# Comparison of $Ca^{++}$-regulated Events in the Intestinal Brush Border

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ABSTRACT The intestinal epithelial cell and specifically the cytoskeleton of the brush border are thought to be controlled by micromolar levels of free calcium. Calcium-binding proteins of this system include intestinal calcium binding protein (CaBP), calmodulin (CaM), villin, and a 36,000-mol-wt protein substrate of tyrosine kinases. To assess the sequence of events as the intracellular $Ca^{++}$ level rises, we determined the amount of CaM and CaBP in the intestinal epithelium by western blotting and tested the $Ca^{++}$ binding of CaM and CaBP by equilibrium dialysis. The $Ca^{++}$-dependent actin severing activity of villin was analyzed in the presence of physiological CaM levels and increasing calcium concentrations. In addition, we analyzed the $Ca^{++}$ levels required for interaction between CaM and the microvillus 110,000-mol-wt protein as well as fodrin and the interaction between a polypeptide of 36,000 mol wt (P-36) and actin. The results suggest that CaBP serves as the predominant $Ca^{++}$ buffer in the cell, but CaM can effectively buffer ionic calcium in the microvillus and thus protect actin from the severing activity of villin. CaM binds to its cytoskeletal receptors, 110,000-mol-wt protein and fodrin differently, governed by the free $Ca^{++}$ and pH. The interaction between P-36 and actin, however, appears to require an unphysiologically high calcium concentration ($10^{-4}$ to $10^{-3}$ M) to be meaningful. The results provide a coherent picture of the different $Ca^{++}$ regulated events occurring when the free calcium rises into the micromolar level in this unique system. This study would suggest that as the $Ca^{++}$ rises in the intestinal epithelial cell an ordered sequence of $Ca^{++}$ saturation of intracellular receptors occurs with the order from the lowest to highest $Ca^{++}$ requirements being CaBP < CaM < villin < P-36.

The actin-based microfilament system is tightly regulated by calcium ions both in muscle and nonmuscle cells. The classic example is skeletal muscle in which $Ca^{++}$ interacts with troponin C to confer $Ca^{++}$ sensitivity on the interaction between actin and myosin (35). Smooth muscle and nonmuscle cells provide a different system in which $Ca^{++}$ binds to calmodulin (CaM),[1] which can then activate myosin light chain kinase which, in turn, activates myosin ATPase by phosphorylation (31).

In addition to directly activating the actomyosin-based contractility system, CaM has been shown to interact with a number of other actin-binding proteins. CaM interacts with a protein termed caldesmon $Ca^{++}$ dependently to regulate its association with actin filaments (51). CaM also binds to the actin-binding proteins nonerythroid spectrin (fodrin) $Ca^{++}$-dependently (20–22), and the intestinal microvillus side-arm protein (110,000 mol wt) $Ca^{++}$-independently (16, 33). The function of these CaM-binding activities is currently unknown, yet since the CaM level in the microvillus bound to the 110,000-mol-wt protein (110 protein) has been estimated at 0.4 mM (18), it is thought to serve as a $Ca^{++}$ buffer, protecting the structural integrity of the microfilament bundle from the effect of villin. Villin, a 95,000-mol-wt protein of the microvillus core, is a $Ca^{++}$-binding protein (18), which, at low $Ca^{++}$ concentrations, bundles actin filaments (4, 17, 41, 42); but when the free $Ca^{++}$ rises to micromolar level or above, villin will sever actin filaments (4, 10, 41), leading to membrane vesiculation (6). Villin can also nucleate actin filament assembly, binding to and capping the barbed end of filaments (15) in a manner similar to gelsolin (66), a widespread $Ca^{++}$-binding protein first isolated from macrophages.

Recently, another actin-binding protein has been isolated from vesicles derived from microvilli of porcine intestine (13).

[1] *Abbreviations used in this paper:* CaBP, the 28,000 avian intestinal high affinity calcium-binding protein; CaM, calmodulin; 110 protein, 110,000-mol-wt protein; P-36, complex of polypeptides with subunit molecular weights of 36,000.



THE JOURNAL OF CELL BIOLOGY · VOLUME 100 MARCH 1985 754-763

It is comprised of a complex of polypeptides with subunit molecular weights of 36,000 (P-36) and 10,000 (P-10). P-36 was shown to be identical to the P-36 that serves as a substrate for the tyrosine protein kinase in Rous sarcoma virus–transformed cells (12, 13). Both the selective extraction with EGTA as well as re-binding experiments with actin and fodrin suggest that it interacts with cytoskeletal components $Ca^{++}$-dependently, in line with its distribution in cultured cells. This was taken to indicate a potential $Ca^{++}$-regulation of the actin/P-36/fodrin network in the cortical cytoplasm (13).

In addition to these $Ca^{++}$-regulated events, the intestine contains another high-affinity $Ca^{++}$-binding protein (CaBP). Like CaM (8, 39), CaBP is small (28,000 mol wt), acidic, and binds multiple calcium ions with high affinity (53, 54, 62–64). Furthermore, its expression is coupled to the levels of the hormone 1,25-dihydroxy–vitamin D3 (2, 54, 62), and it has been postulated to regulate the ability to take up dietary calcium (64). CaBP has been localized throughout the cytoplasm of intestinal epithelial cells (55), including the microvillus region.

In our study, we attempted to reconcile the different $Ca^{++}$ regulated events of the nonmuscle cytoskeleton, using the brush border cytoskeleton of intestinal epithelial cells as a model. We compared the $Ca^{++}$ activation of six brush border proteins: villin, CaM, the intestinal CaBP, fodrin, the 110 protein, and P-36. We determined the level of CaBP and CaM in the intestine and directly compare the amount of bound $Ca^{++}$ of both CaM and CaBP at increasing free $Ca^{++}$ levels. We tested the ability of CaM to protect actin filaments from the severing by villin-$Ca^{++}$. We used a direct CaM-binding assay to study the $Ca^{++}$ concentration necessary for binding of CaM to fodrin and the 110 protein. Finally, we have analyzed the $Ca^{++}$-sensitivity of P-36 binding to actin filaments and relate this to other $Ca^{++}$-regulated events. Physiological implications are discussed.

## MATERIALS AND METHODS

*Isolation of Proteins:* Bovine brain CaM was isolated as in Gopalakrishna and Anderson (27). Antibodies were elicited against performic acid oxidized CaM as described (60) and purified by antigen affinity chromatography on native CaM coupled to cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). Avian CaBP was isolated by the method of Friedlander and Norman (14). For antibody preparation, 300-$\mu$g aliquots of native CaBP in complete Freund's adjuvant were injected into guinea pigs and rabbits. After two further injections at 3 and 5 wk in incomplete Freund's adjuvant, the animals were bled out at 6.5 wk by cardiac puncture while anesthetized.

Villin was isolated essentially as described by Bretscher and Weber (4) as reported previously (15). Fodrin was isolated from bovine brain by a modification of the published method (22). The 110 protein was partially purified by the method of Glenney and Glenney (24). The purified P-36/P-10 complex was tested for reactivity in Western blots using an antibody raised against P-36 from fibroblasts (a generous gift from Dr. Jon Cooper, Salk Institute).

*Determination of the CaM and CaBP Levels in Intestine and Other Tissues by Western Blots:* Mucosal scrapings from six locations (20 cm apart) along the length of the adult chicken small intestine, in addition to other tissues, were weighed, 4 vol of water were added, and the samples were vigorously sonicated. An equal volume of boiling two times–concentrated SDS sample buffer was then added and the samples further boiled 5 min and clarified by centrifugation in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) operated for 20 min at 24 psi. Aliquots were removed for protein determination using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) with BSA as standard. Samples were adjusted to 8 mg protein/ml and 10-$\mu$l aliquots were then run on 1 mm thick SDS PAGE (13% gels). A serial dilution of known amounts of purified CaM and CaBP were run in adjacent slots for comparison. After running the gel at 37 V overnight, the gel was used for transfer to nitrocellulose for 0.75 to 1 h at 200 mA using the Bio-Rad transfer apparatus in 192 mM glycine, 25 mM Tris, 20% methanol (46). The nitrocellulose was then air-dried for 1 h and baked at 65°C under reduced pressure for 4–5 h. The filter was blocked in 4% BSA in phosphate-buffered saline (PBS) for 0.5 h, incubated in antibody (10 $\mu$g/ml affinity-purified anti-CaM; a 1:500 dilution of guinea pig anti-CaBP serum or both), sealed in plastic bags and incubated at 37°C for 2 h with agitation. The blots were rinsed in phosphate-buffered saline containing 0.2% Tween 20 (Sigma Chemial Co., St. Louis, MO) with five to ten changes over 15 min and then further incubated for 1 h at 37°C in phosphate-buffered saline containing 4% BSA, 0.2% Tween 20, and $5 \times 10^5$ cpm $^{125}$I-protein A per milliliter. Blots were then washed with phosphate-buffered saline/Tween for 1 h with numerous changes and air-dried. After autoradiography (generally 2–8 h of exposure using an intensifying screen at −70°C) the nitrocellulose was carefully aligned with the autoradiogram and the bands at the CaM and CaBP position were excised and counted in a gamma counter. A standard curve based on the CaM and CaBP standards run in adjacent lanes was found to be linear, and the amount in whole cell extracts was directly compared. In some cases known amounts of the CaM and CaBP standards were added directly to the lysate to insure that this additional amount could be detected if present.

*Equilibrium Dialysis:* The binding of $Ca^{++}$ to CaM and CaBP simultaneously at different free $Ca^{++}$ concentrations was performed by a modification of the method of Potter et al. (46). A liter of dialysis buffer, consisting of 10 mM imidazole, 0.1 M KCl, 3 mM $NaN_3$, 2 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.2 mM EGTA, 0.2 mCi $^{45}Ca^{++}$ (29 mCi/mg $Ca^{++}$; ICN, Irvine, CA) was adjusted to pH 7.0 and the free $Ca^{++}$ tested with a $Ca^{++}$-selective electrode (Orion 93 series with model 90-02 reference electrode). $CaCl_2$ from a 1 M stock solution was added and the free $Ca^{++}$ and pH were monitored continuously. The pH was maintained at 7.00 with 1 N NaOH and after each addition of $Ca^{++}$, 100 ml was removed and used for equilibrium dialysis. Each flask was used for dialysis with 0.5-ml aliquots of CaM and CaBP (each at 2 mg/ml). After dialysis overnight at room temperature, the aliquots from inside and outside the dialysis bags were counted. The protein content was also measured after dialysis using the Bio-Rad protein assay with purified CaM as standard assuming an extinction coefficient of $A_{280}$ (1%) = 2.1.

*Assay of Actin-severing Activity:* Actin was purified by the method of Spudich and Watt (49) followed by chromatography on Sephadex G-150. An aliquot was desalted into 2 mM $NaPO_4$, pH 7.5, by G-25 chromatography and 20 $\mu$g was labeled with 100 $\mu$Ci Bolton-Hunter reagent (ICN). After the reaction, labeled actin was chromatographed, using a G-25 column in 2 mM Tris, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.2 mM dithiothreitol, pH 8.0. Labeled actin was then mixed with unlabeled actin to 2 mg/ml and polymerized by addition of KCl to 0.1 M and $MgCl_2$ to 2 mM. Actin filaments were harvested by centrifugation in an air-fuge (100,000 *g*, 20 min). Actin was resuspended to 5 mg/ml in 50 mM KCl, 2 mM $MgCl_2$ by brief sonication. Aliquots (5 $\mu$l) were then added to a CaM/villin/$Ca^{++}$ buffer prepared as described below in a total volume of 50 $\mu$l in air-fuge tubes. After incubation for 1 h at room temperature, actin filaments were again harvested by centrifugation (100,000 *g*, 20 min) and supernatant and pellet fractions counted in a Beckman gamma counter.

The CaM/villin/$Ca^{++}$-solutions were prepared as follows. CaM was freed of $Ca^{++}$ by dialysis first against 10 mM EGTA and then by extensive dialysis vs. 10 mM imidazole, pH 7.0. A solution containing 8 mg/ml CaM, 0.5 mg/ml villin, 50 mM imidazole, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM ATP was assayed for free $Ca^{++}$ using the $Ca^{++}$ selective electrode, 45 $\mu$l were removed and added to the actin solution as described above. Microliter amounts of $CaCl_2$ from 10 mM or 0.1 M stock solutions were added, the solution mixed well, tested for free $Ca^{++}$, and an aliquot removed to test the severing activity as described above. This procedure was repeated for 10 samples. The entire assay was repeated on two separate days using fresh reagents and re-calibrating the electrode as described (1).

*CaM-binding assays:* CaM was labeled with Na$^{125}$I with insolubilized glucose oxidase and lactoperoxidase using enzymobeads (Bio-Rad Laboratories) and repurified by affinity chromatography on phenyl-Sepharose (26). The solution was then adjusted to 2 mg/ml BSA and dialyzed against 5 mM imidazole, pH 7.0. $^{125}$I-CaM-binding to native fodrin or 110 protein was assayed using a Bio-dot apparatus (Bio-Rad Laboratories). Nitrocellulose filters (Schleicher & Schull, Inc., Keene, NH) were wetted in 0.5 M NaCl, 10 mM imidazole for 10 min, excess liquid was blotted off, and the filter then placed in a 96-well filtration device. 100 $\mu$l of fodrin or the 110 protein at 67 $\mu$g/ml in $H_2O$ were applied to the well and allowed to filter through by gravity (~1 h) followed by 100 $\mu$l of 50 mg/ml BSA. $^{125}$I-CaM (0.1 $\mu$g in 100 $\mu$l 10 mM imidazole, 100 mM KCl, 1 mM $MgCl_2$, 2.5% BSA, 1 mM EGTA and $CaCl_2$ to give the specified free $Ca^{++}$ concentrations as determined with the $Ca^{++}$ selective electrode at pH 7.0) was applied to the filter and allowed to percolate through by gravity. The filter was rinsed with 200 $\mu$l buffer and the nitrocellulose filter then taken out of the apparatus, excess fluid was blotted off and after drying, the nitrocellulose spots were cut out and counted. Wells in which fodrin or 110 protein were omitted were always run in parallel provide control values and usually subtracted to determine specific binding. Triplicate determinations

at each point were always made.

In some cases, the pH was varied at a specified free $Ca^{++}$ level in testing the interaction between CaM and its receptor. In these experiments, the solutions contained 10 mM Tris, 10 mM imidazole, 10 mM sodium acetate in addition to 1 mM EGTA, 0.1 M KCl, 1 mM $MgCl_2$, 2.5% BSA, and enough $CaCl_2$ to give the specified level of free $Ca^{++}$. The pH was adjusted beginning at pH 5.8 with NaOH and the free $Ca^{++}$ was monitored with the $Ca^{++}$ selective electrode. The $Ca^{++}$-selective electrode was insensitive to hydrogen ions within this range according to the specifications provided by the manufacturer. Aliquots were taken at different pH values, 1 μl of $^{125}$I-CaM was added to 100 μl of the test solution and applied to the filter containing 110 protein or fodrin. The filters were rinsed with the same solutions without $^{125}$I-CaM.

*Interaction of P-36 with Actin:* P-36 was purified from bovine intestine essentially as described by Gerke and Weber (13). P-36 was phosphorylated by incubating P-36 (at 0.4 mg/ml) in 10 mM imidazole, 100 mM KCl, 1 mM $MgCl_2$, and 1 μM ATP (200 μCi $^{32}$P-ATP) for 1 h at 37°C with immunoprecipitated $p60^{src}$ from Rous sarcoma virus–transformed chick fibroblasts. The antisera to p60 was as described (48) and immunoprecipitates using this antibody were a generous gift from Mark Kamps and Bart Sefton (Salk Institute). Previous studies have shown that P-36 is phosphorylated by p60 at the same site in vitro as in vivo (12). After incubation, the Pansorbin containing p60 was collected by centrifugation at 10,000 *g*, 10 min and the free ATP was separated by chromatography on Sephadex G-25. The P-36 peak was further dialyzed vs. 10 mM imidazole, pH 7.3. Aliquots were subjected to NEPHGE (45) and the $^{32}$P-labeled P-36 was found to migrate just to the acidic side of Coomassie Blue–stained P-36. Actin binding, using unlabeled or $^{32}$P-labeled P-36, was performed as described before (14, 16, 24). Equivalent amounts of supernatant and pellet fractions from a high-speed centrifugation were run on SDS-PAGE and subjected to autoradiography. The bands corresponding to P-36 were cut out and counted. Unlabeled P-36 was quantitated by densitometry.

*Other Methods:* The $^{45}Ca^{++}$ overlay was performed as described (37) using electrophoretic transfer and filter treatment as described above. Actin-binding assays were performed as described previously (15, 17, 22). Free $Ca^{++}$ concentrations were determined using a $Ca^{++}$ selective electrode (Orion Research, Inc., Cambridge, MA) as described (40, 46). As noted in those publications, as well as in specifications provided by the supplier, >90% accuracy is found at free $Ca^{++}$ levels of $\geq 5 \times 10^{-7}$ M.

## RESULTS

### *CaM and CaBP*

Studies on the $Ca^{++}$ regulation in the intestinal epithelium have focused on the intestinal CaBP, a small, acidic protein with a high affinity for multiple calcium ions (2, 54, 62). Because these studies would have overlooked microvillus

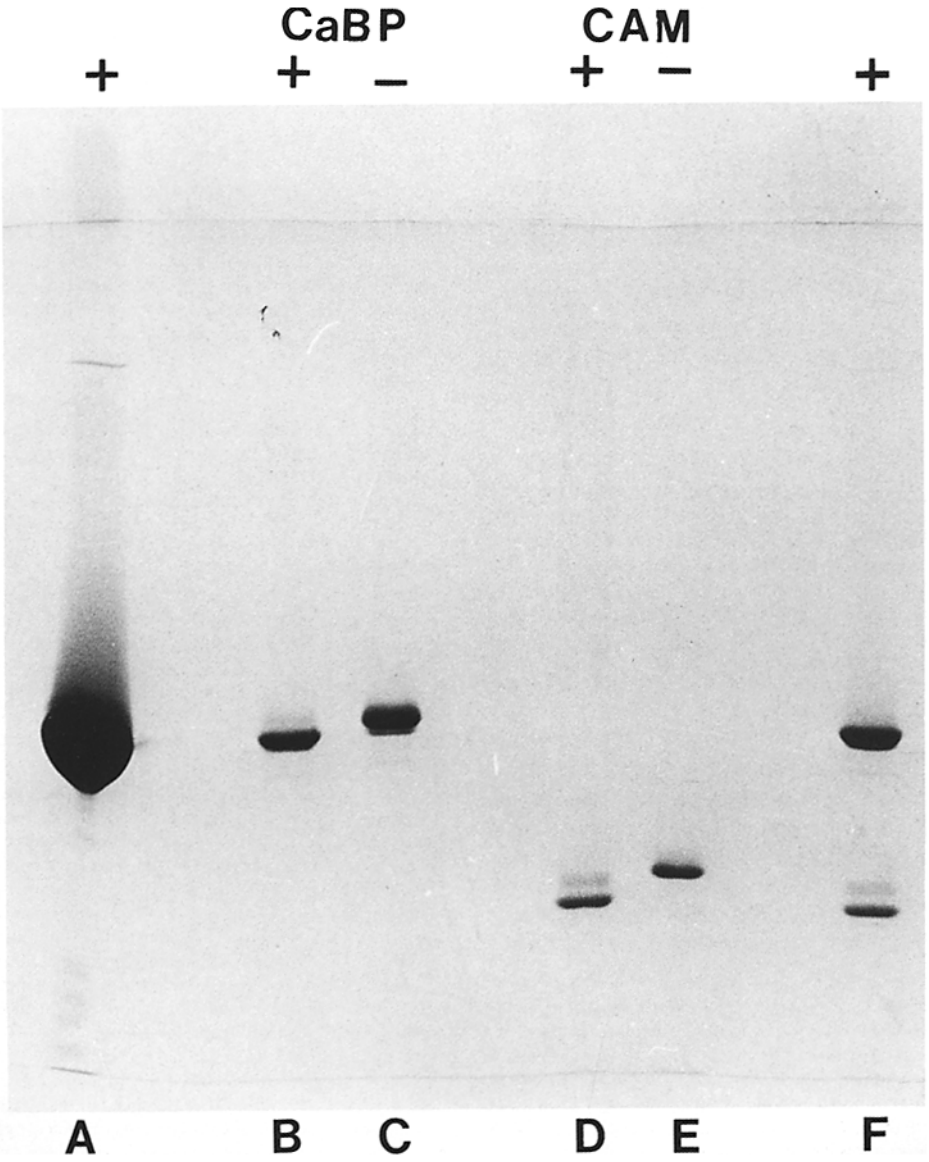


FIGURE 1 CaBP and CaM analyzed by SDS PAGE in the presence or absence of $Ca^{++}$. Purified CaBP (*A–C*), CaM (*D* and *E*), or a mixture of the two (*F*) were adjusted to 1 mM $CaCl_2$ (+) or 5 mM EGTA (−) in SDS sample buffer run on a 15% gel, stained with Coomassie Blue and destained. Note the shift in mobility of CaBP (*B* vs. *C*) as compared with calmodulin (*D* and *E*).

CaM, as most is not readily soluble (18, 34), we compared directly the amounts of CaM and CaBP in the intestine as well as their relative $Ca^{++}$-binding affinity. As shown in Fig. 1, CaBP is easily separable from CaM on SDS-PAGE. Like CaM, there is a noticeable shift to a slightly higher apparent molecular weight induced by removal of calcium ions observed on SDS gels. This shift in molecular weight allowed us to identify the CaBP in column fractions during the purification procedure given the fact that we observed only one protein with this property. Others, however, have not observed such a $Ca^{++}$-dependent shift in molecular weight of mammalian intestinal CaBP (10,000 mol wt) or kidney CaBP (28,000 mol wt) (9).

Recent studies have demonstrated that some CaBP can be directly identified after SDS PAGE by probing a nitrocellulose replica with $^{45}Ca^{++}$ (37). When we applied this technique to proteins of the intestine (Fig. 2), a protein migrating at the molecular weight of CaBP was clearly the most heavily labeled band. We also observed a $^{45}Ca$-decorated component with the mobility of CaM both in whole intestinal cells and brush border cytoskeletal proteins. Although it has been shown that villin also binds $Ca^{++}$ (17), we have found that villin is only poorly labeled by this overlay procedure.

To compare directly the amount of CaM and CaBP in intestinal epithelial cells, we used Western blotting and comparison to known amounts of pure protein run in adjacent lanes (59). We have taken this approach since much of the CaM remains bound to its receptor (the 110 protein) even in the presence of EGTA. Our initial attempts to use this procedure for quantitation were unsuccessful because (*a*) CaM and CaBP are transferred out of the gel and through the nitrocellulose rapidly and (*b*) both proteins are washed off the nitrocellulose membrane during antibody and protein A incubations. To circumvent these problems, we used short times of electrophoretic transfer, followed by baking the filter in an oven, and reducing the times of incubation with antibody and protein A. Using these modifications, we were able to reproducibly detect CaM and CaBP in total cell extracts of intestine as well as other tissues. As shown (Fig. 3; Table I), the amount of CaM and CaBP does not vary dramatically from one segment of the intestine to the other, with CaBP being present in substantially larger amounts in all segments of the intestine tested. Although mucosal scrapings used for these determinations do not represent a homogeneous population of intestinal epithelial cells, it should be noted that intestinal epithelial cells are prepared for immunofluorescence microscopy by a similar procedure after formaldehyde fixation (3). Thus, contamination by other cell types would be expected to be minimal. In addition, because CaBP is only in epithelial cells (55) whereas CaM is more broadly distributed, therefore, other cellular contamination would contribute to CaM but not CaBP. Thus the actual ratio of CaBP to CaM in intestinal epithelial cells may be even higher than calculated here. Consistent with previous results (56), we find high levels of CaBP only in kidney and intestine, whereas CaM was present in intestine in higher amounts than most other tissues (except for brain) and was enriched in the brush border cytoskeletal fraction.

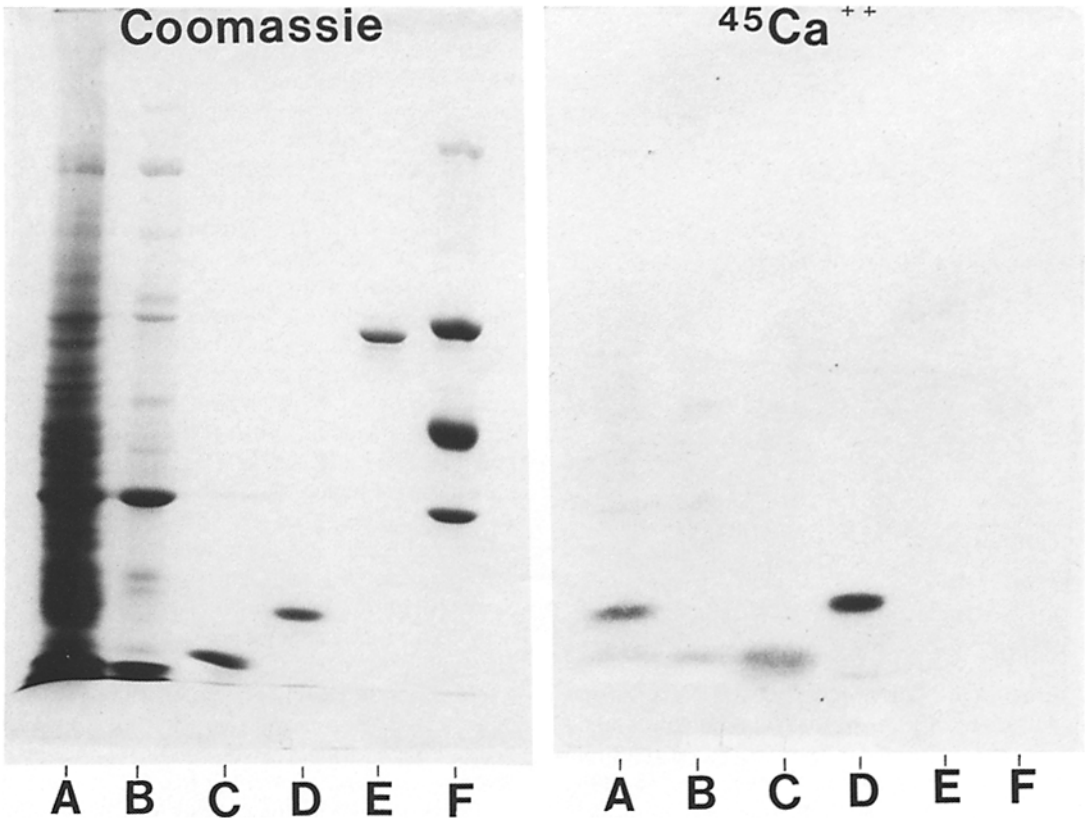


FIGURE 2 Detection of calcium-binding proteins in intestine. Whole intestinal cells (*A*), brush border cytoskeletal proteins (*B*), purified CaM (*C*), CaBP (*D*), villin (*E*), or a mixture of alpha-actinin, BSA, and actin (*F*) were resolved by SDS PAGE (12% acrylamide) and either stained with Coomassie Blue (*left*) or transferred to a nitrocellulose filter. The filter was incubated with buffer containing $^{45}Ca$ and, after unbound $^{45}Ca$ was washed off, the $Ca^{++}$-binding proteins detected by autoradiography (*right panel*). Note the decoration of bands in the total cell extract corresponding to CaBP and CaM.

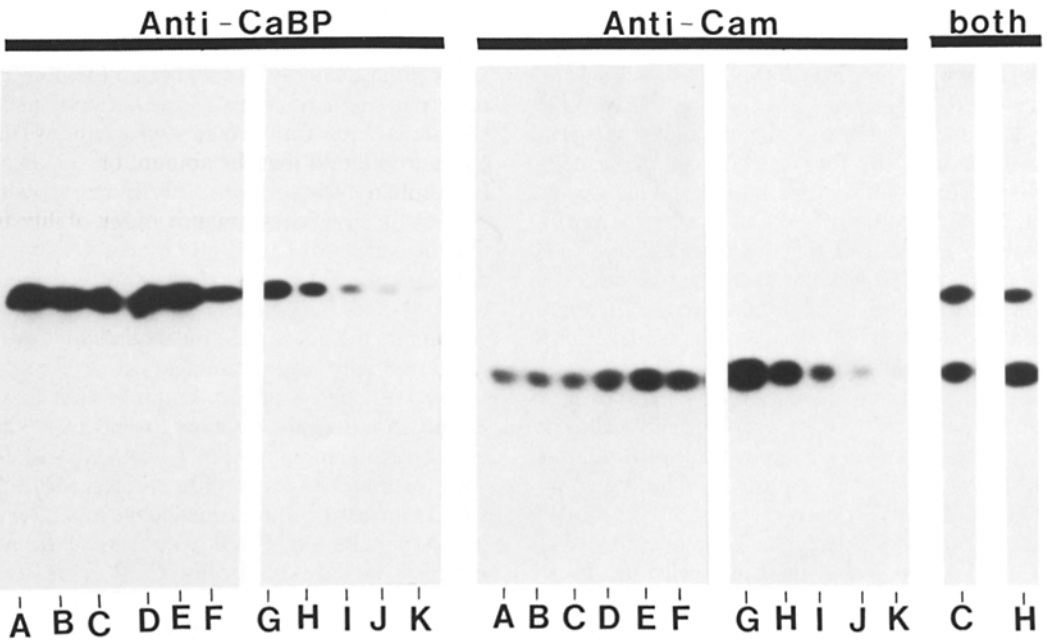


FIGURE 3 Detection and quantitation of CaM and CaBP in intestine by Western blotting. Total cell proteins (80 μg/lane) of intestinal epithelial cells from different positions along the length of the chick small intestine (*A* through *F* corresponding to proximal through distal positions) were resolved by SDS PAGE (15% gel) and transferred to nitrocellulose. CaM plus CaBP standards were run in adjacent lanes of the gel. ([*G*] 0.95 μg CaM + 1.15 μg CaBP; [*H*] 0.48 μg CaM + 0.58 μg CaBP; [*I*] 0.24 μg CaM + 0.29 μg CaBP; [*J*] 0.12 μg CaM + 0.14 μg CaBP; [*K*] 0.06 μg CaM + 0.07 μg CaBP). The nitrocellulose filter was probed with guinea pig anti-CaBP (1:500 dilution), anti-CaM (10 μg antibody/ml), or both antibodies, as indicated above each panel. Reactivity was detected using $^{125}$I-protein A and autoradiography.

TABLE I

*Amount of CaM and CaBP in Avian Tissues*

| Tissue | CaM* | CaBP |
|---|---|---|
| | *μg/mg total protein* | |
| Int-1 (Proximal) | 2.2 | 23.2 |
| Int-2 | 2.5 | 27.0 |
| Int-3 | 2.5 | 26.1 |
| Int-4 | 3.4 | 25.8 |
| Int-5 | 4.8 | 33.6 |
| Int-6 (Distal) | 3.8 | 13.0 |
| BBCS | 29.0 | <0.3 |
| Brain | 7.4 | <0.3 |
| Kidney | 1.3 | 7.6 |
| Heart | 0.4 | <0.3 |
| Liver | 0.6 | <0.3 |

* Expressed as μg CaM or CaBP per milligram protein as determined from Western blots and comparison to purified known amounts of CaM or CaBP run in adjacent wells of the SDS gel (see Fig. 3 for details). Total cell protein was determined by the Bio-Rad protein assay (Bio-Rad Laboratories) using BSA as standard. Int, Intestine; BBCS, brush-border cytoskeletal proteins.

## *Hierarchy of $Ca^+$-binding by CaM, CaBP, and Villin*

Since the three $Ca^{++}$ binding proteins CaM, CaBP, and villin are known to bind $Ca^{++}$ with dissociation constants in the micromolar range (18, 29, 63, 65), we asked the question: Given an increased in free cytosolic $Ca^{++}$, what would be the order of $Ca^{++}$ saturation? First, we tested the amount of $Ca^{++}$ bound to either CaM or CaBP by equilibrium dialysis under conditions of defined free $Ca^{++}$ using $Ca^{++}$/EGTA buffers and monitoring the free $Ca^{++}$ with a $Ca^{++}$ selective electrode. As shown in Fig. 4*A*, the amount of $Ca^{++}$ bound to CaM rises gradually over a rather broad free $Ca^{++}$ range approaching a saturation of 4 mol $Ca^{++}$/mol CaM. This curve exactly parallels the binding curve found previously by Potter et al (46). While using the same solutions for dialysis, however, CaBP bound more $Ca^{++}$ per mole protein at lower free $Ca^{++}$ levels and leveled off at 3 mol bound $Ca^{++}$ per mole CaBP at $5 \times 10^{-6}$ M free $Ca^{++}$. Given that CaBP is present in much larger amounts than CaM (see above), we replotted the data to reflect the relative $Ca^{++}$-binding contribution of CaM and CaBP in the proximal segment of the intestine (Fig. 4*B*). Clearly, under low $Ca^{++}$ conditions most of the cellular $Ca^{++}$ would be bound to CaBP rather than CaM.

Villin is also known to bind $Ca^{++}$ ions in the micromolar range. Rather than directly compare $Ca^{++}$ bound by equilibrium dialysis, we have chosen to monitor one of the $Ca^{++}$-dependent effects of villin (severing of actin filaments) as a function of free $Ca^{++}$ using a $Ca^{++}$/CaM buffer. Since we had previously estimated the CaM level in the microvillus at $4 \times 10^{-4}$ M (18), we chose this level to buffer the free $Ca^{++}$. As shown in Fig. 5, CaM effectively serves as a $Ca^{++}$ buffer and thereby protects actin from the severing activity of villin. Since it has been shown previously that CaM does not interact with villin, actin, or a combination of the two (18, 42), the ratio between CaM and villin or actin is not important. Fig. 5 represents the results of a straightforward two part experiment: (*a*) measuring the free $Ca^{++}$ at increasing total $Ca^{++}$ and a constant CaM level; and (*b*) monitoring the villin severing activity at increasing free $Ca^{++}$ (which was buffered by CaM). Using physiological CaM concentrations, the *total* $Ca^{++}$ rose to $10^{-3}$ M before the *free* $Ca^{++}$ reached the $10^{-5}$ M level needed for villin's severing activity to be expressed. This shows that CaM can act as buffer in the microvillus region where CaM is concentrated and the total $Ca^{++}$ level could be expected to be high due to $Ca^{++}$ uptake from the diet.

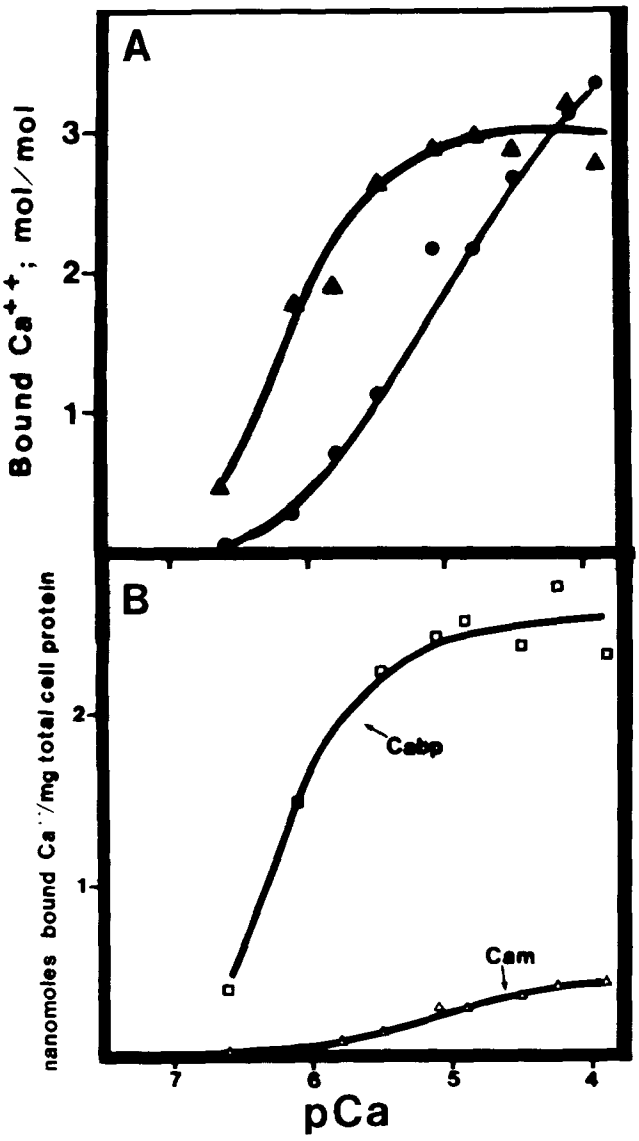


FIGURE 4 $Ca^{++}$-binding by CaM and CaBP at different levels of free $Ca^{++}$. Solutions were made up using $Ca^{++}$/EGTA buffers and $^{45}Ca$ and the free $Ca^{++}$ checked with the $Ca^{++}$-selective electrode. CaM (●) and CaBP (▲) were subjected to equilibrium dialysis for 14 h at 22°C and aliquots from inside and outside the dialysis bags were counted in a liquid scintillation counter. Protein concentration was redetermined after dialysis and plotted by the amount of $Ca^{++}$ bound to CaM or CaBP at a given free $Ca^{++}$ level. The data is replotted in *B* to reflect the relative amount of CaM and CaBP in the proximal segment of the intestine (see Table I) and the $Ca^{++}$ binding contribution of each.

## *CaM Binding to Fodrin and the 100 Protein*

We have previously shown that CaM binds both to the spectrin family of proteins (20, 21, 25) and to the 110 protein (16). To study this interaction in quantitative terms and assess the $Ca^{++}$ level necessary for binding, we used a solid-phase assay with native fodrin or 110 protein bound to nitrocellulose. Although a variant of fodrin (TW260/240) is found in avian brush borders, the CaM-binding activity is the same both in the gel overlay assay (16) as well as the technique employed here (not shown). When we applied this assay to brush border cytoskeletal proteins (Table II), we found that only the 110 protein and fodrin bound $^{125}I$-CaM. We were surprised to find that the 110 protein protein bound more than fourfold more CaM in the presence of $Ca^{++}$ than EGTA. It was shown previously (16, 33) using an $^{125}I$-CaM gel overlay assay that the 110 protein bound CaM in the presence or absence of free $Ca^{++}$. When we analyzed this interaction further, we found that the amount of $^{125}I$-CaM bound to the 110 protein or fodrin was directly related to the free $Ca^{++}$ (Fig. 6). A significant amount of variability in 110 protein binding was found between the three assays reported here. This variability, however, was not found with the fodrin-CaM interaction even though the two assays were run in parallel at the same time. Interestingly the amount of CaM bound to fodrin reached a peak at $5 \times 10^{-5}$ M free $Ca^{++}$ and was reduced up to 50% at even higher free $Ca^{++}$ levels.

Since we had not observed a $Ca^{++}$ dependence of CaM–110 protein interaction previously and this interaction was quite variable, we explored the other conditions that could affect it such as pH. As shown in Fig. 7 the interaction between the 110 protein and CaM was found to be strikingly dependent on pH as well as the free $Ca^{++}$ level. Since in a $Ca^{++}$/EGTA buffer, the free $Ca^{++}$ is critically dependent on small changes in pH, both free $Ca^{++}$ and free $H^+$ were simultaneously monitored with a $Ca^{++}$-selective electrode and a standard pH ($H^+$)-selective electrode when making up these solutions. This result suggests that the 110 protein–CaM interaction may be regulated by small local changes in pH, $Ca^{++}$, or both.

## *$Ca^{++}$-dependent Interaction of P-36 and Actin*

Because we were interested in analyzing the $Ca^{++}$ levels required for various events in the intestinal brush border cytoskeleton, we have repeated the actin binding experiments reported by Gerke and Weber (12) for P-36. P-36 is the intestinal analogue of the fibroblast protein that serves as a substrate for tyrosine protein kinases and also co-localizes with nonerythroid spectrin (fodrin) (25). We have confirmed the original observation that P-36 (and associated P-10) binds to actin filaments and this interaction requires $Ca^{++}$ (Fig. 8). Significant binding, however, is only achieved in the $10^{-4}$ to $10^{-3}$ M free $Ca^{++}$ range. Interestingly, p-36 phosphorylated in vitro by $p60^{src}$ displays the same $Ca^{++}$-sensitivity of binding to actin filaments as the nonphosphorylated form (Fig. 8).

## DISCUSSION

Various events are regulated by calcium in the intestinal epithelium. These include (but are not necessarily restricted to) the binding of $Ca^{++}$ to intestinal CaBP, the $Ca^{++}$-dependent interaction between CaM and its receptors, the actin-filament nucleation and severing activity of villin and possibly the interaction between the recently identified P-36 and its targets.

To understand the sequence of events induced by a rise in the free calcium ions (see Fig. 9), it is necessary to use an accurate means of maintaining and monitoring the free $Ca^{++}$. Although $Ca^{++}$/EGTA buffers have been widely used for this purpose, significant deviations from the calculated free $Ca^{++}$ can occur, especially at the higher end of the physiological range (1, 40, 47). Variability can be due, for instance, to variations in the purity of EGTA (40) or in small changes in pH (1, 47). To circumvent these problems, we used a calcium-selective electrode for all measurements (1) while carefully monitoring the pH. A number of important observations concerning the calcium regulation in the microvillus have resulted from this type of analysis.

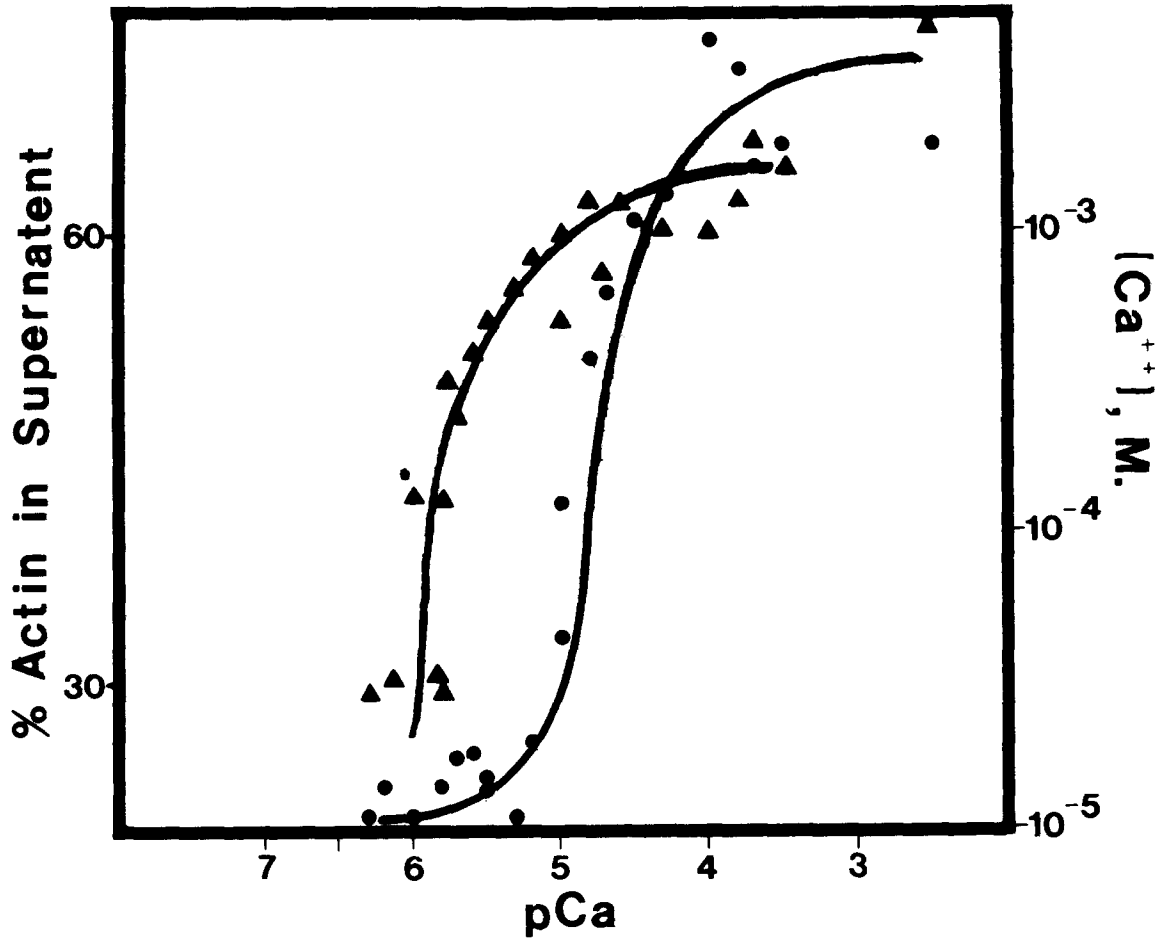


FIGURE 5 Testing the effect of increasing $Ca^{++}$ on the villin severing activity in the presence of physiological levels of CaM. To solutions of villin (0.5 mg/ml) and calmodulin (8 mg/ml) in 50 mM imidazole, we added 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM ATP (pH 7.0) microliter amounts of $CaCl_2$ from a 100 mM or 10 mM stock solution. The free $Ca^{++}$ was then tested with the $Ca^{++}$ electrode (plotted vs. the total $Ca^{++}$ in solution [▲]), 45 μl was removed and added to 5 μl of a solution of polymerized actin, which included trace amounts of $^{125}$I-labeled actin. After incubating 30 min at 22°C, the solution was centrifuged in an air-fuge at 22 psi for 30 min at 4°C. Supernatant (nonsedimentable) actin was separated from pelleted actin and counted in a gamma counter. The percent nonsedimentable was then plotted vs. the pCa (●). Control experiments showed that no effect on sedimentable actin was observed without villin present. Note that the total calcium rose to $10^{-3}$ M before the free $Ca^{++}$ rose to ~$10^{-5}$ M to allow the villin severing activity to be expressed.

TABLE II

*Binding of $^{125}$I-CaM to Brush Border Proteins*

| Protein | $Ca^{++}$ | EGTA |
|---|---|---|
| | *cpm bound* | |
| 110 | 61,800 | 13,300 |
| Villin (95) | 2,200 | ND |
| Fimbrin (68) | 2,200 | 3,400 |
| Actin (43) | 1,300 | 2,400 |
| Fodrin(240) | 8,282 | 1,327 |
| α-Actinin (100) | 1,800 | 2,300 |

Equal amounts (7 μg) of the proteins (in triplicate) were spotted onto nitrocellulose filters and after blocking with BSA, each well was treated with ~100,000 cpm $^{125}$-CaM (~0.01 μg). The nitrocellulose was washed with buffer, dried, and spots were cut out and counted in a gamma counter. *ND*, Not determined. *cpm*, Counts per minute. Molecular weights, × $10^{-3}$.

## Regulation of Cytoplasmic Ionized Calcium

What is the physiological regulator of free (ionized) calcium levels in the intestinal cell cytoplasm? It is generally accepted that in skeletal muscle, the sarcoplasmic reticulum serves as the regulator of $Ca^{++}$ and thereby regulate muscle contraction (see reference 35). In nonmuscle cells, it has been assumed that mitochondria serve to control the free $Ca^{++}$ level (7). Given the relatively low affinity of mitochondria for $Ca^{++}$, especially in the presence of physiological $Mg^{++}$ concentrations, however, this function has been challenged recently (52). Instead, attention has turned to the endoplasmic reticulum as a regulator of $Ca^{++}$ in nonmuscle cells, similar to its function in muscle.

For intestinal epithelial cells, a different mechanism for the regulation of free cytoplasmic $Ca^{++}$ should be considered. In the mature erythrocyte, for instance, which lacks both endoplasmic reticulum and mitochondria, it has been suggested that CaM functions as a $Ca^{++}$ buffer (61). Similarly we have previously suggested that CaM serves as a $Ca^{++}$ buffer in the microvillus of intestinal epithelial cells (18). This suggestion was based on (*a*) the immunofluorescence localization pattern (16, 30), which showed CaM to be localized in the apical (brush border) portion of the cell, and (*b*) an estimation of the amount of CaM present in the microvillus (18, 34), calculated to be some 0.4 mM (18). We have further tested this hypothesis in the present study and found that in the presence of physiological CaM concentrations, the total $Ca^{++}$ can rise to the millimolar range before the free $Ca^{++}$ becomes

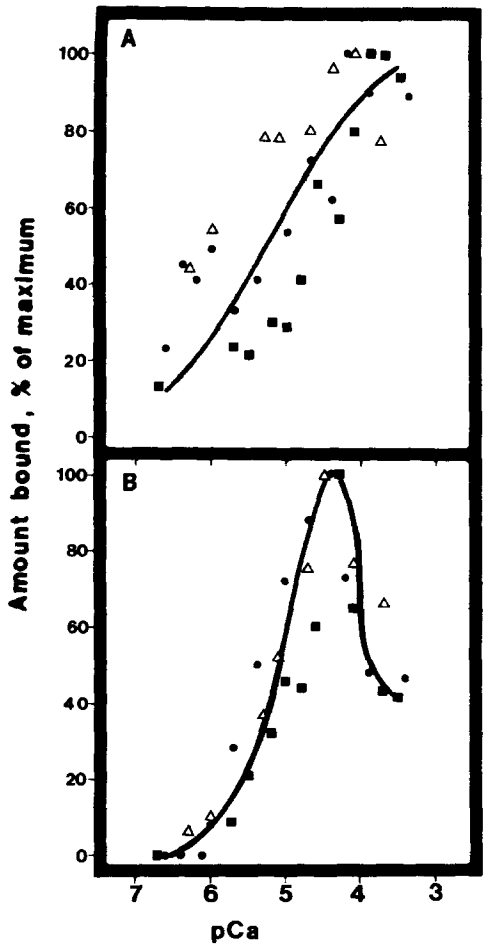


FIGURE 6 Binding of $^{125}$I-CaM to the microvillus 110 protein and fodrin. The 110 protein (*A*) or fodrin (*B*) were insolubilized on nitrocellulose membranes and after blocking unreacted sites with BSA, $^{125}$I-CaM in solutions of the specified free calcium were applied. After the CaM solution filtered through the membrane, the nitrocellulose was washed with buffer alone, dried, cut out, and counted in a gamma counter. The different symbols represent assays performed on different days using freshly made up buffers. Each point is the average of duplicate determinations. Note the increase in binding of CaM to the 110 protein and the increase, then drop in binding to fodrin as the free $Ca^{++}$ is increased.

high enough for the villin severing activity to be expressed (Fig. 5).

In the rest of the chick intestinal epithelial cell, however, the predominant $Ca^{++}$-binding protein is not CaM, but the 28,000-mol-wt CaBP. Although first identified in 1966 (62), the function of CaBP has not been fully elucidated. It has been shown to be regulated by the hormone 1,25-dihydroxy-vitamin D3 and was thought to be involved in the calcium-uptake process (64). Although studies have shown that the initial stimulation of $Ca^{++}$ uptake in vitamin D3-depleted chicks given a dose of 1,25-dihydroxy-vitamin D3 preceded the induction of CaBP (53, 54), the possibility remains that sustained $Ca^{++}$ uptake requires CaBP (2). Three points from the present study seem noteworthy concerning the intestinal CaBP in normal birds. First, CaBP is present in significantly larger amounts than CaM throughout the length of the intestine (Fig. 4; Table I). In this context, it is of interest that in the rat CaM is present in higher amounts than the lower molecular weight CaBP, which is concentrated in the proxi-

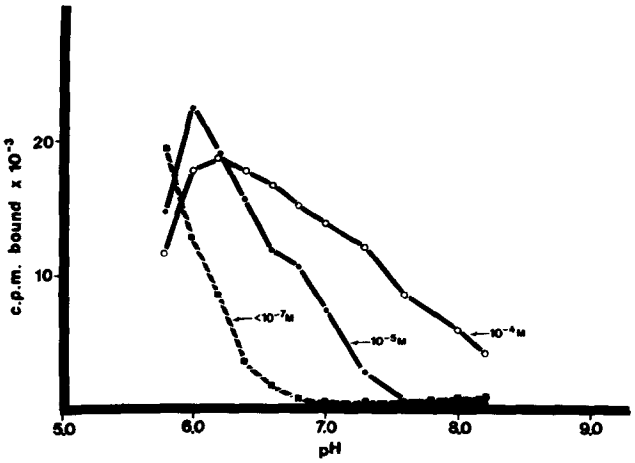


FIGURE 7 Effect of pH on the interaction between CaM and the 110 protein. The 110 protein was bound to nitrocellulose and treated with $^{125}$I-CaM as described in the legend to Fig. 6. In this set of experiments, the pH was varied while maintaining a constant free $Ca^{++}$ level (using the $Ca^{++}$ electrode) as indicated on the graph. Note the interdependence of free $Ca^{++}$ and pH on the binding of CaM to the side arm protein (110 protein).

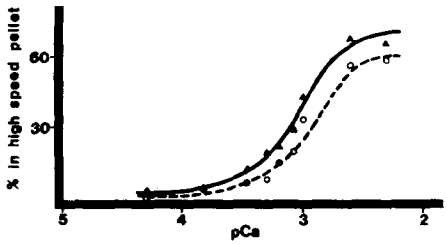


FIGURE 8 Binding of the P-36 to actin filaments in the presence of different free $Ca^{++}$ levels. P-36, either unlabeled (▲) or labeled with $^{32}$P (○) as described in Materials and Methods, were mixed with actin filaments (0.5 mg/ml) and 0.5 mg/ml BSA (to prevent nonspecific binding to the tube) in 10 mM imidazole, 100 mM KCl, 0.5 mM dithiothreitol, and 2 mM $MgCl_2$ with the specified level of $CaCl_2$. After a 30-min incubation, the F-actin was harvested by high speed centrifugation. Supernatant and pellet fractions were adjusted to equivalent volumes of SDS sample buffer and analyzed on a 7.5–17.5% gradient gel and stained with Coomassie Blue. Unlabeled P-36 in the F-actin pellet was quantitated by densitometry and $^{32}$P-labeled P-36 by cutting out and counting the P-36 region of the gel. Note that little P-36 is associated with actin at $10^{-4}$ M $Ca^{++}$.

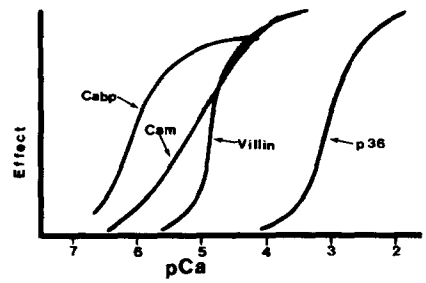


FIGURE 9 Summary of the calcium-dependent effects in the intestine as a function of the free $Ca^{++}$ level. CaBP and CaM represent the direct binding of $Ca^{++}$ to these proteins (see Fig. 4), villin represents the $Ca^{++}$ sensitivity of the actin severing activity and P-36 K the $Ca^{++}$ level needed to detect its interaction with actin.

mal part (duodenum) of the intestine (57). It should be noted that the CaM levels estimated in the present study are in agreement with other estimates using a different technique (28). Secondly, CaBP has a higher affinity than CaM for $Ca^{++}$. This implies that in the cytoplasm of the intestinal cell, free $Ca^{++}$ would probably be controlled by CaBP rather than CaM. This is especially obvious when the relative $Ca^{++}$-binding contributions of CaM and CaBP in one section of intestine are plotted (Fig. 4*B*). Thirdly, the difference in $Ca^{++}$ affinity of CaM and CaBP as well as the difference in location (CaM in the microvillus vs. CaBP throughout the cell) suggests that if both of these two molecules are involved in dietary $Ca^{++}$ uptake, then there could be a net transfer of $Ca^{++}$ from the region of high CaM (microvillus) to the region of high CaBP (cell body) simply by the downhill flow of ions. $Ca^{++}$ would then need to be transported out into the blood by an uphill (energy-requiring) mechanism. Such a $Ca^{++}$ pump has been detected in the basolateral membranes from mammalian intestinal cells (44).

The intestinal epithelial cell may then be (necessarily) different in the way cytoplasmic $Ca^{++}$ is controlled, dictated by the unusual requirement to provide a vectorial flow of $Ca^{++}$ through the cell. In this respect, there is undoubtedly a fundamental difference between intestine and, for instance, liver, in which these high levels of CaBP are not found (Table I). In this latter situation an organelle such as the endoplasmic reticulum could provide a reversible $Ca^{++}$ sequestering activity which could respond to hormonal or other factors. The intestine, by using high CaBP levels, may no longer have the ability to quickly respond to such stimuli. It should be noted that in the apical (brush border) region of intestinal epithelial cells, mitochondria and endoplasmic reticulum are absent. The free $Ca^{++}$ in this zone, then, would be controlled by CaM, known to be in the microvillus region (see above), and by CaBP, which has been localized in all regions of the intestinal cell (55), as expected for a truly soluble protein, and is not excluded from the brush border.

## CaM Interaction

In the intestine, CaM can activate myosin light chain kinase (5, 38) and $Ca^{++}$-ATPase (44) and alter fluid secretion (36) as well as activate various enzymatic activities found in other cells (8, 39). Previous studies have shown that CaM interacts with the two brush border cytoskeletal proteins TW260/240 and 110 protein. In chicks, the common 240,000-mol-wt subunit of the spectrin-like proteins (21) binds CaM $Ca^{++}$ dependently (21, 22), an activity apparently lost in the evolution of mammalian spectrin (25). Although the function of this interaction is unknown, a recent study has shown that CaM together with another factor can regulate the interaction of fodrin with actin (50). This is rather unexpected, since the CaM-binding site has been localized close to the center of the fodrin tetramer (58), whereas the actin-binding site is closer to the ends (22).

It is interesting that as the free $Ca^{++}$ rises from $10^{-6}$ to $5 \times 10^{-5}$ M, the amount of CaM bound to fodrin increases, but then decreases $>5 \times 10^{-5}$ M. Other studies have shown that the ordered binding of multiple calcium ions to CaM can be translated into different effects (29). It may be that CaM binds to fodrin when 1–3 $Ca^{++}$ sites are filled, but the binding of a fourth $Ca^{++}$ results in a reduced CaM-fodrin affinity. The physiological significance, however, is unknown, because the free $Ca^{++}$ would not be expected to rise to this level (see below).

Previous studies have shown that CaM can bind in the absence of free $Ca^{++}$ to the 110 protein, consistent with the observation that CaM is not released from microvilli core filaments by EGTA (18, 34). It was unexpected, then, to find a $Ca^{++}$ dependence of the interaction between CaM and the 110 protein and raises the possibility of a $Ca^{++}$ regulation of the 110 protein activity (actin binding, membrane association, or other as yet unidentified enzymatic activity). The pH effect of the CaM–110 protein interaction appears especially important. Small differences in the pH were found to alter the $Ca^{++}$ sensitivity of this interaction (Fig. 7). Since CaM does not change its $Ca^{++}$-binding activity in this pH range (11), then it would appear that a functional group on the 110 protein or CaM, which is important for this interaction, is titrated within the 6.5 to 7.5 pH range. A qualitative difference in the binding of CaM to the 110 protein between vitamin D–depleted (rachitic) and vitamin D–treated chicks had previously been observed (33). It will be of interest to extend these studies to more quantitative studies such as those reported. Such studies are in progress.

## $Ca^{++}$-dependent Function of Villin

Villin binds to actin filaments $Ca^{++}$ dependently. Villin can nucleate or sever actin filaments at high $Ca^{++}$ (4, 15, 16, 41, 42), but bundles actin in low $Ca^{++}$ (4, 7, 41). We had previously proposed that CaM in the microvillus serves to sequester $Ca^{++}$ and protect the actin filament bundle from the severing activity of villin (18). That the villin mediated actin breakdown is not normally used in mature intestinal epithelial cells is apparent from the examination of electron micrographs of intestine (32, 43). When the villin severing activity is induced in isolated brush borders, actin filament breakdown and membrane vesiculation result (6). In sections of normal intestine, however, this is not observed. Clearly, long thin intact microvillus bundles are the rule, which suggests that villin is serving as a bundling protein under these conditions and, therefore, that the free $Ca^{++}$ is $<10^{-5}$ M. This does not mean that villin's $Ca^{++}$ dependent activity is never expressed and it could allow the removal of microvillus membrane by vesiculation (6) as suggested previously.

## Does P-36 Bind to Actin and Fodrin under Physiological Conditions?

Previous studies have shown that P-36 (12) binds to actin and fodrin in the presence of high $Ca^{++}$ (13). This was in line with the co-localization of fodrin and P-36 in fibroblasts (26) and suggested a $Ca^{++}$ regulation of such a network. The levels required for such an interaction, however, were extremely high, although it was pointed out one other intracellular enzyme ($Ca^{++}$-activated protease) required these levels. In the intestine, however, it is doubtful that the ionized calcium ever approaches the millimolar level and, in fact, is probably below the level of the molecular weight for $Ca^{++}$ binding to villin, $\sim 10^{-5}$ M (see above). It is possible that some other factor or ion stabilizes this interaction and high $Ca^{++}$ can mimic this effect or shift the calcium sensitivity to higher levels. In this regard, however, we find that P-36 phosphorylated on tyrosine retains the same calcium sensitivity as unphosphorylated P-36 (Fig. 8). In the intestine, then, $Ca^{++}$ regulation of the binding of P-36 to actin (or fodrin) probably does not occur.

The authors kindly thank Jon Cooper for the gift of anti-P-36 antibodies and Mark Kamps and Bart Sefton (Salk Institute) for the $p60^{src}$ immunoprecipitates.

This work was supported by a grant from the National Institutes of Health.

*Received for publication 16 July 1984, and in revised form 15 October 1984.*

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