In ileal absorptive cells, carbachol inhibits NaCl absorption and its component brush border Na\(^+/\)H\(^+\) exchanger, acting via basolateral membrane receptors. This carbachol effect involves (i) activation of brush border phosphatidylinositol 4,5-bisphosphate-specific phospholipase C (PLC) activity and brush border but not basolateral membrane translocation of PLC-\(\gamma_1\) (Khurana, S., Kreydiyyeh, S., Aronzon, A., Hoogerwerf, W. A., Rhee, S. G., Donowitz, M., and Cohen, M. E. (1996) Biochem. J. 313, 509–518); and (ii) brush border tyrosine kinase(s) because mucosal but not serosal addition of the tyrosine kinase inhibitor genistein prevents the carbachol-induced inhibition of NaCl absorption and brush border Na\(^+/\)H\(^+\) exchange. In the present work we identify a pool of villin (a brush border actin-binding protein) in the microvillus membrane fraction of rabbit ileum; this pool of villin is tyrosine-phosphorylated and associates with brush border membrane PLC-\(\gamma_1\). Villin is present both in the Triton X-100-soluble and -insoluble fractions of the brush border. The Triton X-100-soluble pool is approximately 4-fold smaller than the brush border pool of villin that is present in the Triton X-100-insoluble fraction. Only the villin present in the Triton X-100-soluble fraction of ileal villus brush border associates with PLC-\(\gamma_1\) and is tyrosine-phosphorylated. Carbachol increases the tyrosine phosphorylation of villin rapidly (as early as 30 s) and transiently. Carbachol also increases the amount of tyrosine-phosphorylated villin that associates with PLC-\(\gamma_1\). These studies demonstrate that carbachol effects on NaCl absorption are accompanied by an increase in brush border PLC-\(\gamma_1\) association with villin and an increase in tyrosine phosphorylation of villin. To study the role of cytoskeletal rearrangement in carbachol-induced inhibition of NaCl absorption, we used the F-actin stabilizing drug jasplakinolide. Jasplakinolide prevents the carbachol inhibition of ileal NaCl absorption. This suggests that F-actin severing is necessary for carbachol to inhibit ileal villus NaCl absorption. Since villin is known to sever actin, these studies suggest a role for villin in the signaling cascade that begins at the basolateral membrane with carbachol binding to its receptor and ends at the apical membrane in inhibition of NaCl absorption.

We have previously shown that the muscarinic-cholinergic agonist carbachol acts on rabbit ileal villus absorptive cells to inhibit NaCl absorption and brush border Na\(^+/\)H\(^+\) exchange (1). This carbachol effect involves a basolateral membrane cholinergic receptor, the activation of which affects signaling pathways at the opposite pole of the cell, at the brush border. Carbachol increases brush border but not basolateral membrane PIP\(_2\)-specific PLC activity by causing both the translocation to and activation of PLC-\(\gamma_1\) in the brush border (BB) (2). In addition a BB membrane tyrosine phosphorylation event is essential for this inhibition of BB Na\(^+/\)H\(^+\) exchange and for the activation of BB PLC-\(\gamma_1\) (2). The nature of the tyrosine kinase involved and the substrates tyrosine-phosphorylated in the carbachol effects are not known. The ileal brush border Na\(^+/\)H\(^+\) exchanger, NHE3, has not been shown to be tyrosine-phosphorylated, and although BB PLC-\(\gamma_1\) is tyrosine-phosphorylated, carbachol does not increase the tyrosine phosphorylation of PLC-\(\gamma_1\) (2).

In the present study we describe another carbachol-induced BB tyrosine phosphorylation event that occurs in the BB but not the basolateral membrane. We demonstrate that BB PLC-\(\gamma_1\) associates with villin, an actin-binding BB-specific cytoskeletal protein. Carbachol increases the association of BB villin with PLC-\(\gamma_1\) and increases the tyrosine phosphorylation of villin. Our study also provides evidence that cytoskeletal remodelling is involved in the regulation of intestinal Na\(^+\) absorption. This is the first report demonstrating the tyrosine phosphorylation of villin; it is also the first report demonstrating the association of villin with PLC-\(\gamma_1\).
Methods—Distal ileum from New Zealand White male rabbits (2–2.5 kg) was used for all experiments. Rabbits were sacrificed by intravenous nembutal overdose. Ileum was then removed, rinsed with 0.9% saline, opened along the mesenteric border, and exposed in vitro to control or carbachol (1 μM). The tissue was preincubated for 10 min at 37 °C, washed with 95% O2, 5% CO2 in Ringer’s/HCO3-containing 10 mM glucose and 1 μM indomethacin to inhibit prostaglandin synthesis. Carbachol was then added to one set of tissues, and the incubation was continued for 30 s or 1 min. The tissues were then chilled, and control and carbachol-treated samples were processed in parallel.

Preparation of BB Membranes—At the end of the above incubation, ileal sheets were chilled on iced Petri dishes, and mucosa was lightly scraped off with glass slides. BB were prepared by differential centrifugation and double Mg2+ precipitation as described previously (2). The final BB pellets were resuspended in 20 mM Tris buffer, pH 7.5, containing 15 mM HEPES, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1% mannitol to the mucosal bathing fluids at the time of incubation conditions in which the signal was not saturated.

Electrolyte Transport in Vitro—The methods used to measure active ileal electrolyte transport have been described previously (1, 2). In brief, ileal mucosa with muscularis propria removed was mounted as a flat sheet between two Lucite modified Ussing chambers having an aperture of 1.13 cm², oxygenated, and maintained at 37 °C. Transmural potential difference, short-circuit current (Isc), conductance, and unidirectional fluxes of Na+ and 36Cl were determined. An automatic volume-controlled perfusate for fluid resistance between the potential difference-sensing bridges and provided continuous short-circuiting of the tissue. Unidirectional fluxes of Na+ and/or Cl- were measured 20–100 min after addition of isotope by using 22Na and 36Cl on tissue matched to differ in conductance by not more than 25%.

Usually eight pieces of ileum from a single animal were studied simultaneously. Unless specified, the bathing solution consisted of Ringer’s/HCO3, composed (in mM) of 115 NaCl, 25 NaHCO3, 2.4 K2HPO4, 0.4 KH2PO4, 1.2 CaCl2, 1.2 MgCl2; the pH was 7.4 after gassing with 95% O2, 5% CO2. Glucose (10 mM) was added to the serosal and 10 mM mannitol to the mucosal bathing fluids at the time of mounting the tissue. The effect of carbachol (0.3 μM) added to the serosal surface was determined over two 20-min flux periods in solvent control-treated (0.02% Me2SO) ileum or tissue exposed to jasplakinolide (5 μM) added to the ileal mucosal surface for 40 min before carbachol addition.

Immunoprecipitation of PLC-γ and Villin—BB (250 or 100 μg of protein) were extracted with a solution containing 1% Triton X-100, 20 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM sodium orthovanadate, 50 mM NaF, and 1 mM phenylmethylsulfonyl fluoride for 15 min at 4 °C. PLC-γ or villin were immunoprecipitated from the soluble extracts as described (2) by using monoclonal antibodies to either PLC-γ or villin. To determine the presence of PLC-γ in the Triton X-100-insoluble fraction, the pellet from the extract above was dissolved in RIPA buffer (see “Experimental Procedures”). The immunoprecipitated proteins were separated by SDS-PAGE and Western analysis done for PLC-γ and Villin—BB (250 or 100 μg of protein) were extracted with a solution containing 1% Triton X-100, 20 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM sodium orthovanadate, 50 mM NaF, and 1 mM phenylmethylsulfonyl fluoride for 15 min at 4 °C. PLC-γ or villin were immunoprecipitated from the soluble extracts as described (2) by using monoclonal antibodies to either PLC-γ or villin. To determine the presence of PLC-γ in the Triton X-100-insoluble fraction, the pellet from the extract above was dissolved in RIPA buffer (see “Experimental Procedures”). The immunoprecipitated proteins were separated by SDS-PAGE and Western analysis done with anti-vav and anti-villin antibodies.

RESULTS

A Tyrosine-phosphorylated BB Protein Associates with BB PLC-γ—Several BB proteins have increased tyrosine phosphorylation in response to carbachol. Those have been identified but do not include NHE3 or PLC-γ. Consequently we tested the hypothesis that an intermediate tyrosine-phosphorylated protein associates with PLC-γ. Rabbit ileal mucosa was treated in vitro with carbachol (1 μM) for 1 min, a time when PLC activity is maximally stimulated in the BB, or for 30 s, an earlier time point when PLC activity is 20% higher than PLC activity in BB from control and carbachol-exposed BB. Tissue was solubilized, and PLC-γ was immunoprecipitated from both the Triton X-100-soluble fraction and Triton X-100-insoluble fraction. As shown in Fig. 1, A and B, all the PLC-γ, both under control conditions and in carbachol-treated ileum were present in the Triton X-100-soluble fraction of the BB, representing the membrane pool. No PLC-γ was present in the Triton X-100-insoluble pool. Carbachol increased the amount of PLC-γ in the BB by 20% (p < 0.05, n = 5) at 30 s (Fig. 1A). Carbachol increased the BB PLC-γ 2-fold at 1 min, as reported earlier and as shown in Fig. 1B (2). These results indicate that the entire pool of PLC-γ present in the ileal villus BB is associated with the membrane and not with the BB cytoskeleton.

To look for tyrosine-phosphorylated proteins associating with PLC-γ, BB were made from control and carbachol-treated ileum (1 μM, 30 s or 1 min). PLC-γ was immunoprecipitated from both control and carbachol-exposed BB. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose, and Western analysis was done using an anti-phosphotyrosine polyclonal antibody. A 95-kDa tyrosine-phosphorylated protein associated with PLC-γ (Fig. 2). Although this protein was tyrosine-phosphorylated under basal conditions, a 30-s exposure to carbachol caused a significant increase (2-fold, p < 0.01, n = 8) in the tyrosine phosphorylation of this 95-kDa protein. At 30 s, the amount of PLC-γ present in the carbachol-treated BB is 20% higher than control BB (Fig. 1A), and the BB PIP2-specific PLC activity is 20% higher than control BB, as reported earlier (2). This suggests that the increase in tyrosine phosphorylation of this 95-kDa protein cannot be explained by increase in amount of BB PLC-γ alone. At 1 min of carbachol exposure, the amount of tyrosine phosphorylation of this 95-kDa protein is similar in control and carbachol-exposed BB (Fig. 2). At this point there is at least 2-fold more PLC-γ present in the carbachol-treated BB (Fig. 1B). Therefore, the increase in tyrosine phosphorylation of this 95-kDa protein is very transient.

The 95-kDa Tyrosine-phosphorylated Protein Is Villin—The 95-kDa protein was examined with antibodies to several known signaling molecules of the same size including Vav and the BB cryoskeletal protein villin. PLC-γ was immunoprecipitated and Western analysis done with anti-vav and anti-villin anti-
bodies. Co-immunoprecipitations determined that the 95-kDa proteins were not Vav. In contrast, the cytoskeletal protein villin co-immunoprecipitated with PLC-\(\gamma_1\) (Fig. 3). Equal amounts of BB proteins (250 \(\mu\)g) from control and carbachol-treated tissue (1 \(\mu\)M, 1 min) were solubilized in buffer containing 1% Triton X-100 and immunoprecipitated with antibodies to PLC-\(\gamma_1\), villin, and phosphotyrosine. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-villin monoclonal antibodies. This experiment is representative of three with similar results.

As seen in Fig. 3, all three antibodies immunoprecipitated a 95-kDa protein, identified with the monoclonal antibody to villin. This is the first demonstration of the presence of a BB pool of villin in the Triton X-100-soluble fraction and thus not associated with the cytoskeleton. The amount of villin present in the Triton X-100-soluble pool which associates with PLC-\(\gamma_1\) is smaller than the total amount of villin present in the Triton X-100-soluble fraction of the BB. As illustrated in Fig. 3 approximately one-third of the villin present in the Triton X-100-soluble pool of the BB associates with PLC-\(\gamma_1\). The increase in tyrosine phosphorylation of villin is associated with an increase in the amount of villin that associates with PLC-\(\gamma_1\). At 30 s there is 2-fold more villin associated with PLC-\(\gamma_1\) (\(p < 0.05, n = 4\)) (Fig. 4). This suggests that at 30 s more tyrosine-phosphorylated villin associates with PLC-\(\gamma_1\). To determine if the entire pool of tyrosine-phosphorylated villin present in the Triton X-100-soluble fraction associates with PLC-\(\gamma_1\), the following experiment was performed. PLC-\(\gamma_1\) was immunoprecipitated from the BB Triton X-100-soluble pool, and after removing the immunoprecipitated PLC-\(\gamma_1\), the remaining Triton X-100-soluble fraction was immunoprecipitated with anti-phosphotyrosine antibodies. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and Western analysis done using anti-villin antibodies. As shown in Fig. 5, the fraction of the Triton X-100-soluble pool that does not associate with PLC-\(\gamma_1\) does not contain tyrosine-phosphorylated villin. These data suggest that the mechanism for interaction between villin and PLC-\(\gamma_1\) may be mediated through the tyrosine phosphorylation of villin.

**The Cytoskeleton-associated Pool of Villin Is Not Tyrosine-phosphorylated**—BB from control and carbachol-treated ileum (1 \(\mu\)M, 1 min) were solubilized in buffer containing Triton X-100 (as described under “Experimental Procedures”), and PLC-\(\gamma_1\) was immunoprecipitated (Ipt) from the Triton X-100-soluble fraction. The remainder of the Triton X-100-soluble pool was reprecipitated with anti-phosphotyrosine antibodies. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and Western analysis (Blot) done with anti-villin monoclonal antibodies. This figure shows results from two different BB preparations. This experiment is representative of three with similar results.
Villin Is Tyrosine-phosphorylated and Associates with PLC-γ₁

FIG. 6. A pool of villin exists in the Triton X-100-soluble fraction of the BB, not associated with the cytoskeleton. A, BB from control and carbachol-treated ileum (1 μM, 1 min) were solubilized in buffer containing Triton X-100 (as described under “Experimental Procedures”), and PLC-γ₁ was immunoprecipitated (Ip) from the Triton X-100-soluble fraction. The immunoprecipitated PLC-γ₁ (left 4 lanes) and the remaining Triton X-100-insoluble fractions (right 4 lanes) were separated by SDS-PAGE, transferred to nitrocellulose, and Western analysis was done using anti-villin monoclonal antibodies. This figure shows results from two different BB preparations. This experiment is representative of three with similar results. This figure shows the amount of villin present in the Triton-soluble fraction that associates with PLC-γ₁ and the villin present in the Triton-insoluble fraction. B, the blot in A was stripped and reprobed with polyclonal antibodies to phosphorytyrosine (P-Tyr).

more villin in the Triton X-100-insoluble fraction, it is not tyrosine-phosphorylated. These data show that the pool of villin present in the Triton X-100-soluble fraction and immunoprecipitated with PLC-γ₁ is much smaller than the villin pool present in the Triton X-100-insoluble fraction and that only the villin that is associated with PLC-γ₁ is a substrate for tyrosine kinases.

To determine the relative amounts of villin present in the Triton X-100-soluble and -insoluble pools, 100 μg of BB was solubilized in buffer containing 1% Triton (as described under “Methods”). The Triton X-100-soluble and -insoluble pools were separated by SDS-PAGE, and Western analysis was done using anti-villin monoclonal antibodies. As seen in Fig. 7, there is 4-fold more villin present in the Triton X-100-soluble fraction as compared with the villin present in the Triton X-100-insoluble fraction (p < 0.05, n = 3). The latter includes both the villin that associates with PLC-γ₁ (as shown in Fig. 6A) and the pool that does not associate with PLC-γ₁.

Role of Cytoskeletal Rearrangement in the Carbachol-induced Inhibition of Ileal NaCl Absorption—Villin belongs to a family of Ca²⁺-regulated actin-binding proteins that nucleate, cap, or sever actin filaments (3, 4). At high Ca²⁺ concentrations villin severs actin filaments. Villin has also been shown to associate with polyphosphoinositides, especially PIP₂ (also the substrate for PLC-γ₁) (5). In vitro studies have also suggested that the major effect of PIP₂ on villin is to inhibit its ability to sever actin filaments (6). Since carbachol causes the activation of BB PLC-γ₁, we hypothesized that this would lead to a localized decrease in the amount of PIP₂ and an increase in Ca²⁺ close to the BB. Both these signaling events would lead to an activation of the actin severing property of villin, which then might regulate the inhibition of NHE3. Our hypothesis then suggests that if the actin filaments are stabilized and cannot be severed by villin, carbachol would not be able to inhibit ileal NaCl absorption.

To test this hypothesis, the F-actin stabilizer jasplakinolide was used (7). Jasplakinolide stabilizes F-actin similar to phalloidin but is more membrane-permeant (8). Ileum was treated with jasplakinolide added to the mucosal surface for 40 min prior to the addition of carbachol. As seen in Fig. 8, serosal addition of carbachol (0.3 μM) inhibited ileal NaCl absorption as reported earlier (1, 2). We have previously shown that carbachol inhibits neutral NaCl absorption and BB Na⁺/H⁺ exchange, the latter as early as 5 min, with the effect persisting at least 40 min following carbachol exposure (1, 2). For studies measuring neutral NaCl absorption in intact tissue, (22)Na and (36)Cl fluxes were measured at steady state (20 min following carbachol exposure). Starting 20 min after carbachol addition, two 20-min flux studies were performed, a time during which ileal Iₑ was constant and slightly increased compared with that during two 20-min basal flux periods in the same tissue before the addition of carbachol. Serosal addition of carbachol caused a statistically significant decrease in mucosal-to-serosal and net Na⁺ and Cl⁻ fluxes (Fig. 8). In ion flux experiments, a negative sign indicates net secretion and a positive sign indicates net absorption (Jₐ represents the difference between unidirectional fluxes and represents active transport). There was no significant change in the serosal-to-mucosal Na⁺ fluxes, but there was a significant increase in the serosal-to-mucosal Cl⁻ fluxes. These data show, as reported earlier, that carbachol decreases ileal active Na⁺ and Cl⁻ absorption and increases Cl⁻ secretion. The effects of jasplakinolide on the transport effects caused by serosal carbachol were determined (Fig. 8). The addition of mucosal jasplakinolide prevented the carbachol effects to decrease mucosal-to-serosal and net Na⁺ and Cl⁻ fluxes. Thus in the presence of jasplakinolide, carbachol-induced inhibition of NaCl absorption (as indicated by decreased mucosal-to-serosal and net NaCl absorption (Jₑ) is prevented. However, the carbachol-induced increase in serosal-to-mucosal Cl⁻ flux was not significantly inhibited. These data show that in ileum pretreated with jasplakinolide the carbachol-induced inhibition of NaCl absorption does not occur, although Cl⁻ secretion is not prevented.

Jasplakinolide added to the ileal mucosal surface (5 μM) did not alter basal active electrolyte transport (Fig. 9). Also, the p-glucose-stimulated increase in Na⁺ absorption (Na⁺-glucose cotransport) was not altered by jasplakinolide (3.0 ± 0.5 versus 2.9 ± 0.5 μeq/cm² h in control and jasplakinolide-treated tissue, respectively, n = 6; not significant).
 FIG. 8. Decrease in ileal NaCl absorption by carbachol is prevented by pretreatment with jasplakinolide. Ileal mucosa was exposed under voltage-clamped conditions to 0.3 μM carbachol on the serosal surface, and the effect was determined over a 20-min flux period with determinations of mucosal-to-serosal (Jm) and serosal-to-mucosal (Js) fluxes of [36Cl]− and [3Na]−. Studies were performed in the absence or presence of jasplakinolide (0.02% Me2SO solvent control) and in tissue pretreated for 40 min with jasplakinolide (5 μM) on the mucosal surface. Data shown represent the effect of carbachol on ileal Na+ and Cl− transport in the absence (black bars) and presence (cross-hatched bars) of jasplakinolide and represent changes in _Isc_ fluxes, and conductance (G) 20–40 min after carbachol addition compared with same parameters during two 20-min flux periods in same tissue before the addition of carbachol. Results are means ± S.E.; n = 6, n = number of animals studied. _Isc_ and fluxes are expressed in μEq/cm² h, and conductance (G) is expressed in mS/cm². *p* values compare jasplakinolide effect with solvent control (paired t test). NS, not significant.

**DISCUSSION**

We previously demonstrated that carbachol-initiated signal transduction that inhibits NaCl absorption in intestinal epithelial cells is highly asymmetrical. Carbachol acts on intestinal epithelial cells via basolateral membrane receptors and is linked to inhibition of NaCl absorption and of brush border Na+/H+ exchange, which is part of this Na+ absorptive process (1). Previously recognized steps in carbachol-initiated signal transduction at the ileal brush border include translocation of protein kinase C to the BB along with an increase in PIP2-specific PLC activity, a short-lived effect (2). The asymmetrical aspect of signal transduction previously recognized was that there was no increase in basolateral membrane protein kinase C activity or amount or PLC-γ1 activity or amount after carbachol treatment (1, 2). In this study we provide evidence of another asymmetric aspect of signal transduction, since carbachol tyrosine phosphorylates villin, a protein specifically present in the BB and absent from the basolateral membrane.

We previously demonstrated that BB PLC-γ1 was activated in response to carbachol by a tyrosine kinase-dependent mechanism; however, there was no increase in tyrosine phosphorylation of PLC-γ1 itself in response to carbachol (2). This tyrosine kinase effect was necessary for the carbachol-induced increase in BB PLC-γ1 amount, the increase in BB PIP2-specific PLC activity, and for the inhibition of the Na+/H+ exchanger by carbachol (2). We speculated that PLC-γ1 could be recruited to the BB by associating with a BB-anchored tyrosine-phosphorylated protein (2). We now show that carbachol increases tyrosine phosphorylation of a BB pool of villin, and this pool of villin associates with PLC-γ1. This is the first observation of villin associating with PLC-γ1, and is also the first report of villin being tyrosine-phosphorylated.

The entire PLC-γ1 that translocates to the BB following carbachol exposure is present in the Triton X-100-soluble fraction, representing the membrane-associated fraction. Thus we determined if there was a pool of villin in the Triton X-100-soluble fraction of the BB. Until now, villin was considered only to be a structural protein present in the cytoskeletal core of the BB and to be involved in the morphogenesis of the BB. However, knock-out studies of villin called the latter into question since the small intestine was structurally normal in these mice (9). These results suggest that BB villin may have other functions. We showed that villin is present in three pools in the BB. The great majority is present in the BB cytoskeleton, but there are in addition two smaller pools of villin in the Triton X-100-soluble fraction of the BB, which are separated as being associated with PLC-γ1 or not associated with PLC-γ1. The three BB pools are also separated based on tyrosine phosphorylation of villin, with only the PLC-γ1-associated pool being tyrosine-phosphorylated. Even though 4-fold more villin is present in the Triton X-100-insoluble fraction, it is not tyrosine-phosphorylated. This suggests that part of the pool of villin associated with the membrane is a substrate for BB tyrosine kinases, whereas the villin associated with the cytoskeleton and the villin in the soluble fraction not associated with PLC-γ1 are not. The rapid and transient nature of the carbachol-induced increase in tyrosine phosphorylation of villin associated with PLC-γ1 suggests it is involved in early aspects of signaling. We speculate below how villin tyrosine phosphorylation might activate PLC-γ1 and vice versa how activation of PLC-γ1 could affect villin.

Carbachol causes a significant (2-fold *p* < 0.05, *n* = 4) increase in the amount of villin that associates with PLC-γ1 (Fig. 4), and there is a 2-fold increase in the amount of the tyrosine phosphorylation of this pool of villin (Fig. 2). This suggests that while carbachol causes no net increase in the tyrosine phosphorylation of individual villin molecules associated with PLC-γ1, the number of villin molecules tyrosine-phosphorylated, present in the Triton X-100-soluble fraction, and associated with PLC-γ1 is increased by carbachol. This also suggests that the increase in the tyrosine-phosphorylated villin molecules leads to their association with PLC-γ1, since there is no tyrosine-phosphorylated villin present in the BB other than what associates with PLC-γ1. What accounts for the interaction between tyrosine-phosphorylated villin and PLC-γ1 is not known. One possibility is that this might be mediated through the SH2 group of PLC-γ1. In fact sequence analysis demonstrates that villin contains the (p)Y-hydrophobic-X-hydrophobic motif.
phalloidin (8), and actin filaments may be more stable with stabilizing actin filaments and displacing phalloidin from F-actin containing SH2 domains and thus participate in signaling cascade involvement in cytoskeletal rearrangement.

Villin belongs to a family of Ca\(^{2+}\)-regulated actin-binding proteins that nucleate, cap, or sever actin filaments. Unlike other proteins of this family, at low Ca\(^{2+}\) concentrations villin induces the formation of tightly packed microfilament bundles (11). At calcium concentrations above 5 \(\mu\text{M}\) in vitro, villin severs actin filaments. Villin has been shown to associate with polyphosphoinositides, especially PIP\(_2\). In vitro studies have demonstrated that the major effect of PIP\(_2\) on villin is to inhibit its ability to sever actin filaments (6). Differential activation of severing and nucleating activities in response to changes in the concentration of Ca\(^{2+}\) and polyphosphoinositides, which are often immediate consequences of cell stimulation, could place villin directly in the pathway between receptor activation and cytoskeletal remodeling. We have shown that the pool of villin that is intimately associated with the microvillus membrane undergoes changes in tyrosine phosphorylation as part of a signaling process that begins at the basolateral membrane and terminates in the microvillus membrane. In this instance basolateral carbachol initiates a signaling cascade that leads to changes in the BB including the tyrosine phosphorylation of villin and its association with PLC-\(\gamma_1\). The activation of PLC-\(\gamma_1\) decreases the amount of PIP\(_2\) and this would be expected to remove the inhibitory effect of PIP\(_2\) on the actin severing property of villin, thus allowing villin to sever actin filaments. PLC-\(\gamma_1\) activation also generates inositol 1,4,5-trisphosphate, which increases intracellular free calcium levels, and would also activate the actin severing property of villin.

We hypothesize that tyrosine phosphorylation of villin may inhibit its actin bundling property, although this remains to be demonstrated. In several other actin-bundling proteins, such as dematin and band 4.9, phosphorylation has been shown to inhibit their actin bundling property (12, 13). The consequence of all these carbachol-induced signaling events described above would be to inhibit the actin bundling and promote the actin severing property of villin, which would disrupt the structure of the microvillus cytoskeleton or increase the amount of short F-actin filaments.

To begin addressing the functional significance of this process in intestinal cells, we used the F-actin stabilizer jasplakinolide. In vitro and in vivo, jasplakinolide has been shown to stabilize actin filaments and displace phalloidin from F-actin (7). In addition, it is more plasma membrane-permeable than phallolidin (8), and actin filaments may be more stable with jasplakinolide than phallolidin under high Ca\(^{2+}\) concentrations (7). As shown in Fig. 8, carbachol-induced inhibition of NaCl was not seen in ileum that had been pretreated with jasplakinolide. Our interpretation of this result is that filaments stabilized by jasplakinolide are more resistant to Ca\(^{2+}\)-dependent cleavage by villin, and this could explain the reversal of carbachol-induced inhibition of NaCl absorption by jasplakinolide. In recent years there has been increasing evidence of the association of ion transport proteins with actin-binding proteins (14, 15) and the involvement of cytoskeletal remodeling in the regulation of vectorial transport (16, 17). More recently a study by Berdiev et al. (18) demonstrated the stimulatory effects of short F-actin filaments on the rat epithelial sodium channel in planar lipid bilayers. Although this direct effect of short F-actin filaments on channels may be one mechanism of regulating transport proteins, we suggest another possible mechanism involving a remodeling of the microvillar core and possibly endocytosis of NHE3.

Our studies do not establish a role for villin in mediating carbachol inhibition of NaCl absorption. However, the temporal and anatomic similarity of carbachol-induced changes in ileal NaCl absorption and changes in villin tyrosine phosphorylation suggest that the two could be linked. How could the actin severing property of villin inhibit NHE3? We previously showed that nearly all described protein kinase regulation of NHE3 is by changes in \(V_{\text{max}}\) (19); and we recently showed that protein kinase C inhibits NHE3 in the BB of a polarized intestinal cell line, Caco-2, by decreasing the number of transporters in the plasma membrane (20). We suggest that inhibition of ileal villus NaCl absorption by carbachol may be due to a decrease in the amount of NHE3 present in the microvillus membrane. Our results are consistent with the suggestion that carbachol induces an increase in the actin severing property of BB villin resulting in the disassembly of the microvillar cytoskeleton, which could lead to one of the following. First, disruption of NHE3 recycling. It is generally assumed that targeting of membrane proteins to the lysosomes from the apical surface is minimal in differentiated enterocytes in the adult intestine and that the bulk of the proteins get recycled between the plasma membrane surface and intracellular recycling pool (21). The disruption of the microvillar cytoskeleton could disrupt the process of recycling, thus decreasing the amount of NHE3 remaining in the apical membrane in the presence of carbachol. Whether the removal of NHE3 from the membrane (endocytosis) or insertion into the membrane (exocytosis) in this process of recycling is affected is unknown. This is a reasonable hypothesis since it is becoming increasingly evident that several transporters are regulated by exocytic insertion or endocytic retrieval from the cell surface (22–24). Second, the disassembly of the cytoskeleton could cause the microvilli to fragment into vesicles (25), which would decrease the microvillar length or surface area. This could decrease the number of NHE3 molecules present at the microvillar surface, thus decreasing NaCl absorption. Incubating isolated BB in solutions containing high Ca\(^{2+}\) causes such BB vesiculation (26, 27); similarly, parathyroid hormone or ionomycin cause a rapid (1 min) and dramatic shortening of renal microvilli (28). Phallolidin inhibits this vesiculation (29, 30). These observations then suggest a simple mechanism for vesiculation, by stimulation of the actin severing property of villin. This may be a general mechanism for ileal villus cells to rid the cell of pathological agents including enteropathogens, reduce metabolic demands on the cell, and account for the high membrane turnover rate in normal cells. Our data suggest that structural rearrangements serve as a prerequisite for functional adaptation of transport processes. Agonist-induced morphologic changes are consistent with, and may represent a particular case of, the currently developing concept of transepithelial “cross-talk” between basolateral and luminal membranes.

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