BRUSH-BORDER CALMODULIN

A Major Component of the Isolated Microvillus Core

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ABSTRACT

Calmodulin is present in brush borders isolated from intestinal epithelial cells and is one of the major components of the microvillar filament bundle. Calmodulin was purified from either demembranated brush borders or microvilli by a simple boiling procedure. The boiled supernate derived from the microvillus cores contained one major polypeptide of 20,000 daltons. The supernate from the brush-border preparation contained the 20,000-dalton subunit and a second protein of 30,000 daltons. The 20,000-dalton subunit has been identified as calmodulin by several criteria: (a) heat resistance, (b) comigration with brain calmodulin on alkaline urea gels and SDS gels, both cases in which the 20,000-dalton protein, like calmodulin, exhibits a shift in electrophoretic mobility in the presence of Ca++, and (c) 4-5-fold activation of 3',5'-cyclic nucleotide phosphodiesterase in the presence but not the absence of Ca++. With a cosedimentation assay it was determined that brush-border calmodulin does not bind directly to actin. In the presence of Ca++ (>5 × 10^-7 M) there was a partial release of calmodulin from the microvillus core, along with a substantial conversion of microvillus actin into a nonpelletable form. The dissociation of calmodulin was reversed by removal of Ca++. If microvillus cores were pretreated with phalloidin, the Ca++-induced solubilization of actin was prevented, but the partial dissociation of calmodulin still occurred. The molar ratio of calmodulin:actin is 1:10 in the demembranated brush border and 1:2-3 in the microvillus core. No calmodulin was detected in the detergent-solubilized brush-border membrane fraction.

Movements of microvilli on the apical, brush-border surface of cells lining the intestine may facilitate the absorptive functions of this epithelium. In vivo observations of brush-border motility have been recorded (32, 38), although adequate documentation of these technically difficult observations is still lacking. Studies on the isolated brush border indicate that this organelle is a highly organized, motile apparatus comprised of actin, myosin, and various associated proteins that are organized in a functional configuration analogous to that determined for actin and myosin in striated muscle (5, 25-27). In vitro experiments on contractile models of the brush border have helped to reveal possible molecular mechanisms for microvillar movement (25, 30). The addition of MgATP and micromolar levels of free calcium ions to demembranated brush borders isolated from chicken intestine causes rapid retraction of the microvillus cores into and through the terminal web (25). Presumably, this contractile mechanism coupled to a relaxation mechanism, perhaps involving the extensive linkages of the microvillus cores to the plasma membrane (6, 24, 25, 27, 28), is responsible for cyclic movements of microvilli in vivo.

We hope to determine the molecular basis for the observed calcium sensitivity of this contrac-
tion, which most probably is actomyosin mediated (25, 26). There are a variety of regulatory mechanisms of actomyosin interaction, working separately or in tandem, which could involve calcium control. These include so-called actin-linked regulation by troponin-like proteins (see references 13 and 22 for reviews) and myosin-linked regulation through a calcium-activated myosin light-chain kinase (see reference 14 for a review) or through direct activation of the myosin as observed for myosins in molluscan muscle (36). Studies on purified brush-border myosin point to the involvement of a light-chain kinase because the properties of this myosin are virtually identical to those of other vertebrate, nonmuscle myosins (26), most of which have been shown to require phosphorylation to exhibit actin activation of Mg-ATPase activity.

As a first step in the investigation of calcium regulation of microvillar motility, we have explored the possibility that the ubiquitous calcium-binding protein calmodulin may be present in the brush border. Calmodulin is the Ca" regulatory subunit of the myosin light-chain kinase (12, 14, 34) and may also be a subunit in a protein complex analogous to troponin in nonmuscle cells (1, 14) and may also be a subunit in a protein complex analogous to troponin in nonmuscle cells (1, 14) as well as a regulatory subunit in a whole host of other enzymatic activities in eukaryotic cells (7, 9, 13, 14, 18, 22, 40). This includes the possible function of calmodulin as a regulatory component in intestinal secretion (17).

MATERIALS AND METHODS

Isolation of Brush Borders and Microvilli

Brush borders were prepared from chicken intestinal epithelium by the procedure of Mooseker et al. (26). Microvilli were separated from brush borders by procedures modified from Booth and Kenny (3) and Bretschler and Weber (4). To make microvilli, the final pellet of brush borders was suspended in 4 vol of solution A (75 mM KCl, 5 mM MgCl2, 1 mM ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA), 4 mM NaN3, 0.1 mM dithiotreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM imidazole, pH 7.3). Additional MgCl2 was added to bring the final concentration to 15 mM. The suspension was homogenized in a 15-ml Dounce homogenizer with a tight-fitting pestle. Release of microvilli was monitored by light microscopy. After 30-50 strokes, the suspension was centrifuged for 10 min at 2,000 g. The pellet contained brush borders; microvilli were collected from the supernatant by a 10-min centrifugation at 31,000 g. These low- and high-speed centrifugations were repeated once on the resuspended pellet of microvilli to remove any contaminating brush-border fragments.

One of the reviewers of this paper suggested the possibility that the NaN3 used in our isolation protocol could destroy a variety of enzymes to which calmodulin might bind. However, the distribution of calmodulin in the brush-border and microvillus preparations isolated in the absence of NaN3 was identical to that of our usual protocol. Nevertheless, brush borders prepared without azide are much more susceptible to proteolysis during storage.

Preparation of Brush-Border Calmodulin

Brush borders and microvilli were demembranated by suspension in 1% Nonidet P-40 (Particle Data Inc., Elmhurst, Ill.) in solution A (27). The brush borders were pelleted at 2,000 g for 10 min, and microvillus cores at 31,000 g for 15 min. This process was repeated several times to insure complete removal of membranes. The pellets were suspended in 10 mM imidazole, pH 7.3, and placed in a boiling water bath for 5 min. The solubilized membrane fraction from the first detergent wash was also boiled. These suspensions were then centrifuged for 30 min at 31,000 g and the supernates were frozen.

Preparation of Calmodulin and Calmodulin-depleted Phosphodiesterase from Brain

A fraction enriched in calmodulin was prepared from either fresh or frozen bovine brain by the procedures of Cheung (10) and Lin et al. (20).

The procedure of Cheung and Lin (11) was used for the preparation of cyclic 3',5'-nucleotide phosphodiesterase. Phosphodiesterase activity was measured using a two-step method from Cheung and Lin (11). The assay mix consisted of 0.01 M Tris-HCl, pH 7.5, 1.0 mM MgCl2, 2.0 mM 3',5'-cAMP, and either 0.1 mM CaCl2 or 1.0 mM EGTA. Inorganic phosphate was measured by the method of Taussky and Schorr (37).

Other Methods

Actin was purified from chicken breast muscle by the procedure of Spudich and Watt (35). Protein concentrations were determined by the method of Lowry et al. (21). Polyacrylamide gel electrophoresis in the presence of SDS was performed by the methods of Fairbanks et al. (16) and Laemmli (19). Gel electrophoresis in the presence of 5 M urea was performed with an electrode buffer of 80 mM glycine, 20 mM Tris-HCl, pH 8.5. Protein samples were made in the same buffer containing 8 M urea and either 0.1 mM CaCl2 or 5 mM EGTA. Gels were stained by the method of Fairbanks et al. (16) and were scanned at 633 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). Protein peaks were quantitated by cutting and weighing. Light micrographs of purified microvilli were made on Kodak SO 115 35-mm film with a Zeiss Axiomat equipped with differential interference contrast optics. Electron microscopy of microvillus cores was performed by the method of Begg et al. (2).

RESULTS AND DISCUSSION

Identification of Brush-Border Calmodulin

Calmodulin was identified in preparations of demembranated brush borders and microvillus cores prepared from isolated microvilli (Fig. 1) but was not present in preparations of the detergent-solubilized brush-border membrane. Suspensions of these fractions were heated to 90°C for 5 min, and the high-speed supernates were analyzed for
the presence of calmodulin. The brush-border supernate contains two prominent major polypeptides of apparent molecular weights of 20,000 and 30,000 daltons (Fig. 2). The larger subunit is probably tropomyosin (25), another heat-stable protein, and the 20,000-dalton subunit has been identified as calmodulin (see below). The supernate from the microvillus preparation contains one major polypeptide, the 20,000-dalton subunit (Fig. 2). In both brush-border and isolated microvillus preparations, this extraction procedure results in a quantitative release of the 20,000-dalton subunit into the high-speed supernate, suggesting that the 20,000-dalton band contains a single polypeptide. A similar analysis of heated supernates prepared from the solubilized brush-border membrane fraction indicated that no 20,000-dalton subunit is released from the brush border during membrane removal. This result indicates a tight association between calmodulin and some as yet unidentified component(s) of the isolated brush-border motile apparatus. The approximate molar ratio of calmodulin to actin, based on scans of Coomassie Blue-stained gels, is 1:10 in the demembranated brush border and 1:2-3 in the isolated microvillus core. Calmodulin is a major component of the isolated microvillus core, but we cannot exclude the possibility that calmodulin is present in the terminal web region of the brush border as well. Removal of microvilli from brush borders is not quantitative, and only relative values for amounts of calmodulin in these structures can be obtained with this approach. Nor can we exclude the possibility that some of the calmodulin or other proteins may be redistributed or lost during brush border isolation, a problem inherent in any cell fractionation procedure. Nevertheless, the results we have obtained for the protein constituents of the brush border and isolated microvillus are highly reproducible, evidence in support of the conclusion that calmodulin, in vivo, is tightly associated with the contractile apparatus of the brush border at low levels of free Ca++. 

The 20,000-dalton subunit purified from the brush border has been identified as calmodulin by several criteria. Like calmodulin, the brush border subunit is a heat-resistant protein. Another characteristic of calmodulin is its differential electrophoretic mobility in the presence vs. absence of Ca++ on polyacrylamide gels containing either urea (15) or SDS (8). This same anomalous behavior is exhibited by the 20,000-dalton brush-border subunit on urea (Fig. 3) or on SDS gels. On Fairbanks (16) tube gels, which contain EDTA, calmodulin and the 20,000 dalton subunit have an apparent molecular weight slightly larger than the 19,000-dalton myosin light chain (26) (Fig. 2), but, on Laemmli (19) slab gels, the 20,000-dalton subunit is resolved as single bands at either 20,000 or 17,000 daltons or as a closely spaced band. 

Figure 1 Microvilli purified from brush borders of the chicken intestine. Differential interference contrast light micrograph.
doublet of both bands. This variation is correlated with the presence or absence of Ca"+ in the protein sample applied to the gel (Figs. 4 and 5). Another diagnostic for calmodulin is the Ca"+-sensitive activation of 3',5'-cyclic nucleotide phosphodiesterase (40). Although other calcium-binding proteins such as troponin C activate phosphodiesterase, the concentrations needed are several 100-fold higher than that of calmodulin (1, 40). The 20,000-dalton subunit purified from either brush-border or microvillus preparations activates, in a saturable way, phosphodiesterase activity in the presence but not the absence of calcium. We have observed, on the average, a 4-5-fold activation of phosphodiesterase by the putative brush-border calmodulin preparations in the presence of Ca"+. Results from one such experiment are listed in Table I. We conclude from these results that the 20,000-dalton subunit of the microvillar filament bundle is calmodulin.

Interaction of Brush-Border Calmodulin with F-actin
The ratio of calmodulin to actin (1:2-3) in the isolated microvillus core raises the possibility that calmodulin interacts directly with the actin filaments of the microvillus. No protein other than actin is present in amounts sufficient to interact with calmodulin in molar ratios of calmodulin: binding protein that are less than or equal to 1. To test the possibility that calmodulin binds directly to actin filaments, brush-border calmodulin was mixed with F-actin purified from chicken skeletal muscle using solution conditions identical (i.e., solution A) to those under which calmodulin remains tightly bound to the microvillus core. When the F-actin-calmodulin mixture was centrifuged at 100,000 g for 90 min, calmodulin remained in the supernate, along with some actin, but no calmodulin was present in the F-actin pellet (Fig. 4). The same result was obtained when 0.2 mM CaCl2 was substituted for EGTA in the incubation solution (results not shown). It seems likely, then, that calmodulin is attached indirectly to the actin filaments of the microvillus through its interaction with one or several of the other proteins of the microvillus core, which include subunits of 70,000, 95,000, and 105,000 daltons (Fig. 5) (see also references 6 and 24).
FIGURE 5 Effect of Ca²⁺ microvillus cores. (A) Extraction of MV cores with solutions containing >10⁻⁶ M Ca²⁺ results in partial solubilization of actin (A), 70,000- and 95,000-dalton subunits, and calmodulin. Removal of Ca²⁺ before centrifugation (10⁻⁴ + EGTA) results in reassociation of the calmodulin, most of the 95,000-dalton subunit, and some actin into the pellet (P) fraction. (B) As in A, except that MV cores were preincubated with phalloidin (0.05 mg/ml) before Ca²⁺ extraction. The Ca²⁺-induced solubilization of actin and 95,000-dalton subunit is inhibited, but the partial release of calmodulin and 70,000-dalton still occurs. Addition of EGTA before centrifugation (10⁻⁴ + EGTA) causes reassociation of calmodulin and 70,000-dalton into pellet fraction.

Effects of Calcium on Calmodulin Binding and Microvillus Core Structure

To determine whether Ca²⁺ might cause a release of calmodulin from the microvillus core, detergent-treated microvilli were extracted with solutions containing various levels of free Ca²⁺ using Ca²⁺-EGTA buffers (29). After a 30-60 min incubation, the microvilli were centrifuged at 80,000 g for 90 min, conditions which should pellet the microvillus cores and most free actin filaments. The resulting supernates and pellets were analyzed by SDS gel electrophoresis and phosphodiesterase assay (Fig. 5a). At levels of Ca²⁺ above 5 x 10⁻⁷ M there was a partial release of calmodulin (~30%) into the supernate. However, there was also substantial solubilization of microvillus actin, the 70,000-, and the 95,000-dalton subunits. Reextraction of the pellet fractions with fresh Ca²⁺ solutions did not result in additional solubilization of calmodulin or other microvillar proteins (results not shown). Removal of Ca²⁺ by the addition of EGTA before centrifugation (Fig. 5) results in a reassociation of the calmodulin into the pellet fraction, along with much of the 95,000-dalton subunit. The amount of actin and 70,000-dalton

| Source of calmodulin | Concentration | EGTA | Ca²⁺ |
|----------------------|--------------|------|------|
| PDE alone (basal activity) | | 29 | 36 |
| PDE + brain | 600 | 38 | 108 |
| PDE + BB | 220 | 46 | 157 |
| PDE + BB | 0.8 | 38 | 151 |
| PDE + MV | 80 | 16 | 146 |
| PDE + MV | 0.8 | 40 | 153 |

For each assay, 0.2 mg/ml calmodulin-depleted phosphodiesterase (PDE) was used. See text for assay conditions. Calmodulin fractions from brain, brush border (BB), and microvilli (MV) were prepared as described in the text. Saturation of the PDE with calmodulin occurred at ~1.0 µg calmodulin/ml of assay.

TABLE I

Activation of 3',5'-Cyclic Nucleotide Phosphodiesterase by Brush-Border Calmodulin
FIGURE 6 Effects of Ca\(^{++}\) on the microvillar filament bundle. Electron micrographs of thin sections through pellets of microvillus cores after extraction with Ca\(^{++}\). See Fig. 5 and the text for details of experimental procedure. (a) \(10^{-9}\) M Ca\(^{++}\). The microvillus cores are intact. (b) \(10^{-7}\) M Ca\(^{++}\). Substantial disruption of the microvillar bundles has occurred, suggesting a loss of bundle cross-links. (c) \(10^{-4}\) M Ca\(^{++}\). Filament bundles have been completely dispersed and straight, "normal-looking" actin filaments are absent. Instead, microfilamentous meshworks are observed. (d) \(10^{-4}\) M Ca\(^{++}\). EGTA was added before centrifugation to lower the Ca\(^{++}\) concentration to <\(10^{-6}\) M. Straight actin filaments and some cross-linking of filaments into bundles are observed, suggesting that the disruptive effects of Ca\(^{++}\) on microvillus structure may be reversible. Bar, 0.2 \(\mu\)m. \(\times\) 80,000.
calmodulin with the isolated microvillus core. Al-
determine the role of Ca" in the association of
versed by the removal of Ca"(Fig. 6).
elevated Ca" levels which may be partially re-
duced transformation of microvillus actin into the
pelletable form was completely inhibited, along with the release of the 95,000-dalton subunit. The partial release of calmodulin and the 70,000-dalton subunit still occurred, although there was, in some experiments, a slight reduction in the amount of calmodulin released by Ca" treatment.

Preliminary ultrastructural analysis of these preparations is consistent with the biochemical results in that we have observed complete disruption of filament bundles and filament structure at elevated Ca" levels which may be partially reversed by the removal of Ca" (Fig. 6).

The original intent of these experiments was to determine the role of Ca" in the association of calmodulin with the isolated microvillus core. Although the addition of Ca" does result in a partial release of calmodulin from the microvillus core, the specificity of this effect is difficult to determine given the drastic disruptive effects of Ca" on microvillus structure. However, the results obtained with phalloidin-treated microvillus cores indicate that the Ca"-dependent release of calmodulin and the structural changes induced by Ca" are distinct phenomena (which may or may not be related). Thus, the partial dissociation of calmodulin is not the result of a general solubilization or proteolysis of the microvillus core activated by Ca", although we cannot determine from these results whether the dissociation is a direct or an indirect effect. For example, calmodulin may be bound to one of the other proteins released (95,000- or 70,000-dalton) during Ca" extraction of microvillus cores. The calmodulin that is not released into the supernate by Ca" treatment is quite resistant to further extraction with Ca", suggesting that at least two classes of calmodulin binding sites exist in the isolated microvillus core. The possibility that the calmodulin in the pellet fraction is simply denatured is unlikely because normal levels of phosphodiesterase activation were observed using these pellet fractions (results not shown). It is tempting to postulate a role for calmodulin in the structural and biochemical transformation of microvillus cores induced by Ca", but further experimentation will be required to test this exciting possibility.

Conclusions

Our results establish the presence of calmodulin in the isolated brush border and its tight association with the cytoskeletal elements of this organelle. These observations add to the growing body of evidence that calmodulin specifically associates with various components of the cytoskeleton such as neurofilaments (31), the mitotic apparatus (14), and actin-containing stress fibers in cultured cells (14).

Although we know essentially nothing concerning the function of calmodulin in the brush border, our results do add support to the hypothesis that the Ca" sensitivity of brush-border contraction is mediated through the activation of a myosin light-chain kinase by calmodulin. Even though our results are consistent with the notion that most of the calmodulin is located in the microvillus, not in the terminal web, where actomyosin interaction takes place, there may be a dissociation of calmodulin from binding sites in the microvillus as the level of Ca" rises above 10^-7 - 10^-6 M. Thus, under conditions that activate contraction, calmodulin may be free to diffuse into the terminal web region.

Although calmodulin may function in the regulation of brush-border myosin, it seems likely that this protein has other functions as well. We have already mentioned a possible role for calmodulin in regulating the organization of actin in the microvillus. Other possibilities include the control of any number of the various enzymes associated with the microvillus membrane. One exciting possibility is the involvement of calmodulin in the vitamin D-dependent transport of Ca" (33). Perhaps calmodulin acts as a carrier in the vectorial transport of calcium through the intestinal epithelium. At the very least, the large amount of calmodulin in the microvillus could serve as a calcium buffer in a region of the intestinal epithelial cell where other Ca" sinks, such as mitochondria and smooth endoplasmic reticulum, are absent.

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