Stabilization of F-Actin Prevents cAMP-Elicited Cl⁻ Secretion in T84 Cells

Michael Shapiro, Jeffrey Matthews, Gail Hecht, Charlene Delp, and James L. Madara

Gastrointestinal Pathology Division, and the Departments of Pathology and Medicine (Gastroenterology), Brigham and Women's Hospital and Harvard Medical School; the Department of Surgery, Beth Israel Hospital; and the Harvard Digestive Diseases Center, Boston, Massachusetts 02115

Abstract

T84 cells, a human intestinal epithelial cell line, serve as a model of electrogenic Cl⁻ secretion. We find that cAMP-elicited Cl⁻ secretion in T84 cells is accompanied by a marked redistribution of F-actin in the basolateral portion of the cell. To prevent this F-actin redistribution and thereby assess its importance to Cl⁻ secretion, we have defined simple conditions under which this model epithelium can be loaded with nitrobenzoxadiazole (NBD)-phalloidin. This reagent binds F-actin with high affinity thus stabilizing the F-actin cytoskeleton by preventing depolymerization, an event necessary for dynamic reordering of actin microfilaments. NBD-phalloidin loading is not cytotoxic as assessed by lactic dehydrogenase release, protein synthesis, transepithelial resistance, and the ability of the loaded cells to pump Na⁺ in an absorptive direction in response to the apical addition of a Na⁺ ionophore. However, cAMP-elicited redistribution of F-actin and the cAMP-elicited Cl⁻ secretory response are both markedly impaired in NBD-phalloidin preloaded T84 cells. In contrast, the carbachol-elicited Cl⁻ secretory response (Ca⁺⁺ mediated) is not attenuated by NBD-phalloidin preloading nor is it accompanied by redistribution of F-actin. These findings suggest that the cAMP-elicited cytoskeletal redistribution we describe is an integral part of cAMP-elicited Cl⁻ secretion in T84 cells. (*J. Clin. Invest.* 1991; 87:1903-1909.) Key words: cytoskeleton • plasma membrane

Introduction

All epithelia possess a complex cytoskeleton. One major component of the cytoskeleton is actin which often exists in a polymeric form, so-called F-actin microfilaments. In intestinal epithelia, the F-actin-containing cytoskeleton has three prominent components: parallel filaments, which comprise the core of microvilli; an apical, circumferential, perijunctional belt that associates with myosin and is contractile; and a less well characterized microfilamentous cortical meshwork along the basolateral membrane (1-4). Classically the actin-based cytoskeleton was thought to be primarily responsible for maintenance of cell shape in epithelia. However, recent information

This work was reported in abstract form at the Annual Meeting of the American Gastroenterological Association, May 1990, San Antonio, TX.

Address correspondence to Dr. James Madara, Department of Pathology, Brigham and Women's Hospital, 20 Shattuck Street, Room 1423, Boston, MA 02115.

Received for publication 12 July 1990 and in revised form 4 February 1991.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/91/06/1903/07 \$2.00

Volume 87, June 1991, 1903-1909

suggests that the microfilamentous cytoskeleton may influence transepithelial transport events. For example, activation of Na⁺ coupled nutrient transporters of intestinal absorptive cells results in altered tight junction permeability, an alteration thought to be due to increased tension within the perijunctional actin-myosin ring (5). Actin has also been reported to be associated with major transport proteins such as H⁺K⁺ ATPase in parietal cells (6) although the functional significance of such associations are unclear.

In the course of studying the phenotypic response of T84 cells (a human intestinal epithelial cell line used as a model for intestinal Cl⁻ secretion and barrier function [7-9]) to the secretagogue cAMP, we observed that a striking redistribution of the F-actin cytoskeleton occurs in parallel to the secretory response. To examine the potential relevance of this cytoskeletal response to Cl⁻ secretion, we have defined conditions under which epithelial cells can be loaded with nitrobenzoxadiazole (NBD)-conjugated phalloidin while maintaining confluency and avoiding cytotoxicity. This agent binds to actin microfilaments with high affinity and inhibits microfilaments from being depolymerized (10). Blocking depolymerization of microfilaments prevents depolymerization/repolymerization cascades and thus provides a mechanism of "rigidifying" the actin-based cytoskeleton. We find that prevention of the cAMP-elicited redistribution of F-actin prevents the Cl⁻ secretory response usually elicited by this nucleotide. Since, under these conditions, T84 cells remain as monolayers that maintain both barrier function and their ability to pump Na⁺, we speculate that the cytoskeletal response to cAMP is integral to Cl⁻ secretion in this model. In contrast to the cAMP response, carbachol-elicited Cl⁻ secretion is not accompanied by a reorganization of F-actin nor is it inhibited by NBD-phalloidin preloading.

Methods

The human intestinal epithelial cell line, T84, was used to prepare confluent monolayers on glass or on permeable collagen-coated supports as previously described (11). Monolayers were allowed to grow to steady-state resistance values (8). In some experiments collagen-coated permeable supports had a surface area of 2 cm² (8); however, to circumvent the otherwise prohibitive costs of NBD-phalloidin, collagen-coated permeable supports of 0.33 cm² were used for several experiments. The monolayer morphology, the response to secretagogues, and the effect of NBD-phalloidin preloading on the responses measured, did not vary between these two monolayer sizes (data not shown). Transepithelial solute fluxes and measurements of resistance and short circuit current (I_{sc}), a measure of Cl⁻ secretion in T84 cells, were obtained as before (7, 9, 11).

Protein synthesis, as measured by leucine incorporation, was performed according to the methods of Critchlow et al. and Kastyo et al.

1. *Abbreviations used in this paper:* I_{sc}, short circuit current; NBD, nitrobenzoxadiazole.

(12, 13). Lactic dehydrogenase (LDH) release was measured as before (11). For fluorescence studies, monolayers were fixed and labeled with rhodamine-phalloidin as previously described in detail (11). F-actin content was measured by the method of Howard and Oresajo (14).

NBD-phalloidin and rhodamine-phalloidin were obtained from Molecular Probes Inc., Junction City, OR, dried under pure nitrogen gas, and reconstituted in media or buffer (i.e., without vehicle). Nystatin was used from a stock solution of 5 mg/ml in methanol and was obtained from Sigma Chemical Co., St. Louis, MO. Radioisotopes were obtained from New England Nuclear, Boston, MA, and all other reagents were obtained from Sigma. Statistical analysis was performed by paired Student's *t* test or analysis of variance (ANOVA) as specified.

Results

Electrical response to cAMP/analysis of paracellular pathway.

As shown in Fig. 1, elevation of intracellular cAMP elicits an increase in Isc that has been shown by others to represent a Cl^- secretory current (9, 15, 16). After addition of 1 mM 8-bromo-cAMP (the standard condition of cAMP stimulation unless otherwise noted), Isc rose to peak at $22.5 \pm 1.9 \mu\text{A}/\text{cm}^2$ by 30 min. This secretory response was subsequently maintained over the next 30 min (Fig. 1). In parallel to the increase in Isc, resistance decreased (63 ± 3 vs. $91 \pm 1\%$ of baseline value for cAMP exposed vs. controls, respectively, at 30 minutes, $P < 0.05$). Since we have previously shown that resistance across T84 monolayers can be modulated by alterations in tight junction permeability, putatively due to alterations in the perijunctional actin-myosin ring (5, 17, 18), and since cAMP has been shown to alter paracellular permeability characteristics in other epithelia (19–21), we initially questioned whether the observed resistance changes were due to cytoskeletally mediated changes in tight junctions. This hypothesis which was, in fact, the original basis for initiating the experiments reported here was subsequently proven false: first, transepithelial mannitol fluxes did not increase after cAMP ($104 \pm 8\%$, $106 \pm 5\%$, $149 \pm 14\%$, vs. $113 \pm 10\%$, $116 \pm 7\%$, $136 \pm 11\%$, for three consecutive 20-min flux periods for control and cAMP-stimulated monolayers, respectively, $P = \text{NS}$, total $n = 15$); second, and similarly, serosal to mucosal ^{22}Na flux was not enhanced following cAMP (107 ± 9 , $108 \pm 5\%$, $128 \pm 10\%$ vs. $112 \pm 7\%$, $108 \pm 8\%$, $128 \pm 11\%$ for three consecutive 20-min flux periods relative to baseline monolayer for control and cAMP, respectively, $P = \text{NS}$, total $n = 19$); and last, as will be detailed, and in contrast to other states in which T84 cell tight junction permeability is altered (11, 17), no changes in F-actin distribution in the area of the perijunctional actin-myosin ring were identified following cAMP exposure. These findings are consistent with the hypothesis that the resistance decrease seen after cAMP exposure is

Figure 1. Isc response to 1 mM 8-bromo-cAMP in T84 cells. Isc, a measure of Cl^- secretion in T84 monolayers (9), peaks after 30 min and persists over the ensuing 30 min. Isc is of relatively small magnitude in the control group

(vehicle only). (n for each point = 6–12; cAMP and control groups significantly different $P < 0.01$ by analysis of variance, F value = 58.7).

due to a transcellular Cl^- current, not to a change in the paracellular pathway.

Effects of cAMP on F-actin distribution and content. As shown in Fig. 2, cAMP induced regionally restricted and profound alterations in F-actin distribution in T84 cells. These changes were readily apparent 30 min after stimulation and were maintained over the following 30-min period in parallel to the cAMP-induced Isc response shown in Fig. 1. As shown in the left-hand panels of Fig. 2, which are taken in the plane of the apical perijunctional actin-myosin ring, cAMP did not elicit an alteration in perijunctional ring F-actin, a finding that is in agreement with the lack of effect of cAMP on junctional permeability (vide supra). In contrast, as shown on the right-hand panel, which shows optical sections of F-actin distribution in the basal pole of the cell, cAMP alters the distribution and appearance of basolateral F-actin microfilaments. In control monolayers, F-actin in the basal pole of the cell consists of a finely dispersed array of homogenous microfilaments. After cAMP exposure, F-actin at this basal site is displaced to the cell periphery as thickened bundles that surround the cleared central zone. In a blinded review of randomly obtained photomicrographs (59 from cAMP and 60 from control), we were able to use the above differences in basolateral F-actin distribution to correctly identify the treatment group in 87% of micrographs. In two subsequent groups of experiments graded for the presence of thickened, peripherally margined basolateral F-actin, blinded analyses of randomly obtained photomicrographs showed: 72% and 93%, respectively, of photographs of cAMP-stimulated monolayers have these changes as opposed to 11% and 20% of controls (data for 30- and 60-min experiments, respectively, total of 186 micrographs and 23 monolayers were used in these analyses). It also appeared that the evolution of the cAMP-elicited cytoskeletal change paralleled the change in Isc: 38% of images were judged to contain the above changes at a time when the Isc response was half maximal ($n = 52$; half maximal effort at 15 min, $\mu\text{Amp cm}^{-2}$ under unstirred conditions).

We next sought to determine whether the basolateral redistribution of F-actin elicited by cAMP was accompanied by a net change in cellular F-actin content (e.g., net depolymerization). F-actin content was determined in eight monolayers stimulated with cAMP (1 mM 8-bromo-cAMP, 1 mM theophylline) and compared with that in seven control monolayers. Although the specific F-actin binding of cAMP monolayers was only 76% of controls, this comparison did not show a significant difference from control values (39 ± 3 vs. $53 \pm 8 \times 10^{-3} \mu\text{g}$ protein for cAMP and control, respectively, $P = 0.09$).

Stabilization of F-actin cytoskeleton by NBD-phalloidin preloading. Several approaches were used to load cells with NBD-phalloidin. The transient permeabilization procedure used by Barak and colleagues (10) in mesenchymal cells was found not to be applicable since it was associated with a marked reduction in transepithelial resistance. By modifying techniques employed in nonepithelial cells, we eventually found that simple coincubation of epithelial monolayers with this reagent eventually led to its internalization as had been described for loading nonepithelial cells with nontoxic concentrations of NBD-phalloidin (22). We coincubated monolayers for 1–5 h in media containing NBD-phalloidin concentrations ranging from 0.1 to 10 μM . This simple coincubation permitted NBD-phalloidin uptake and association with cytoskeletal F-actin as shown in Fig. 3. Since the NBD-phalloidin concen-

Figure 2. Fluorescent localization of F-actin in T84 monolayers, viewed en face, in control monolayers (*top*) and in monolayers exposed for 30–60 min to 1 mM 8-bromo-cAMP and 1 mM theophylline (*bottom*). Different optical planes of focus are obtained using Nomarski optics. In the left panels, the plane of focus is at the apical membrane and the perijunctional ring of actin (*arrowheads*) is seen. The fine flocculated actin centrally in the cell at this level represents microvillus-associated F-actin as well as F-actin underlying the apical membrane. The distribution of F-actin in the apical pole of the cell is not discernibly affected by cAMP. In the right panel, the optical plane of focus is at the basal pole of T84 cells. Here the fine, randomly dispersed pattern of F-actin in composing the basolateral cytoskeletal cortex of controls (*top right*) is replaced by thickened bundles (*arrowheads*) of peripherally relocated F-actin with central clearing (*asterisk*) after cAMP exposure (*bottom right*). Such changes in F-actin in the basal aspect of T84 cells were observed to occur within 30 min of exposure with 1 mM 8-bromo-cAMP and 1 mM theophylline. ($\times \sim 1,800$)

tration was low, it was not surprising that the labelling of the cytoskeleton seen after loading was weak and readily quenched during observation. We found we could load monolayers with no discernible toxicity by incubating them for 1.5–5 h with 0.5 μ M NBD-phalloidin. As shown in Table I, NBD-phalloidin

loading did not increase LDH release or diminish [3 H]leucine incorporation into protein. Significantly, transepithelial resistance, a general electrical parameter that also serves as an extremely sensitive indicator of toxicity, was also unaffected by NBD-phalloidin loading (Table I). Lastly, as shown in the ta-

Figure 3. En face photomicrograph of F-actin distribution within a T84 monolayer seen after coincubation with 1 μ M NBD-phalloidin. NBD, a weak and readily quenched fluorophore, allows fluorescent visualization of the distribution of the loaded phalloidin. As would be expected for the distribution of F-actin, in the apical pole of the cell, perijunctional rings are identified (arrowhead). Thus, simple coincubation of T84 cells with NBD-phalloidin permits entry of this probe into the cytosolic space where it binds F-actin.

ble, loaded cells were able to actively pump Na^+ as judged by their steady-state Isc response to the apically added Na^+ ionophore nystatin (500 U/ml). In separate experiments it was determined that the concentration of vehicle used (methanol) did not independently affect monolayer resistance or secretory response (data not shown). It should be noted that all the data in the table were collected from monolayers loaded for 3–6 h although, as detailed below, a shorter loading period may be sufficient to negate the cAMP-elicited cytoskeletal (and Cl^- secretory) response.

cAMP exposure fails to remodel F-actin or stimulate Cl^- secretion in T84 monolayers preloaded with NBD-phalloidin. As shown in Fig. 4, the dramatic cAMP-elicited rearrangement of F-actin in the basal portion of T84 cells is attenuated by preloading cells with NBD-phalloidin. This inhibitory effect was semi-quantitatively assessed in one group of experiments: photographs were obtained from areas of control and cAMP exposed monolayers (which demonstrated F-actin patterns described previously) and compared to photographs taken from unselected areas of cAMP-exposed but NBD-phalloidin preloaded monolayers. Four monolayers from each group were examined. Blinded review of this sample of NBD-phalloidin preloaded and cAMP exposed monolayers yielded images that were judged to be indistinguishable from controls in 44% of micrographs. The remaining 56% of micrographs in this group were identified as exhibiting partial but subtle cAMP-like changes separable from the full cAMP response although not indistinguishable from the “normal” pattern. Thus, NBD-phalloidin preloading profoundly inhibited the cAMP-elicited cytoskeletal response.

As shown in Figs. 5 and 6, NBD-phalloidin preloaded monolayers also had markedly attenuated Isc responses to cAMP. In initial experiments using cells of low passage number

(<60), we found that NBD-phalloidin preloading for as short as 90 min was sufficient for inhibiting the cAMP-elicited rearrange of F-actin (and the Cl^- secretory response). Higher passage number cells may require longer loading periods for NBD-phalloidin loading presumably due to passage-related alterations in plasma membrane permeation by this reagent. As indicated in Table I and further supported by the carbachol data (see below), even several hours of incubation with NBD-phalloidin does not impair the functional viability of monolayers. Hence, one can, in future experiments, use more extended loading periods to make such passage-related loading issues irrelevant.

NBD-phalloidin loading also inhibits cAMP-elicited Cl^- secretion at cAMP concentrations below that at which maximal secretion can occur. At 0.2 mM 8-bromo-cAMP, a dose that elicits 14.5% of the maximal Isc response, NBD-phalloidin preloading still exerts substantial inhibitory effects on Isc (77.3% inhibition as compared with controls, $n = 5$; 5.8 ± 0.9 vs. $22.7 \pm 2.8 \mu\text{A} \cdot \text{cm}^{-2}$ at 12 min for NBD-phalloidin loaded vs. control monolayers).

Carbachol-stimulated Cl^- secretion is not attenuated by NBD-phalloidin loading. To see whether NBD-phalloidin loading also attenuated Ca^{++} -induced Cl^- secretion, we assessed the effects of loading on carbachol-elicited Isc (23). As shown in Fig. 7, loading conditions (here 12–16 h) that greatly attenuated the Isc response to 8-bromo-cAMP, did not effect the Isc response to carbachol. Since the carbachol-elicited Isc is not influenced by NBD-phalloidin preloading, we were interested in seeing whether F-actin rearrangement parallels the rapid Isc response elicited by carbachol. F-actin distribution at the peak of the carbachol-elicited Isc response did not appear qualitatively different from that in control monolayers. This impression was confirmed by semi-qualitative analyses similar to those outlined above which showed no significant expression of the pattern elicited by cAMP with carbachol (11% vs. 9% for control vs. carbachol, respectively, $n = 35$ images for each).

Discussion

We show that, as in Madin-Derby canine kidney (MDCK) cells (24), cAMP elicits a redistribution of F-actin in T84 cells. The link between cAMP and F-actin reordering is unclear although Lamb and colleagues have recently shown in fibroblasts that a cAMP-dependent kinase inhibits myosin light chain kinase ac-

Table I. Effects of NBD-Phalloidin Loading on T84 Monolayers

	Loaded	Control	P
LDH release	15	12	
percentage of total content	(pool of 5)	(pool of 4)	
^3H leucine incorporation	75 ± 8	87 ± 6	NS
dpm/ μg protein	($n = 7$)	($n = 7$)	
Resistance	350 ± 24	301 ± 20	NS
$\text{ohm} \cdot \text{cm}^2$	($n = 23$)	($n = 22$)	
Steady-state Isc response to nystatin	2.5 ± 0.4	3.2 ± 0.4	NS
$\mu\text{A}/\text{cm}^2$	($n = 12$)	($n = 10$)

Figure 4. En face photomicrographs of F-actin localization in the basal pole of T84 cells in control monolayers (left), monolayers exposed to 1 mM 8-bromo-cAMP and 1 mM theophylline for 60 min (center) and in monolayers preloaded with NBD-phalloidin and subsequently stimulated with cAMP as above for 60 min (right). Here the F-actin distribution is highlighted with a rhodamine probe at the end of the experiment to intensify the signal. As can be seen, NBD-phalloidin preloading largely prevents the cAMP-elicited F-actin reordering (see text for quantitation of this inhibition of the F-actin response).

tivity and such inhibition is accompanied by a reordering of cytoskeletal actin (25). More importantly, we show that inhibition of the cAMP-elicited cytoskeletal response also substantially inhibits the cAMP-elicited Cl^- secretory response. Inhibition of these cAMP-elicited cytoskeletal and Cl^- secretory responses occurs under conditions in which T84 cells are able to carry out normal synthetic functions, able to form barriers to passive ion flow, able to pump Na^+ in response to a Na^+ ionophore, and able to secrete Cl^- in response to carbachol, hence demonstrating retention of several cellular functions including general electrophysiological parameters and the specific ability to secrete Cl^- . These findings support the recent speculative hypothesis, recently raised in a review, that the cytoskeleton may be involved in the regulation of Cl^- secretion (26). Such data also suggest that cytoskeletal influence on Cl^- secretion may be agonist specific, occurring with cAMP-mediated but

Figure 5. Time course of Isc response to cAMP in control monolayers (top curve) and in monolayers preloaded with NBD-phalloidin (bottom curve). In contrast to controls, monolayers preloaded with NBD-phalloidin have a

markedly attenuated Isc response to cAMP ($P < 0.01$, ANOVA, $F = 24.15$). Since controls unexposed to cAMP display Isc of 1–3 μAmp (not shown), NBD-phalloidin substantially inhibits the cAMP response. ($n = 6$ and 7 for control and NBD-phalloidin preloaded curves, respectively.) Loading conditions may affect the degree of inhibition of the cAMP response (see text).

not Ca^{++} -mediated Cl^- secretion. The hypothesis linking the cytoskeleton to Cl^- secretion had previously been raised although prior emphasis was on the microtubular rather than the microfilamentous cytoskeleton (27). Such observations raise the possibility that one or more of the membrane-associated proteins known to participate in Cl^- secretion interact with the cytoskeleton. Such putative cytoskeletal-transport protein interactions could be indirect, but this study suggests they are functionally important. Candidate transmembrane transport events that might be so influenced include the apical Cl^- conductance, the basolateral $\text{Na}:\text{K}:\text{Cl}$ cotransport process, the basolateral K conductance, and the basolateral Na^+ pump.

Figure 6. Net Isc response to 30-min stimulation with cAMP in low passage number (< 60) T84 cells preloaded with 0.5 μM NBD-phalloidin for various periods of time. Net Isc in control monolayers (20.7 ± 2.2 $\mu\text{A}/\text{cm}^2$, $n = 12$) pro-

duced by 30-min exposure to cAMP (left column) is significantly greater than the response in Isc achieved in monolayers preloaded NBD-phalloidin for 90 min (6.75 ± 0.7 $\mu\text{A}/\text{cm}^2$, $n = 4$), for 210 min (4.4 ± 0.7 $\mu\text{A}/\text{cm}^2$, $n = 5$), or for 300 min (4.7 ± 0.7 , $n = 11$) ($P > 0.05$, $P < 0.01$, ANOVA, $F = 23.65$). The inhibition seen with 90 min loading is not significantly less than with the longer periods for these low passage number cells. Higher passage cells need longer loading periods to achieve substantial inhibition of cAMP-elicited Cl^- secretion (see text).

Figure 7. NBD-phalloidin preloading attenuates cAMP but not carbachol-elicited Cl^- secretion. Carbachol (10^{-4} M) elicits a short-lived Cl^- secretory response (not paralleled by a rearrangement of F-actin; see text) that is unaffected by NBD-phalloidin preloading. In contrast, paired experiments show the usual inhibition

of 1 mM 8-Br-cAMP-elicited Cl^- secretion by preloading. Furthermore, separate experiments using lower doses of cAMP (0.2 mM) that elicit Cl^- secretory responses of similar magnitude to those elicited by 10^{-4} M carbachol, also show substantial inhibition of Cl^- secretion. $n = 7$ for each carbachol curve. $n = 5-7$ for each cAMP bar. Monolayers were loaded by incubation with NBD-phalloidin for ~ 16 h for these experiments.

Since the cytoskeletal responses observed are basolateral, not apical, it would not be surprising if this were the site at which cytoskeletal influence of cAMP-induced Cl^- secretion is expressed. At this time we cannot rule out the interesting possibility that the effect is at the level of the Cl^- conductances, although, as indicated above, this may be less likely. Experiments to examine the effects of NBD-phalloidin on these specific membrane transport pathways are currently underway.

Studies in other tissues indicate that links between the cytoskeleton and proteins involved in ion transport across the plasma membrane do occur. For example, Nelson and colleagues have shown in MDCK cells that a substantial fraction of Na^+/K^+ -ATPase associates with a cytoskeletal complex that includes ankyrin and fodrin, although here such associations are thought to be of primary importance in establishing the polarized distribution of this protein on the cell surface (28), not in functionally modifying pump activity. In addition, the anion transport protein of chicken erythrocytes is known to be a major site of plasma membrane-cytoskeletal association in this cell although, again, it is not clear if such associations could influence the ion transport characteristics of this protein (29). Lastly, and perhaps most interesting given our findings, Jorgensen and colleagues find that a putative component of the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter in pig kidney can only be extracted under high salt conditions, a result they interpreted as indicating a link between this cotransporter and the cytoskeleton (30).

We also define a simple method by which the F-actin cytoskeleton can be stabilized in an epithelium without exerting detectable toxicity and without disrupting the monolayer. This method, which likely will be adaptable to other epithelia, provides a means of assessing the contribution of F-actin to transport events in confluent monolayers. The interaction of phalloidin with the cytoskeleton is well characterized (31). Phalloidin binds avidly to F-actin ($K_d < 0.1 \mu\text{M}$) and prevents depolymerization of actin oligomers (22, 31). These facts make phalloidin, and the native compound phalloidin, extremely useful F-actin specific probes. In fact, in our study we have used these compounds in two ways other than to load cells: as a fluorescent probe for F-actin localization and as a biochemical probe to quantitate F-actin content of cells, both widely accepted as well characterized methods (11, 14). It was originally

assumed that plasma membranes were impermeant to phalloidin and thus initial studies using this reagent in living cells, as has been done here, relied on microinjection (32) or transient reversible permeabilization (10). More recently, however, it has been noted that NBD-conjugated phalloidin can be loaded into nonepithelial cells by simple coincubation (22, 33). Whether by microinjection, permeabilization, or incubation loading, conditions could be identified in endothelial cells, fibroblasts, and chick embryo cells that allowed F-actin labelling without cytotoxicity (10, 22, 31, 33). As shown by Phillips et al., loading of endothelial cells with nontoxic doses of NBD-phalloidin prevents the thrombin-induced increases in endothelial permeability that appear to occur due to thrombin-induced cytoskeletal contraction (33). We suspect use of this reagent may provide initial insights into the poorly studied but potentially important area of cytoskeletal influences on ion transport in epithelia.

In summary, we describe a cAMP-elicited change in F-actin that parallels the Cl^- secretory response to this mediator in T84 cells. NBD-phalloidin loading of T84 cells can be performed by a simple coincubation without detectable cellular toxicity. The stabilization of F-actin in T84 cells by this technique inhibits both the change in F-actin and Cl^- secretory response typically elicited by cAMP but does not alter other electrical parameters of monolayer function. Lastly, both the rearrangement of F-actin and the inhibition of the Cl^- secretory response seen in NBD-phalloidin preloaded cells does not occur with Ca^{++} -mediated Cl^- secretion induced by carbachol. We speculate that the changes in F-actin produced by cAMP are crucial for the generation of Cl^- secretion by this mediator.

Acknowledgments

We thank Susan Carlson for her expert technical help with preparation of photomicrographs and Virginia Hamel for her help in preparing the manuscript.

This work was supported by National Institutes of Health grants R01-DK 35932 and P01-DK33506 and by an individual National Research Service Award (NRSA) and American Gastroenterological Association Senior Fellow Award to Dr. Shapiro, and by NRSA Training Program DK-08020.

References

1. Mooseker, M. S. 1985. Organization, chemistry and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annu. Rev. Cell Biol.* 1:209-242.
2. Rodewald, R., S. B. Newman, and M. J. Karnovsky. 1976. Contraction of isolated brush borders from the intestinal epithelium. *J. Cell Biol.* 70:541-554.
3. Hagen, S. J., and J. S. Trier. 1988. Immunocytochemical localizations of actin in epithelial cells of rat small intestine by light and electron microscopy. *J. Histochem. Cytochem.* 36:717-727.
4. Madara, J. L., and J. S. Trier. 1987. In *Physiology of the Gastrointestinal Tract*. L. R. Johnson, editor. Raven Press, Ltd., New York. 1209-1250.
5. Madara, J. L., and J. R. Pappenheimer. 1987. Structural basis for physiological regulation of paracellular pathways in intestinal epithelia. *J. Membr. Biol.* 100:149-164.
6. Mercier, F., H. Reggio, G. Devilliers, D. Bataille, and P. Mongeat. 1989. Membrane-cytoskeleton dynamics in rat parietal cells: mobilization of actin and spectrin upon stimulation of gastric acid secretion. *J. Cell Biol.* 108:441-453.
7. Dharmasathaphorn, K., J. A. McRoberts, K. G. Mandel, L. D. Tisdale, and H. Masui. 1984. A human colonic tumor cell line that maintains vectorial electrolyte transport. *Am. J. Physiol.* 246:G204-G208.
8. Madara, J. L., and K. Dharmasathaphorn. 1985. Occluding junction structure-function relationships in a cultural epithelial monolayer. *J. Cell Biol.* 101:2124-2133.
9. Dharmasathaphorn, K., K. G. Mandel, H. Masui, and J. A. McRoberts. 1985. Vasoactive intestinal polypeptide-induced chloride secretion by a colonic epithelial cell line: direct participation of a basolaterally localized Na^+ , K^+ , Cl^- cotransport system. *J. Clin. Invest.* 75:462-471.

10. Barak, L. S., R. R. Yocum, E. A. Nothnagel, and W. W. Webb. 1980. Fluorescence staining of the actin cytoskeleton in living cells with F-nitrobenz-2-oxa-1,3-diazole-phalloidin. *Proc. Natl. Acad. Sci. USA* 77:980-984.
11. Hecht, G., C. Pothoulakis, J. T. LaMont, and J. L. Madara. 1988. *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. *J. Clin. Invest.* 82:1516-1524.
12. Critchlow, J., D. Magee, S. Ito, K. Takeuchi, and W. Silen. 1985. Requirements for restitution of the surface epithelium of frog stomach after mucosal injury. *Gastroenterology* 88:237-249.
13. Kostyo, J. L., and E. Knobi. 1959. The effect of growth hormone on the in vitro incorporation of leucine-2-¹⁴C into the protein of rat diaphragm. *Endocrinology* 65:395-401.
14. Howard, T. H., and C. O. Oresajo. 1985. The kinetics of chemotactic peptide-induced change in F-actin content, F-actin distribution, and the shape of neutrophils. *J. Cell Biol.* 101:1078-1085.
15. Weymer, A., P. Huott, W. Liu, J. A. McRoberts, and K. Dharmasathaphorn. 1985. Chloride secretory mechanism induced by prostaglandin E₁ in a colonic epithelial cell line. *J. Clin. Invest.* 76:1828-1836.
16. Cartwright, C. A., J. A. McRoberts, K. G. Mandel, and K. Dharmasathaphorn. 1985. Synergistic action of cyclic AMP- and calcium-mediated chloride secretion in a colonic epithelial cell line. *J. Clin. Invest.* 76:1837-1842.
17. Madara, J. L., J. Stafford, D. Barenberg, and S. Carlson. 1988. Functional coupling of tight junctions and microfilaments in T84 monolayers. *Am. J. Physiol.* 254:G416-G423.
18. Madara, J. L., D. Barenberg, and S. Carlson. 1986. Effects of cytochalasin D on occluding junctions of intestinal absorptive cells: further evidence that the cytoskeleton may influence paracellular permeability and junctional charge selectivity. *J. Cell Biol.* 102:2125-2136.
19. Duffy, M. E., B. Hainan, S. Ho, and C. J. Bentzel. 1981. Regulation of epithelial tight junction permeability by cyclic AMP. *Nature (Lond.)* 294:451-453.
20. Jacobson, H. R. 1979. Altered permeability in the proximal tubule response to cyclic AMP. *Am. J. Physiol.* 236:F71-F79.
21. Stelzner, T. J., J. V. Weil, and R. F. O'Brien. 1989. Role of cyclic adenosine monophosphate in the induction of endothelial barrier properties. *J. Cell Physiol.* 139:157-166.
22. Barak, L. S., R. R. Yocum, and W. W. Webb. 1981. In vivo staining of cytoskeletal actin by autointernalization of nontoxic concentrations of nitrobenzoxadiazolephalloidin. *J. Cell Biol.* 89:368-372.
23. Dharmasathaphorn, K., and S. J. Pandol. 1986. Mechanism of chloride secretion induced by carbachol in a colonic epithelial cell line. *J. Clin. Invest.* 77:348-354.
24. Mills, J. W., and M. Lubin. 1986. Effect of adenosine 3',5'-cyclic monophosphate on volume and cytoskeleton of MDCK cells. *Am. J. Physiol.* 250:C319-C324.
25. Lamb, N. J. C., A. Fernandez, M. A. Conti, R. Adelstein, D. B. Glass, W. J. Welch, and J. R. Feramisco. 1988. Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. *J. Cell Biol.* 106:1955-1971.
26. O'Grady, S. M., H. C. Palfrey, and M. Field. 1987. Characteristics and functions of Na:K:Cl cotransport in epithelial tissues. *Am. J. Physiol.* 253:C177-C192.
27. Strombeck, D. R. 1973. Colchicine, cycloheximide and serotonin inhibition of intestinal fluid production caused by *Vibrio cholera* toxin. *Life Sci.* 12:211-218.
28. Nelson, W. J., and R. W. Hammerton. 1989. A membrane-cytoskeletal complex containing Na⁺, K⁺-ATPase, ankyrin, and fodrin in Madin-Darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* 108:893-902.
29. Marchesi, V. T. 1985. Stabilizing infrastructure of cell membranes. *Annu. Rev. Cell Biol.* 1:531-561.
30. Jorgensen, P. L., J. Petersen, and W. D. Rees. 1984. Identification of a Na⁺, K⁺, Cl⁻-cotransport protein of M_r 34000 from kidney by photolabeling with [³H]bumetanide. *Biochim. Biophys. Acta* 775:105-110.
31. Cooper, J. A. 1987. Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* 105:1473-1478.
32. Hamaguchi, Y., and I. Mabuchi. 1982. Effects of phalloidin microinjection and localization of fluorescein-labeled phalloidin in living sand dollar eggs. *Cell Motil.* 2:103-113.
33. Phillips, P. G., H. Lum, A. B. Malik, and M. F. Tsan. 1989. Phalloidin prevents thrombin induced increases in endothelial permeability to albumin. *Am. J. Physiol.* 257:C562-C567.