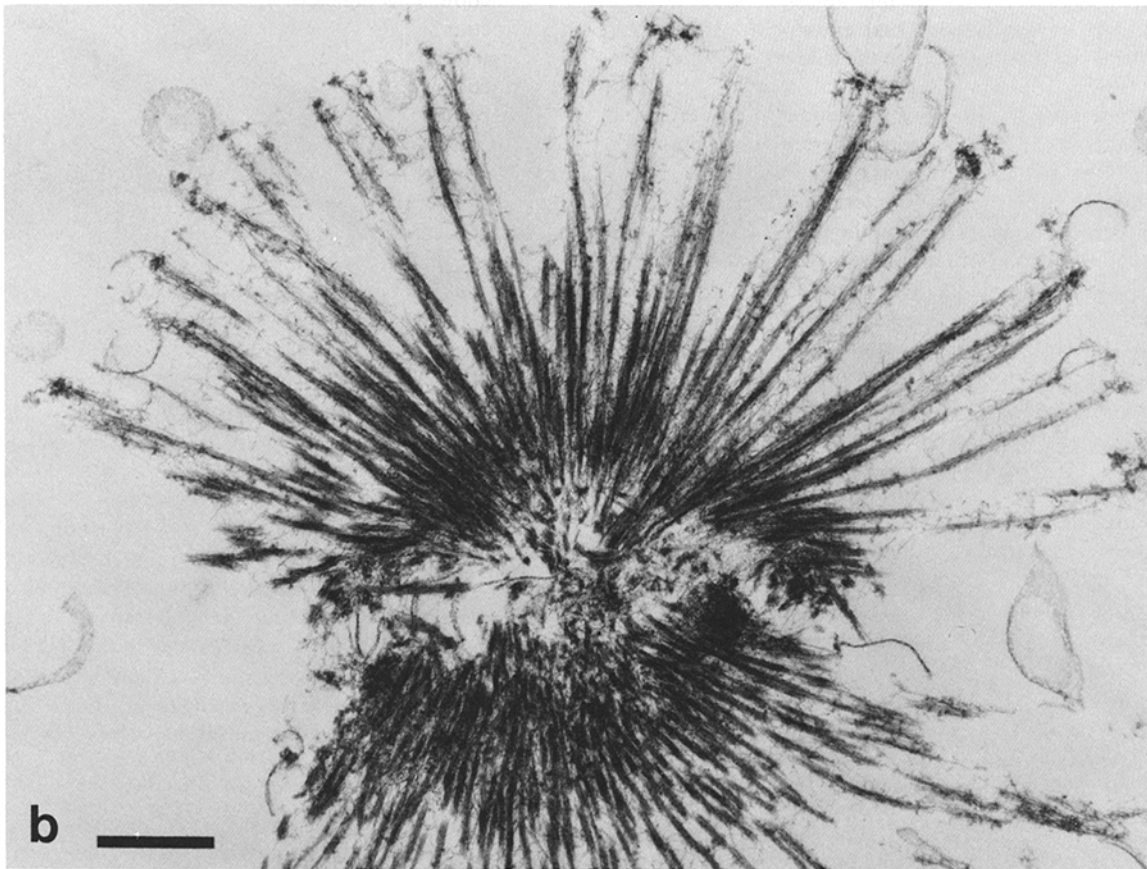
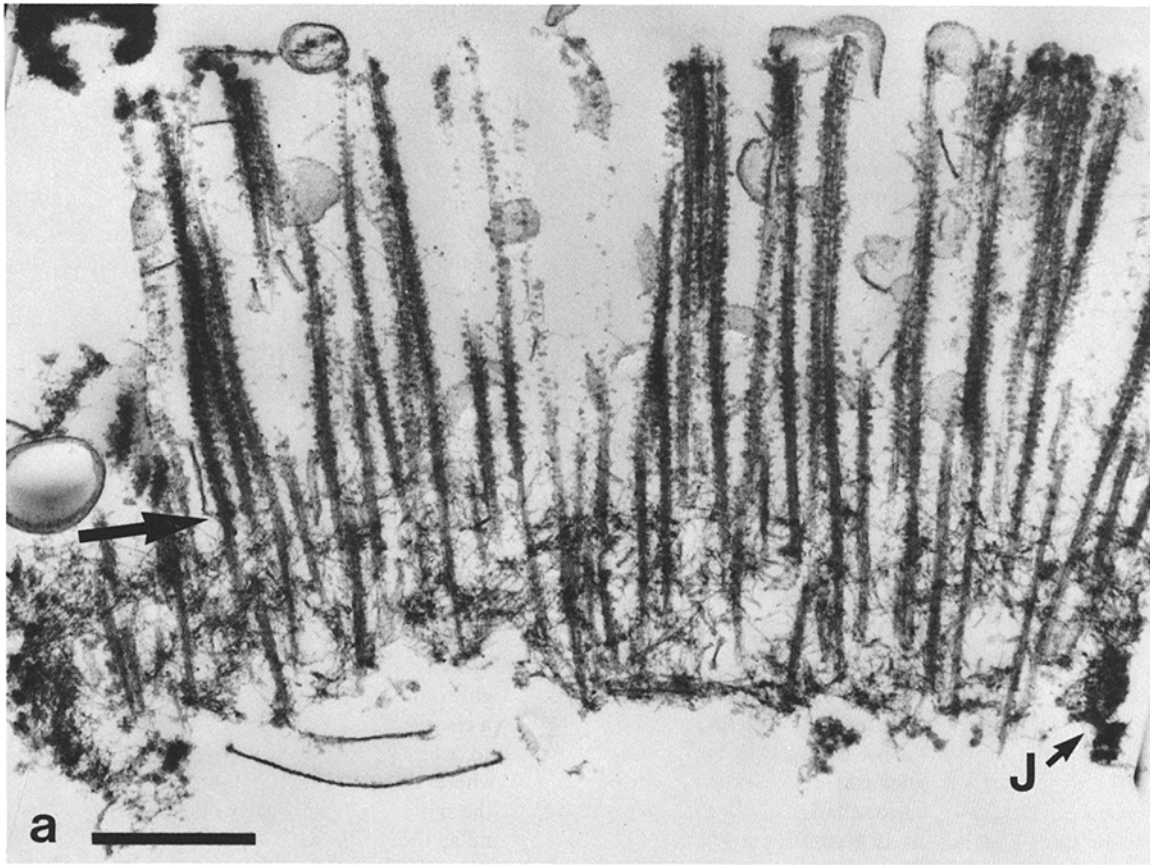
connections between the bases of the microvilli, and where ATP has caused a loss of bridges between the microvillus cores and the membrane (for example, see Fig. 4*d*). Conversely, clearing is much less evident in contracted brush borders where the microvillus membrane connections and core-to-membrane bridges remain essentially intact (Fig. 4*c*—the brush border on the left).

Both morphological changes—constriction of the terminal web and clearing of brush border material from around the bases of the microvilli—also occur in individual brush borders that are not associated with other brush borders in a sheet (Fig. 4*d*). This indicates that the entire motile structure is self-contained within single brush borders and does not depend on intact intercellular junctions or on the association of a brush border with adjacent brush borders.

In the absence of Ca^{++} ($< 10^{-8}$ M free), the effects of ATP are variable, as indicated above. Typically, electron microscopy, like light microscopy, reveals only small changes in the ultrastructure of ATP-treated brush borders (not shown). The structural changes that occur are reminiscent of, but usually not so great as, those that occur with Ca^{++} and ATP. In most preparations incubated with ATP alone, there is some evidence of "pinching-in" at the zonula adherens, because the area that was originally intercellular space becomes enlarged, and there is a slight rounding of the brush borders. In many cases, there is also a clearing of material from the apical end of the terminal web and a loss of microvillus bridges. In occasional preparations where extensive contraction occurs in the absence of Ca^{++} , the morphological changes that occur in ATP alone are the same as those that we have described for Ca^{++} -dependent contraction. This indicates that the mechanics of terminal web contraction are the same whether stimulated by Ca^{++} or not.

Demembrated brush borders also contract, indicating that contraction in isolated brush borders does not depend on the presence of the brush border membrane. During demembration with nonionic detergents, sheets of isolated brush borders are usually disrupted into individual brush borders. However, the overall curvature and cytostructure of the individual brush borders is not extensively changed by demembration. Although higher concentrations of free Ca^{++} ($> 1 \mu\text{M}$) cause microvillus solation, the cytostructure of demembrated brush borders incubated in Ca^{++} ($1 \mu\text{M}$) with no ATP (Fig. 5) resembles that of the membrane-intact brush borders treated with Ca^{++} (see Fig. 3). Very thin filaments interdigitate between the microvillus rootlets from the original level of the plasma membrane to the bases of the rootlets. The original position of the plasma membrane is delineated by the presence of the bridges that once connected the microvillus core to the membrane. These bridges are not present on the microvillus rootlets, below the level of the membrane (54).

FIGURE 5 Terminal web contraction in demembrated brush borders. (a) Demembrated brush borders incubated at 37°C in the presence of $1 \mu\text{M}$ free Ca^{++} and no ATP. At this concentration of Ca^{++} , core solation does not occur, and the cross-filaments which formerly linked the core to the membrane are evident along the length of the core to the point of insertion into the terminal web (arrow). Note the remnant of a junctional complex (J) which may include the circumferential filament bundle formerly associated with the zonula adherens. The ultrastructure of the terminal web region is similar to that in membrane intact brush borders (Fig. 3), except the underlying vesicles are removed by the detergent treatment. Bar, $0.5 \mu\text{m}$. $\times 43,300$. (b) Demembrated brush border incubated at 37°C in the presence of $1 \mu\text{M}$ Ca^{++} and 2 mM ATP. Note the "cupped" appearance of the terminal web region, and the close packing of the "rootlet" ends of the microvillar cores as compared to that in (a), which has presumably resulted from an active contraction of the terminal web region. In addition, partial solation of the tip-ends of core filaments has occurred and the membrane-filament bridges are absent. Bar, $0.5 \mu\text{m}$. $\times 30,000$.



The terminal webs of demembrated brush borders incubated in Ca^{++} ($1 \mu\text{M}$) and ATP contract (Fig. 5) as in membrane-intact brush borders. Moreover, this contraction causes a similar increase in the concentration of terminal web material. Unfortunately, the core-to-membrane bridges are lost from the cores in ATP under conditions that promote this contraction, making it impossible to state conclusively whether the increase in concentration of material in the terminal web is due to an active movement of the material toward the bases of the microvillus rootlets or simply to a lateral concentration of material due to constriction of the terminal web. Also, in the presence of ATP, Ca^{++} ($1 \mu\text{M}$) causes more solation of actin filaments in demembrated brush borders than occurs in the absence of ATP. Nevertheless, contraction of these brush borders in the absence of their membrane indicates that the anchorage for the forces of contraction and the proteins involved in contraction are totally contained within the detergent-insoluble cytostructure.

Phosphorylation of the 20-kDalton Light Chain of Brush Border Myosin

When isolated brush borders, both membrated and demembrated, are incubated at 37°C in ATP, many brush border proteins become phosphorylated (Fig. 6). The phosphorylation of only one of these proteins, however, is significantly increased by Ca^{++} under conditions in which brush borders contract ($+\text{Ca}^{++}$ -TFP). This protein of 20-kdaltons migrates on SDS polyacrylamide gels with the same mobility as the 20-kdalton light chain of brush border myosin and co-purifies with brush border myosin heavy chain when it is isolated from brush borders (results shown below). We have, therefore, identified this brush border phosphoprotein as a brush border myosin light chain. This myosin light chain is also similarly phosphorylated in a Ca^{++} -dependent manner in brush borders isolated from rachitic chickens (see Figs. of reference 37).

Of the other major proteins of the brush border cytostructure that have been previously identified (for review see reference 54)—the 260/240 spectrinlike terminal web proteins (28), myosin heavy chain and its 17-kdalton light chain, actin, calmodulin, as well as the microvillus proteins of 105-kdaltons, 94-kdaltons (flaccin or villin), and 70-kdaltons (fimbrin)—only one appears as though it might be phosphorylated under the conditions of our assay. A protein that is phosphorylated whether Ca^{++} is present or not, comigrates on SDS gels with the 260-kdalton polypeptide of the 260/240 spectrinlike complex. Nevertheless, positive identification of the 260-kdalton phosphoprotein as the 260-kdalton spectrinlike protein has yet to be made. Most of the other proteins that are phosphorylated are minor brush border proteins and the phosphorylation of some of those proteins is detergent-sensitive, indicating that either the phosphoproteins themselves or their kinase is removed by detergent.

In both membrated and demembrated brush borders, phosphorylation of BBMLC₂₀ (Fig. 6) increased greatly in the presence of Ca^{++} ($+\text{Ca}$, $-\text{TFP}$) at 37°C and usually reached a maximum within 5 min. In the absence of Ca^{++} ($-\text{Ca}$, $-\text{TFP}$), BBMLC₂₀ phosphorylation was much less than in the presence of Ca^{++} , but it also increased over the course of 5 min. In the particular experiment shown in Fig. 6, both in the presence and in the absence of Ca^{++} , there was slightly greater BBMLC₂₀ phosphorylation in the membrated brush borders than in the

demembrated ones isolated at the same time, but in other experiments (results not shown) there was little or no difference in their levels of BBMLC₂₀ phosphorylation. Furthermore, we have not detected any BBMLCK activity in the detergent wash (Keller and Mooseker, unpublished observations).

TFP, an antipsychotic phenothiazine that inhibits Ca^{++} -dependent contraction in brush borders (Table I), also inhibits Ca^{++} -dependent phosphorylation of the 20-kdalton light chain of brush border myosin (Fig. 6, $+\text{Ca}^{++}$, $+\text{TFP}$). However, the amount of BBMLC₂₀ phosphorylation that did not require Ca^{++} was also not inhibited by TFP, so that in the presence of TFP and Ca^{++} ($+\text{Ca}^{++}$, $+\text{TFP}$) there was an equivalent amount of BBMLC₂₀ phosphorylation as in the absence of Ca^{++} ($-\text{Ca}^{++}$, $\pm\text{TFP}$). Presumably, Ca^{++} -independent, TFP-insensitive BBMLC₂₀ phosphorylation is due to the presence of MLCK that has been proteolyzed during brush border isolation and incubation at 37°C , and is no longer Ca^{++} -calmodulin-dependent (see Discussion). TFP had no consistent effect on the phosphorylation of any other brush border protein (see also Fig. 1 in reference 52), but in some cases the overall level of phosphorylation was somewhat less in the presence of TFP, regardless of whether Ca^{++} was present or not (for example in Fig. 6, membrated brush borders, $-\text{Ca} + \text{TFP}$ and $+\text{Ca} + \text{TFP}$ and demembrated, $+\text{Ca} + \text{TFP}$).

To positively identify the 20-kdalton protein as a brush border myosin light chain, myosin was isolated by sieve chromatography from demembrated brush borders that had been incubated with Ca^{++} ($1 \mu\text{M}$) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under conditions where the 20-kdalton protein was phosphorylated with ^{32}P (Fig. 7). Although some dephosphorylation of all of the ^{32}P -labeled brush border phosphoproteins occurred during fractionation of the brush borders by sieve chromatography, the ^{32}P -labeled 20-kdalton brush border protein eluted in the same fractions as the 200-kdalton myosin heavy chain (Fig. 7), with an elution volume much less than would be expected of a protein of 20-kdaltons that was not complexed with a larger protein. Also present in these fractions was a 17-kdalton protein that together with the myosin heavy chain and the phosphorylated 20-kdalton protein comprises the native brush border myosin molecule.

Ca^{++} -sensitivity of Myosin Light Chain Phosphorylation

The sensitivity of brush border myosin light chain phosphorylation to Ca^{++} was determined by incubating isolated brush borders in $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and nine different concentrations of free Ca^{++} set with Ca/EGTA buffers between $<10^{-8}$ M and 10^{-4} M. (Fig. 8). The brush borders were incubated at 25°C during this assay to minimize proteolysis and therefore, maximize the Ca^{++} -dependence of BBMLC₂₀ phosphorylation. Phosphorylation of the BBMLC₂₀ was stimulated by concentrations of free Ca^{++} as low as $0.3 \mu\text{M}$ and was maximally stimulated by concentrations of free Ca^{++} of $1 \mu\text{M}$ and above. The phosphorylation of the other brush border proteins was affected little, if at all, by the different concentrations of free Ca^{++} .

Kinetics and Quantitation of BBMLC₂₀ Phosphorylation during Brush Border Contraction

Quantitation of the amount of P_i incorporated into BBMLC₂₀ (moles P_i /moles BBMLC₂₀) in the brush borders of Fig. 6

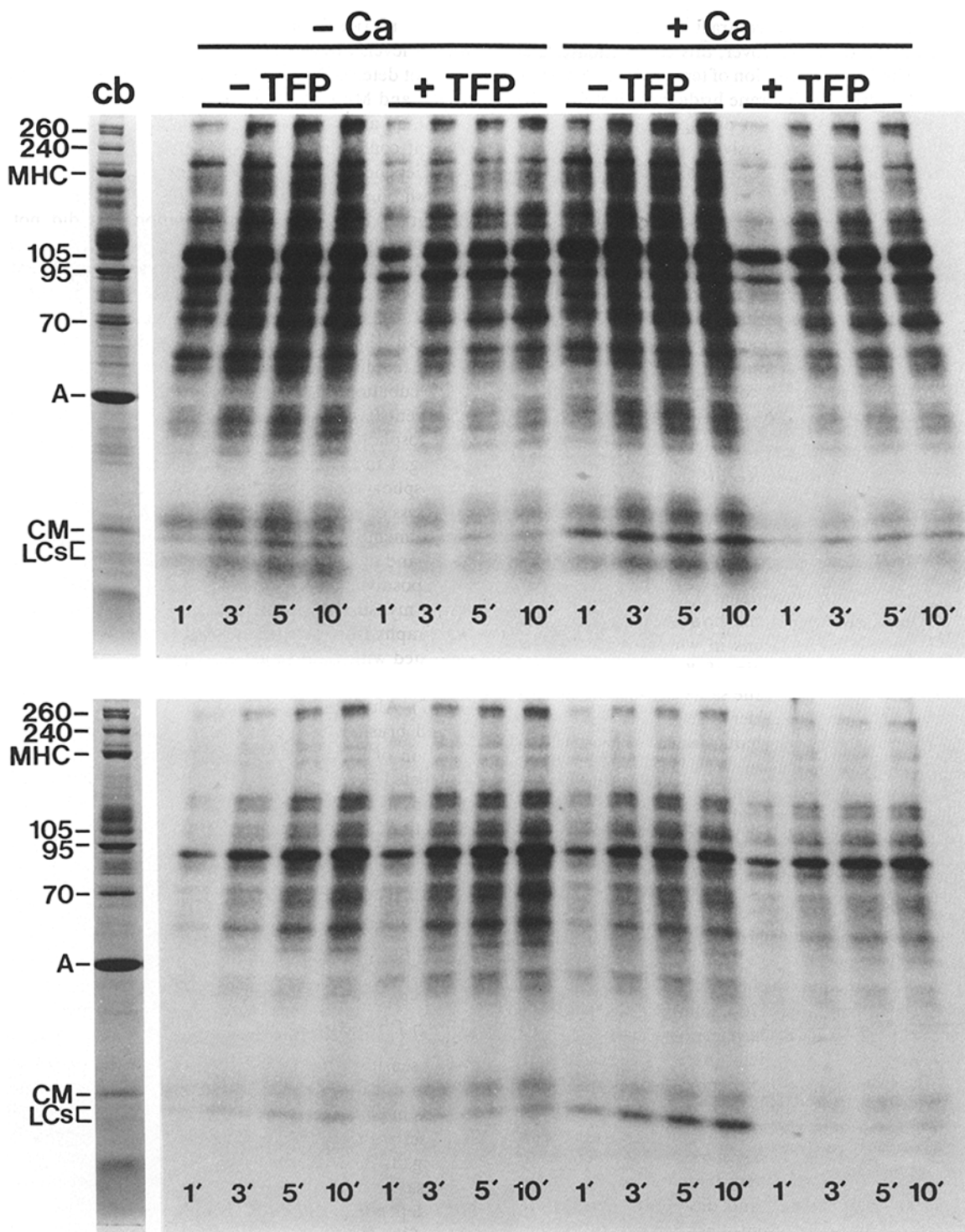


FIGURE 6 Analysis of protein phosphorylation during terminal web contraction in membrane-intact and demembrated brush borders. Brush borders and demembrated brush borders were incubated at 37°C in contraction solutions containing 2 mM [γ - 32 P]ATP in either the presence (+Ca; 1 μ M) or absence (-Ca; 1 mM EGTA) of Ca^{++} , and with (+TFP) or without (-TFP) 0.1 mM trifluoperazine. At the times indicated, aliquots were mixed with SDS and boiled. The samples were then electrophoresed on linear (5-15%) polyacrylamide gradient gels. Shown are autoradiographs of gels containing samples of membrane-intact (top) and demembrated brush borders (bottom). The first lane of each (*cb*) is the staining pattern observed with Coomassie Blue. In the presence of Ca^{++} (+Ca $^{++}$, -TFP), there is a rapid phosphorylation of BBMLC₂₀ to levels which are about twofold that observed in the absence of Ca^{++} (-Ca $^{++}$, -TFP) (see Fig. 9) in both membrane-intact and demembrated brush borders. This Ca^{++} -dependent activation is inhibited by TFP (+Ca $^{++}$, +TFP), although some nonspecific inhibition of protein phosphorylation is also observed. The migration of certain brush border proteins including the 260/240 spectrinlike doublet, (260/240); myosin heavy chain, (MHC); the 105-kdalton, (105), 95-kdalton villin/MV-95k, (95), and 70-kdalton fimbrin, (70), microvillus core proteins; actin, (A); calmodulin, (CM); and the two myosin light chains, (LCs) are indicated.

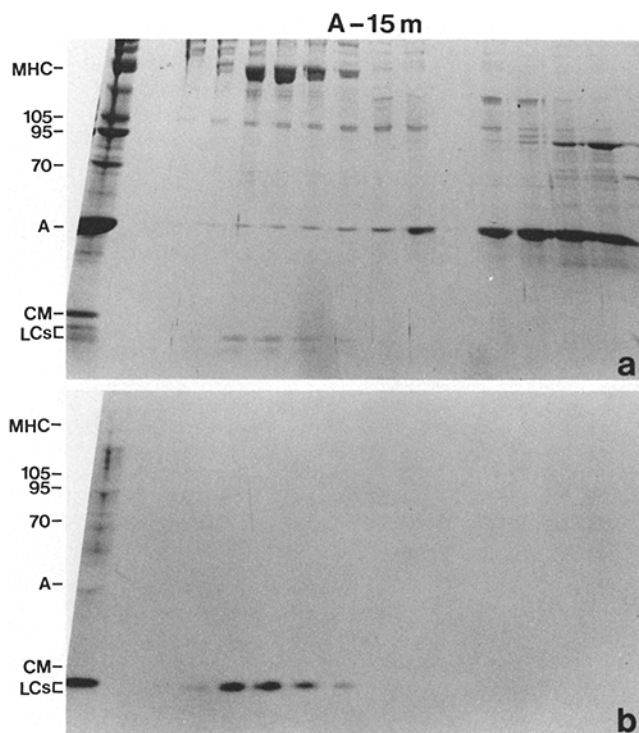


FIGURE 7 Purification of brush border myosin phosphorylated *in situ*. Demembrated brush borders were incubated in the presence of Ca^{++} and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 5 min at 25°C . The brush border proteins were then solubilized in KI-extraction buffer (see Materials and Methods) and chromatographed on a Bio-Gel A-15m gel filtration column. (a) SDS PAGE (5–15% linear gradient) of column fractions stained with Coomassie Blue. The first lane on left contains the solubilized brush border proteins loaded on the column. Partially purified myosin elutes just after the void volume. Molecular weight markers and abbreviations as in Fig. 6. (b) Autoradiograph of the gel in (a). The 20 kdalton protein which exhibits Ca^{++} -sensitive phosphorylation (Fig. 6) elutes with brush border myosin, identifying this protein as the regulatory light chain of myosin (BBMLC₂₀).

reveals that in the presence of Ca^{++} and ATP at 37°C —conditions in which brush borders contract— $>50\%$ of the brush border MLC_{20s} are phosphorylated within 3 min and $\sim 60\%$ are phosphorylated after 10 min. In this experiment, approximately half as many light chains (30–35% of total) were phosphorylated in the absence of Ca^{++} ($-\text{Ca}^{++}$, $\pm\text{TFP}$) or in the presence of Ca^{++} and TFP ($+\text{Ca}^{++}$, $+\text{TFP}$), as in the presence of Ca^{++} ($+\text{Ca}^{++}$, $-\text{TFP}$). As was pointed out previously, the dependence of both brush border contraction and BBMLC₂₀ phosphorylation on Ca^{++} varies from experiment to experiment, probably in relationship to the amount of proteolysis that occurs during brush border preparation. Although there was only a twofold stimulation of the BBMLC₂₀ phosphorylation in the experiment in Fig. 6, in other brush borders incubated at 25°C to maximize Ca^{++} -sensitivity there was greater than a fourfold stimulation of BBMLC₂₀ phosphorylation by Ca^{++} , that was completely sensitive to TFP (Fig. 8, and Fig. 1 in reference 52). Consistent with a greater Ca^{++} -sensitivity of BBMLC₂₀ phosphorylation there appears to have been less proteolysis of brush border proteins in the brush borders incubated at 25°C (Fig. 8, and Fig. 1 in reference 52) than in those incubated at 37°C (Fig. 6), because there is more (relative

to total brush border cytoskeletal protein) of the protease-sensitive 105-kdalton microvillus protein remaining in the brush borders in Fig. 8 than in Fig. 6.

To determine the relationship between both the timing and final level of BBMLC₂₀ phosphorylation and extent of brush border contraction, brush borders were incubated in suspension in the presence of 2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or 2 mM nonradioactive ATP. At specified times, samples of the ^{32}P -labeled brush borders were mixed with SDS-sample buffer and boiled for BBMLC₂₀ phosphorylation determination, and nonradioactive brush borders were fixed for light microscopy to assay for extent of contraction. The results of one experiment are shown in Fig. 9b. Within 1 min, $\sim 32\%$ of the BBMLC_{20s} were phosphorylated and some contraction was evident in most of the brush borders incubated in the presence of Ca^{++} . In brush borders incubated in the absence of Ca^{++} for the same time, there was no detectable contraction and only $\sim 16\%$ of the BBMLC_{20s} were phosphorylated. By 3 min, all of the brush borders in Ca^{++} had contracted to some extent and BBMLC₂₀ phosphorylation in those brush borders had reached 55%. In the brush borders in the absence of Ca^{++} only a small amount of contraction was detectable by 3 min and $\sim 25\%$ of the BBMLC_{20s} were phosphorylated. After 5 min, both BBMLC₂₀ phosphorylation and contraction had almost reached their maximum extents both in the presence and absence of Ca^{++} . All brush borders in the presence of Ca^{++} were tightly contracted, whereas the brush borders in the absence of Ca^{++} were contracted to variable extents, with most of the brush borders at least slightly contracted. Ten minutes of incubation did not significantly increase either BBMLC₂₀ phosphorylation or contraction in either of the conditions.

DISCUSSION

The Ca^{++} -dependent contraction of isolated chicken brush borders that we describe here is reminiscent of the Ca^{++} -independent “pinching-in” of isolated rat brush borders previously described by Rodewald et al. (63). However, improved techniques of specimen preparation for electron microscopy (5, 54, 57) have allowed us to observe and describe brush border contraction in greater detail. Although regulation by Ca^{++} of contractility in intestinal brush borders isolated from rat and chicken may differ, it is now evident that the overall morphology of terminal web contractility in both systems is similar.

Another epithelial system that has been stimulated to contract is pigmented retinal epithelia (58). When extensively glycerinated and incubated with ATP, individual cells within a sheet of retinal epithelium contract. The force for this contraction is attributed to a ring of actin filaments that encircles each of the cells at their apical end. Isolated chicken intestinal brush borders also have a circumferential ring of actin filaments at the level of their zonula adherens (39). It is possible that all of the force necessary for terminal web contraction in brush borders is generated within this ring by interactions between actin and myosin. However, we suggest that interactions between microvillus rootlets and myosin throughout the terminal web might also contribute force for contraction, because myosin is present between the rootlets (19, 33, 36, 56). An isotropic contraction of this network of myosin filaments anchored to microvillus rootlets throughout the terminal web could give the pattern of terminal web contraction that is observed.

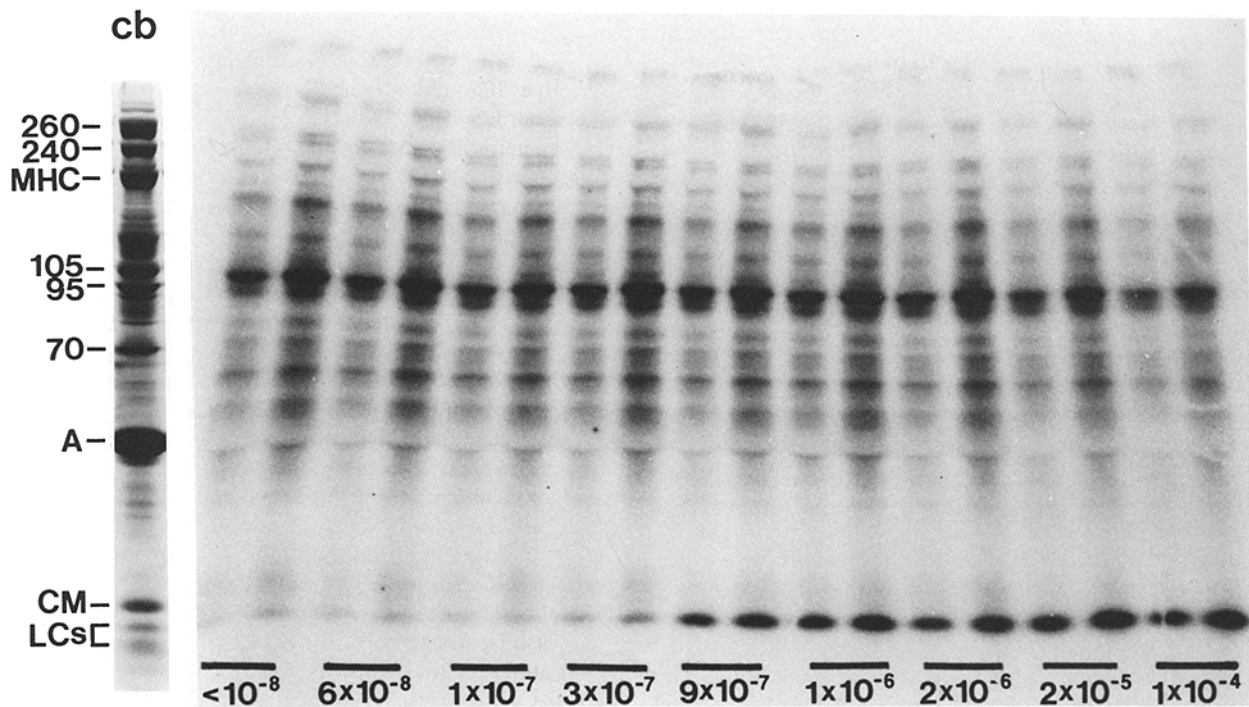


FIGURE 8 Ca^{++} -sensitivity of brush border myosin light chain phosphorylation. Demembranated brush borders were incubated in buffers containing 0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, and nine different concentrations of free Ca^{++} ions ranging from $<10^{-8}$ M-0.1 mM. Samples were boiled in SDS after 30 and 60 s of incubation at 25°C and electrophoresed by SDS PAGE (5-15% linear gradient). Shown is the autoradiograph and one lane from the stained gel (cb). Ca^{++} -activated phosphorylation of BBMLC₂₀ is maximal at $[\text{Ca}^{++}]$ above 1 μM . Abbreviations and molecular weight markers are as in Fig. 6. The calculated $[\text{Ca}^{++}]$ s are indicated at the bottom of the autoradiograph.

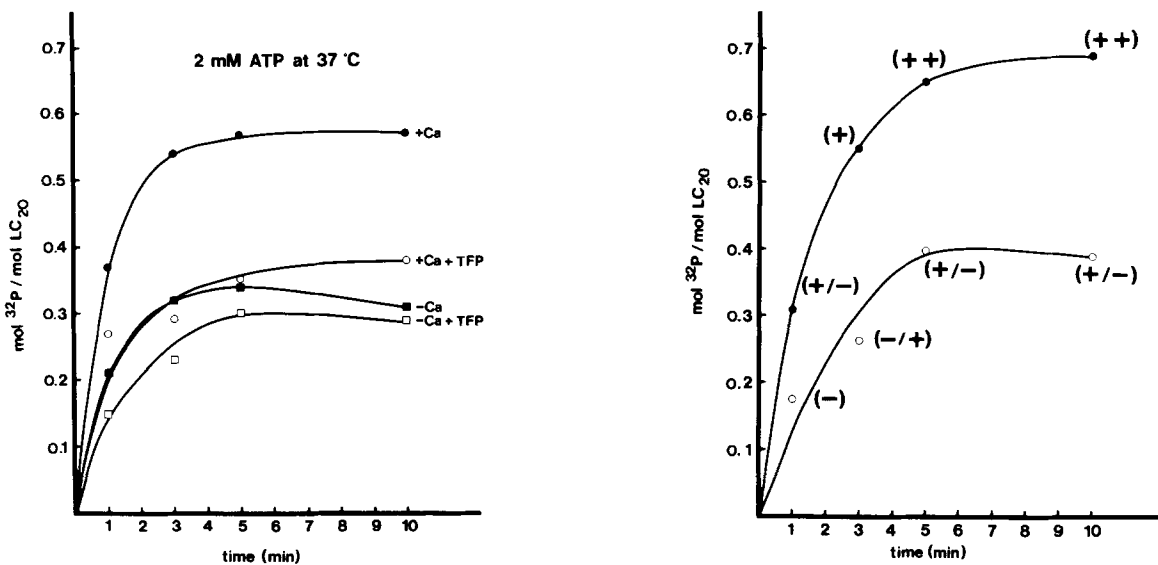


FIGURE 9 Quantitation of phosphorylation of BBMLC₂₀ during terminal web contraction in membrane-intact brush borders. (a) The BBMLC₂₀ bands were excised from the gel of membrane-intact brush borders shown in Fig. 6, and the amount of phosphate incorporated into each band was determined by scintillation counting. The amount of phosphate/mole of BBMLC₂₀ is plotted against time of incubation at 37°C (in min). Abbreviations as in Fig. 6. (b) In parallel experiments, brush borders were incubated at 37°C in 2 mM ATP or in 2 mM $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$. At 1, 3, 5, and 10 min, aliquots of the brush borders were mixed with SDS, boiled, and electrophoresed by SDS PAGE for determination of BBMLC₂₀ phosphorylation. After equivalent times of incubation, brush borders incubated in nonradioactive 2 mM ATP were fixed for light microscopy and scored for contraction within the categories of (-), no contraction; (-/+), a slight amount of contraction detectable in a small percentage of brush borders; (+/-), variable amounts of contraction in most brush borders; (+), contraction detectable in all brush borders; and (++) extensive contraction of all brush borders. The results are presented so that the score for the amount of contraction is adjacent to the determination of BBMLC₂₀ phosphorylation for the parallel sample.

The function of terminal web contraction in brush borders *in vivo* is unknown. If the time course of contraction that we observe in isolated chicken brush borders actually reflects the time course of brush border motility *in vivo*, terminal web contraction is not a rapid event, and probably does not agitate the contents of the intestinal lumen. Contraction of cells in other sheets of epithelia has been postulated to produce changes in shape, not only of the cells themselves, but also of the sheet or of the whole tissue. However, it seems unlikely that contraction in brush borders would be to change the shape of the intestinal epithelium which is already determined by its arrangement on the surface of the intestinal villi. One function for contraction of brush border terminal webs may be to change the morphology of intercellular junctions, as it does in isolated brush borders, thereby affecting the permeability of the intestinal epithelium.

Except for a fanning-out of their array caused by contraction of the terminal web, we have not observed any ATP-dependent movement of microvilli. Nevertheless, some types of microvillar motility, for example, rotation of the bundle of actin filaments to produce a screwing action of the helically arranged bundle-to-membrane cross-bridges within the microvilli, would not be detectable with the techniques of light and electron microscopy that we have used to observe brush borders in this study. Moreover, Ca^{++} -dependent solation of microvillus cores, if coupled to terminal web contraction, may play a vital role in brush border motility *in vivo*. At concentrations of free Ca^{++} greater than $1\ \mu\text{M}$, microvilli in membrane-intact isolated brush borders undergo what, without extensive analysis, appears to be brownian movement before they vesiculate and are lost (Keller and Mooseker, unpublished observations). Their flaccidity under these conditions is most certainly due to solation of their core bundles of actin filaments which, *in vivo*, might be closely regulated and coordinated with contraction of the terminal web. A rise in intracellular free Ca^{++} concentration could trigger both terminal web contraction and microvillus core solation.

Solation of microvilli requires only a rise in Ca^{++} concentrations to $5\text{--}10\ \mu\text{M}$, (although ATP might potentiate solation of microvilli at threshold levels of Ca^{++} , such as $1\ \mu\text{M}$), but terminal web contraction is an active process, also requiring ATP. Isolated brush borders do not contract in solutions in which AMP-PNP or ATP- γ -S are substituted for ATP. Neither AMP-PNP, a non-hydrolyzable analogue of ATP that relaxes rigor in skeletal muscle (18, 80), nor ATP- γ -S, which can be used as a substrate by myosin light chain kinase to phosphorylate myosin light chains (10), can be used as a substrate by actin-activated myosin ATPase (10, 18, 80). ATP, therefore, plays two roles in brush border contraction. One is to provide the energy required for cycling of actin-myosin cross-bridges. The other is to donate the phosphate group for myosin light chain phosphorylation.

The regulation of brush border contraction by Ca^{++} appears to be mediated by calmodulin. TFP, a potent inhibitor of calmodulin activity in a number of systems (44, 48) which can be used to distinguish between MLCK-linked and troponin-C-linked actomyosin regulation (68), inhibits both Ca^{++} -dependent brush border contraction and brush border myosin light chain phosphorylation. We cannot rule out the possibility that TFP inhibits brush border contraction by inhibiting some brush border system unrelated to the myosin light chain kinase (for example, see reference 34), but our results that brush

border contraction is both temporally correlated with, and dependent on, light chain phosphorylation indicate that Ca^{++} probably regulates brush border contraction in a manner similar to the myosin-linked regulation proposed for the control of smooth muscle contraction (1). That is, Ca^{++} binds to calmodulin and together they bind to myosin light chain kinase, stimulating its activity. The activated MLCK then phosphorylates the regulatory light chain of myosin which increases the actin-activated myosin ATPase resulting in force production. Consistent with this scheme is the previous finding that the ATPase activity of myosin isolated from brush borders was not activated by actin (56), presumably because the regulatory light chain of the isolated myosin was not phosphorylated.

The variability between different brush border preparations in the Ca^{++} -dependence of both contractility and myosin light chain phosphorylation is also consistent with this scheme for regulation of brush border contractility. It has been shown previously that when MLCK is partially proteolyzed, it loses its ability to bind Ca^{++} -calmodulin complexes and becomes maximally active in the absence of Ca^{++} (31, 73). Therefore, if brush border myosin light chain kinase is partially proteolyzed during brush border isolation, it might become active even in the absence of Ca^{++} . Moreover, TFP would not inhibit any MLCK activity or contraction that did not depend on Ca^{++} . This could explain why trifluoperazine inhibits only the extent of brush border contraction and myosin light chain phosphorylation that is not stimulated by Ca^{++} . The loss of Ca^{++} -dependence for contraction in brush borders makes it unlikely that there is another Ca^{++} -dependent regulatory system, for example Ca^{++} -dependent myosin (11) or Ca^{++} -dependent thin-filament control (21, 32, 49) unless, of course, that system is also labile and destroyed during brush border preparations.

Because intestinal brush borders are isolated from a digestive organ, inhibiting proteolysis during brush border preparation is a difficult problem. Numerous protease inhibitors have not been effective in controlling proteolysis in this system. One good indicator of the amount of proteolysis that has occurred in the brush borders is the amount of 105-kdalton protein remaining in the preparation. The 105-kdalton protein is one identifiable protein in the brush border that is particularly sensitive to proteolysis (54). Because the 105-kdalton protein is located exclusively in microvilli, it is probably not the BBMLCK. However, when the 105-kdalton protein is reduced in the preparation, there is less dependence of BBMLC₂₀ phosphorylation on Ca^{++} . For example, there is less 105-kdalton protein relative to total brush border cytoskeletal protein in the preparation in Fig. 6 than in Fig. 8, and correspondingly less Ca^{++} -dependence for BBMLC₂₀ phosphorylation. Consequently, it is very likely that variations in the amount of proteolysis of BBMLCK during brush border isolation cause variations in the Ca^{++} -dependence of both contraction and BBMLC₂₀ phosphorylation. The lack of Ca^{++} -regulation of contractility observed by Rodewald et al. (63), in isolated rat brush borders might well have been due to proteolysis of rat BBMLCK during brush border preparation and not to some fundamental difference between rat and chicken brush borders, because the experiments of Rodewald et al. (63) were done before the use of protease inhibitors during the isolation of intestinal brush borders became routine.

Of course, another possibility for the lack of dependence of isolated brush border contractility on Ca^{++} is that the BBMLC₂₀ in isolated brush borders is already phosphorylated.

This could result from myosin that was phosphorylated *in vivo* retaining its phosphate during brush border isolation. If this is the case, then additional phosphorylation *in vitro* would not be necessary for maximal actin-myosin interaction. If the myosin in isolated brush borders is, in fact, already phosphorylated, the Ca^{++} -dependent, TFP-sensitive increase in BBMLC₂₀ increase labeling with ³²P could be the result of an increased turnover of phosphate on the myosin due to the presence of a Ca^{++} -calmodulin-dependent phosphatase, in addition to the kinase. A Ca^{++} -calmodulin-dependent phosphatase has been recently isolated from rabbit skeletal muscle (71), and it appears to be identical to calcineurin in brain (40). In the presence of Ca^{++} in our assays, the number of BBMLC₂₀s that are labeled with ³²P may increase without increasing the percentage of BBMLC₂₀ that is phosphorylated.

Although isolation of brush borders containing phosphorylated myosin is a possibility, some of our experiments indicate that it may be an unlikely one. When assaying phosphorylation in isolated brush borders under conditions where ATP becomes quickly limited, the BBMLC₂₀s are first phosphorylated and then, over time, dephosphorylated (see reference 37, Fig. 5). This loss of label is due to dephosphorylation and not to a loss of the light chain, because addition of fresh [γ -³²P]ATP results in a relabeling of the BBMLC₂₀ (T. C. S. Keller, unpublished observations). Furthermore, successful isolation of brush border myosin with a ³²P-labeled light chain in this study (Fig. 7) required inclusion of NaF (a phosphatase inhibitor) in all of the solutions subsequent to labeling with ³²P. Although these results indicate that it is unlikely that brush borders isolated in the absence of ATP contain phosphorylated myosin, we are currently determining the actual amount of phosphorylated myosin in the brush borders as isolated. Gel systems that readily separate phosphorylated from non-phosphorylated myosin light chain in purified myosins (20, 59) have not successfully resolved the two forms of the light chain in whole brush border samples. Therefore, we are attempting to develop a gel system that will demonstrate the amount of phosphorylation present on light chains in isolated brush borders.

Phosphorylation of BBMLC₂₀, in addition to possibly increasing the myosin's actin-activated ATPase activity, might also affect the assembly properties of the myosin. Phosphorylation of the regulatory light chains of thymus (65, 66), platelet (66), and gizzard myosin (72) stabilizes filaments of those myosins in the presence of ATP *in vitro*. However, in relaxed smooth muscle, myosin is in bipolar filaments even when its light chains are not phosphorylated (70).

The state of myosin in uncontracted isolated brush borders is unknown, but purified brush border myosin does form bipolar filaments (56). Analysis of the terminal webs of mouse brush borders prepared for electron microscopy by a quick-freeze, deep-etch, rotary-replication technique indicates that the filaments that have been shown to be myosin in uncontracted terminal webs are physically no larger than is expected for a myosin dimer, myosin tetramer, or small myosin oligomer (35, 36). As of yet, there is no clear image of myosin in contracted brush borders, but the apparent redistribution of terminal web material in contracted brush borders that we have described may involve assembly of myosin into larger, possibly bipolar aggregates which could interact with antiparallel actin filaments to provide the force for contraction.

In addition to providing the force for contraction, myosin may also play a structural role in brush borders. It has been

recently shown that in the presence of Ca^{++} , arterial smooth muscle can maintain isometric stress even when the regulatory light chain of the myosin is not phosphorylated (17). This "latch" mechanism, as it has been named to suggest an analogy to the "catch" mechanism of mollusc myosin, requires little expenditure of energy to maintain its force, indicating that it may be important in maintenance of structure and tension. Nevertheless, phosphorylation of the MLC₂₀ is necessary for shortening or development of stress, indicating that cycling of actin-myosin cross-bridges requires light chain phosphorylation (17). In the brush border, when myosin is not phosphorylated and actively increasing contraction in the terminal web, it may be able to provide structural support for the microvillus rootlets. In this way, actin-myosin interaction might actually resist passive movement of microvilli when contraction is not occurring (47). If the role of myosin associated with microvillus rootlets is not purely structural, the myosin may also be involved in moving vesicles along those rootlets in the terminal web (for discussion of this possibility see reference 55).

The possibilities that myosin plays both a structural and contractile role and that it changes its state of assembly during contraction emphasizes two very important aspects of the isolated brush border system. These are the integrity and the dynamism of the brush border cytostructure. This cytostructure, as isolated, contains all of the machinery necessary for contraction, and when provided with the proper stimuli— Ca^{++} and ATP—can coordinate that machinery to give directed force. The brush border contractile machinery includes not only actin, which is a major component of the brush border structure, but also myosin, myosin light chain kinase, calmodulin, and possibly a myosin light chain phosphatase. All of these components remain associated with the cytostructure during isolation and demembration of brush borders. Nevertheless, the association of calmodulin and myosin light chain kinase must be such that in the presence of Ca^{++} and ATP, the calmodulin is able to bind to and activate the kinase. Furthermore, activated kinase, which is probably present in brush borders in amounts substoichiometric to myosin, must be able to phosphorylate many myosin molecules. The phosphorylated myosin, which in preliminary experiments we have found becomes "solubilized" from the brush border under contractile conditions (Keller, Howe, and Mooseker, unpublished observations), must then be able to interact with actin to develop the coordinated force necessary for contraction. Finally, the brush border cytostructure may also have a phosphatase associated with it that can dephosphorylate myosin light chains, because when ATP becomes depleted, myosin light chains are dephosphorylated (reference 37, Fig. 5).

Over the past several years, there have been numerous reports demonstrating the existence of Ca^{++} - and calmodulin-dependent myosin light chain kinase activity in a variety of smooth muscle and nonmuscle sources (1, 3, 14, 15, 20, 31, 67, 69, 76, 78). Also, myosin light chain phosphorylation has been directly correlated with increases in actin-activated myosin ATPase activity (1, 11, 43, 75) and with force production in both smooth muscle cells (2, 4, 10, 16, 17) and actomyosin threads spun from purified proteins (42). Furthermore, myosin light chain phosphorylation has been shown to be stimulated in whole cells under conditions that stimulate motility and secretion (24, 25). Nevertheless, we feel that our studies with isolated brush borders are a significant contribution to this growing body of knowledge, because they demonstrate a direct

causal link between myosin light chain phosphorylation and force production in an isolated, but intact, cytoskeletal system.

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Note Added in Proof: See the manuscript by D. R. Burgess in this issue (*J. Cell Biol.* 95:853-863) for a demonstration of terminal web contraction in glycerinated intestinal-epithelial cells.

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