Microfilament-dependent Activation of $Na^+/K^+/2Cl^-$ Cotransport by cAMP in Intestinal Epithelial Monolayers

Jeffrey B. Matthews, Christopher S. Awtrey, and James L. Madara
Division of Gastrointestinal Pathology, Department of Pathology, Brigham and Women's Hospital, and Department of Surgery, Beth Israel Hospital; Harvard Medical School; and the Harvard Digestive Diseases Center, Boston, Massachusetts 02115

Abstract

cAMP-mediated stimulation of Cl^- secretion in the human intestinal cell line T84 is accompanied by significant remodeling of F-actin, and both the secretory and cytoskeletal responses may be largely ablated by previous cell loading with phalloidin derivatives, reagents that prevent dynamic reordering of microfilaments (1991. *J. Clin. Invest.* 87:1903–1909). In this study, we examined the effect of phalloidin loading on the cAMP-elicited activity of the individual membrane-associated transport proteins involved in electrogenic Cl^- secretion. Efflux of ^{125}I and ^{86}Rb was used to assay forskolin-stimulated Cl^- and K^+ conductances, respectively, and no inhibitory effect of phalloidin could be detected. Na^+/K^+-ATPase pump activity, assessed as bumetanide-insensitive ^{86}Rb uptake and the ability of monolayers to generate a Na^+ absorptive current in response to apical addition of a Na^+ ionophore, was not different between control and phalloidin-loaded monolayers. Forskolin was found to stimulate $Na^+/K^+/2Cl^-$ cotransport (bumetanide-sensitive ^{86}Rb uptake) in time-dependent fashion. In the absence of any agonist, cotransporter activity was markedly decreased in phalloidin-loaded monolayers. Furthermore, under phalloidin-loaded conditions, the forskolin-elicited increase in bumetanide-sensitive ^{86}Rb uptake was markedly attenuated. These findings suggest that cAMP-induced activity of Cl^- channels, K^+ channels, and the Na^+/K^+-ATPase are not influenced by F-actin stabilization. However, cAMP-induced activation of the $Na^+/K^+/2Cl^-$ cotransporter appears to be microfilament-dependent, and ablation of this event is likely to account for the inhibition of cAMP-elicited Cl^- secretion seen in the phalloidin-loaded state. Such findings suggest that the $Na^+/K^+/2Cl^-$ cotransporter is functionally linked to the cytoskeleton and is a regulated site of cAMP-elicited electrogenic Cl^- secretion. (*J. Clin. Invest.* 1992. 90:1608–1613.) Key words: intestinal secretion • cytoskeleton • cell culture • cell volume • chlorides

Introduction

Actin-based cytoskeletal networks may influence a variety of important cellular activities through close association with integral membrane proteins, including those involved in ion transport. A notable example of a microfilament-linked transport protein is the band 3 anion exchanger of erythrocytes (1), but similar cytoskeletal associations have also been identified in epithelial cells (2). In the case of epithelial cells, such cytoskeletal associations are thought to be of importance in the establishment of cell polarity for vectorial transport (3). However, it is largely unknown whether the specific transport characteristics of individual integral membrane proteins might be regulated by direct or indirect interactions with cytoskeletal elements.

The T84 cell line, a cultured human intestinal line with cryptlike features, has been widely used as a model for electrogenic Cl^- secretion (4). We recently reported that cAMP-elicited stimulation of Cl^- secretion was paralleled by a striking redistribution of F-actin confined to the basal pole of T84 cells. In monolayers preloaded with NBD-phallicidin[1] (a phalloidin derivative that stabilizes F-actin and prevents dynamic reordering of microfilaments), both the cAMP-elicited Cl^- secretory current and the accompanying phenotypic change in the cytoskeleton were largely ablated (5). NBD-phallicidin loading was shown not to affect transepithelial resistance, protein synthesis, or the ability of cells to pump Na^+. Moreover, the inhibitory effect of NBD-phallicidin on cAMP-induced Cl^- secretion was agonist-specific, since carbachol (Ca^{++}-mediated) Cl^- secretion was preserved and carbachol did not elicit a rearrangement of the cytoskeleton (5). Such data suggest cytoskeletal influence over cAMP-regulated intestinal Cl^- secretion.

Epithelial electrogenic Cl^- secretion occurs via the coordinated action of four specific membrane transport processes. Internalization of salt occurs across the basolateral membrane via the $Na^+/K^+/2Cl^-$ cotransporter (6). The basolateral Na^+/K^+-ATPase provides the Na^+ gradient permitting uptake of salt by the cotransporter, and basolateral K^+ channels provide a K^+ exit pathway (7). Apically positioned Cl^- channels serve as the basis for vectorial electrogenic Cl^- secretion (8). The current consensus view is that electrogenic Cl^- secretion is

Address correspondence to Dr. Jeffrey B. Matthews, Department of Surgery, Beth Israel Hospital, 330 Brookline Ave., Boston, MA 02215.
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1. *Abbreviations used in this paper:* HPBR, Hepes-phosphate buffered Ringer's solution; NBD-phallicidin, a phalloidin derivative that stabilizes F-actin and prevents dynamic reordering of microfilaments.

predominantly regulated at the level of the Cl^- and, to a lesser extent, K^+ channels (8–10).

In this report, we examine the four membrane transport pathways that define Cl^- secretion for evidence of cAMP-elicited and cytoskeletal-influenced regulation. We find that in addition to the known effects of cAMP on Cl^- channel regulation, cAMP agonists also increase $Na^+/K^+/2Cl^-$ cotransporter activity in this cell line. Moreover, in cells preloaded with NBD-phallicidin or the native compound phalloidin, this activation of the basolateral cotransporter is largely attenuated. In contrast, our findings suggest that phalloidin loading does not affect the Na^+/K^+-ATPase pump activity, Cl^- channel activity, or K^+ channel activity.

Methods

T84 cells were maintained in culture and grown on permeable collagen-coated supports (Costar Corp., Cambridge, MA) or 35-mm plastic dishes, as previously described in detail (11).

Preloading of monolayers with NBD-phallicidin or phalloidin. Simple coincubation with 0.5 μM NBD-phallicidin for 2–20 h without previous permeabilization of T84 cells leads to its internalization and association of this reagent with actin (5). Additionally, we now report that the native compound phalloidin (10–33 μM), which is less permeant than NBD-phallicidin, but also far less expensive, can also be loaded into cells by coincubation for up to 24 h. This loading of phalloidin resulted in no detectable change in baseline transepithelial resistance (1,131±98 Ω/cm^2 for monolayers loaded with 33 μM phalloidin for 24 h versus 1,169±101 Ω/cm^2 for control, $n = 17$), and inhibited cAMP-mediated agonist (see Results) but not carbachol-(data not shown) stimulated Cl^- secretion, as previously described for cells loaded with NBD-phallicidin (5), thus demonstrating the specificity and nontoxic nature of these loading conditions. There exists some passage-related variability in optimal loading conditions for NBD-phallicidin and phalloidin (5), and thus, in practice, both positive and negative controls (I_{sc} response to cAMP-mediated agonists and carbachol, respectively) for loading efficiency are performed every three to five passages. Since the effects on electrogenic Cl^- secretion and individual transport pathways reported below were identical for both NBD-phallicidin and phalloidin loading conditions, the results were pooled, and the term "phalloidin-loaded" is used below to refer to both reagents.

Transepithelial electrolyte transport studies. Using a dual voltage clamp (Bioengineering Department, University of Iowa, Iowa City, IA) on confluent monolayers grown on permeable collagen-coated 24-well inserts, transepithelial potential difference, resistance, and short circuit current (I_{sc}) were measured (5, 12). In T84 cells, I_{sc} is an accurate measurements of net Cl^- secretion (11). Electrical measurements were carried out in Hepes-phosphate buffered Ringer's solution (HPBR) containing 135 mM NaCl, 5 mM KCl, 3.33 mM $Na\,H_2PO_4$, 0.83 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM Hepes, pH 7.5. In one set of experiments, a Cl^--free HPBR solution (with gluconate substituting for chloride) was used to assess the ability of cAMP-stimulated monolayers to pump Na^+ in response to an apically applied Na^+ ionophore (nystatin, 500 U/ml).

^{86}Rb and ^{125}I efflux studies. Semiconfluent monolayers on 35-mm dishes were used 2–5 d after plating. The rate constant of ^{125}I and ^{86}Rb efflux was used to estimate agonist-stimulated activation of Cl^- and K^+ channels; it has been validated that in T84 cells, ^{125}I and ^{86}Rb can reliably replace ^{36}Cl and ^{42}K for measurement of efflux via Cl and K channels, respectively (13). Control and phalloidin preloaded monolayers were exposed to HPBR containing 0.5–1 μCi ^{125}I and/or 1–2 μCi ^{86}Rb for 60–90 min. After four rapid washes with 2 ml of unlabeled HPBR, 1 ml of fresh HPBR was placed in the dish. Using a sample-and-replace technique, 1-ml samples were obtained at 1-min intervals and counted by standard scintillation methods. After four baseline samples were obtained, unlabeled HPBR containing the appropriate agonist was used as the replacement solution, and further 1-min samples were obtained. The residual radioactivity in the dish was measured after extraction with 1 ml of 0.1 N NaOH. The rate constant of tracer efflux was calculated as $[\ln(R_2) - \ln(R_1)]/(t_2 - t_1)$, where R_x is the percent radioactivity remaining in the monolayer at time t_x, as described by others (13).

^{86}Rb uptake studies. Semiconfluent monolayers grown on 35-mM dishes were preexposed to HPBR containing the appropriate agonist with or without 10 μM bumetanide for 5–20 min. After aspiration of this solution, the dishes were exposed to HPBR labeled with ~1.5 μCi ^{86}Rb for 1 min. Uptake was terminated by washing four times with an ice-cold solution containing 100 mM $MgCl_2$ and 10 mM TRIS-Cl, pH 7.5. Cellular radioactivity was extracted with 0.1 N NaOH. Protein was determined spectrophotometrically (Pierce Chemical Co., Rockford, IL). A similar protocol was used to specifically study basolateral membrane uptake in confluent monolayers grown on permeable supports in six-well inserts in which the basolateral solution alone contained ^{86}Rb. For these experiments, uptakes were terminated by repeatedly dipping the insert into ice-cold $MgCl_2$ buffer, and the filters were cut out and placed directly into scintillation vials. It has been shown by others (14) that for T84 cells, ^{86}Rb uptake studies yield qualitatively similar results using cells grown either on plastic dishes or permeable supports.

Linearity of uptake of ^{86}Rb under both unstimulated and forskolin-stimulated conditions was confirmed for the initial 5 min after exposure to this isotope ($r = 0.997$, data not shown). Activity of the $Na^+/K^+/2Cl^-$ cotransporter was defined as the bumetanide-sensitive component of ^{86}Rb uptake. This value was derived by subtracting the individual values of ^{86}Rb uptake in the absence of bumetanide from the mean value of the uptake for dishes containing bumetanide. The bumetanide-insensitive component of uptake under these conditions largely reflects Na^+/K^+-ATPase activity, since the basolateral K^+ conductance elicited by cAMP agonists is relatively small (see below). Under forskolin-stimulated conditions and in the presence of 1 mM ouabain, more than 85% of ^{86}Rb uptake is in fact bumetanide-sensitive (data not shown). However, ouabain-preincubation and intracellular Na^+ depletion steps (14) were not used (15, 16) to eliminate the potentially confounding effects of ouabain-induced cell swelling and altered cytosolic ion concentrations on the cytoskeleton and on cotransporter function.

Materials. NBD-phallicidin (Molecular Probes, Inc., Eugene, OR) and phalloidin (Sigma Chemical Co., St. Louis, MO) were stored in a methanol stock solution, dried under pure N_2, and reconstituted in media. Bumetanide (Hoffmann-LaRoche, Inc., Nutley, NJ) and forskolin (Calbiochem, San Diego, CA) were dissolved in DMSO. Radioisotopes were obtained from New England Nuclear (Boston, MA). All other reagents were from Sigma.

Statistical analysis. Results are reported as mean±SE. Student's t test for paired variates and analysis of variance (ANOVA) were used where appropriate.

Results

Electrical response in control and phalloidin-loaded monolayers. Phalloidin-loaded monolayers exhibited a markedly inhibited secretory response to the cAMP agonist forskolin (Fig. 1). Phalloidin loading was found to inhibit the cAMP-mediated Cl^- secretory response throughout the forskolin dose-response curve. For example, at 10 nM forskolin, there is ~ 90% attenuation of the current (1.3±0.1 $\mu A/cm^{-2}$ vs. 12.1±2.2 $\mu A/cm^{-2}$ for phalloidin vs. control, $n = 4$, $P < 0.015$).

Effect of cytoskeletal stabilization on cAMP-mediated stimulation of Cl^- and K^+ conductive pathways. Forskolin increased the rate constant of ^{125}I efflux, reaching a maximum

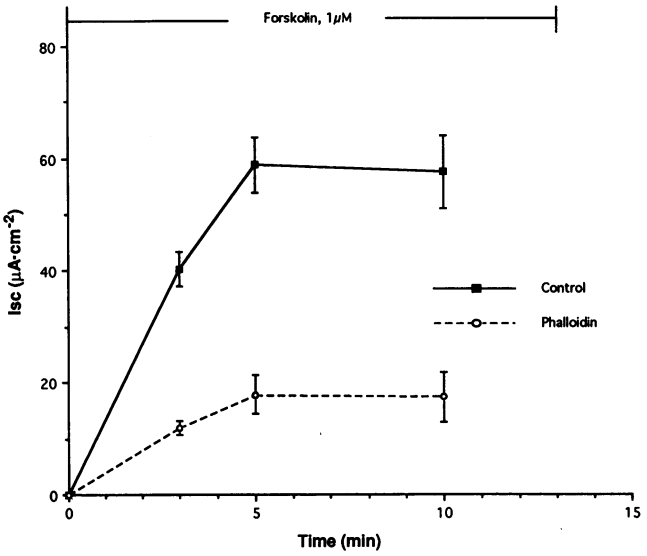

Figure 1. Phalloidin preloading attenuates the Cl^- secretory response of T84 cell monolayers to a cAMP-mediated agonist. Time course of short circuit current (I_{sc}) response to forskolin (1 μM) in phalloidin (*dotted line*) and control (*solid line*) monolayers (n = 11, F = 103.7 by ANOVA, $P < 0.0001$).

within 2–3 min (Fig. 2 *A*). This finding, consistent with Vlengarik et al. (13), indicates that cAMP elicits opening of conductive Cl^- pathways and provides a large anion efflux signal. Forskolin also caused a small but significant increase in the rate constant of ^{86}Rb efflux (Fig. 2 *B*) (13). To magnify the K^+ channel signal to probe its potential sensitivity to phalloidin loading, we also examined ^{86}Rb efflux after exposure to the Ca^{++} agonist carbachol. After carbachol (0.1 mM), the rate constant of ^{86}Rb efflux increased from 0.026±0.004 to 0.092±0.013 min^{-1} (n = 19, $P < 0.001$), consistent with the findings of others (13).

There was no detectable difference in the maximum rate constant or the time course of ^{125}I efflux between control and phalloidin-loaded monolayers in response to forskolin (Fig. 2 *A*), indicating that the large inhibitory effect of phalloidin on the overall secretory response is unlikely to be explained by inhibition of the regulated Cl^- efflux pathway. Phalloidin also exerted no detectable effect on forskolin-stimulated ^{86}Rb efflux (Fig. 2 *B*). To validate the lack of influence of phalloidin loading on K^+ channels, we measured ^{86}Rb efflux in monolayers stimulated by the Ca^{++} agonist carbachol (0.1 mM), which, as shown above, elicits a much larger efflux signal than forskolin. Even under these conditions, phalloidin did not affect ^{86}Rb efflux (0.131±0.035, n = 5, vs. 0.092±0.013, n = 19, NS, maximal rate constants postcarbachol addition for loaded vs. control monolayers, respectively). These data not only show the lack of an effect of phalloidin loading on ^{125}I and ^{86}Rb efflux, but, by indicating unaltered regulation of Cl^- and K^+ channels in the phalloidin-loaded state, further confirm the lack of nonspecific toxic effects of such loading.

Effect of cytoskeletal stabilization on Na^+/K^+-ATPase activity. There was no significant difference in the bumetanide-insensitive component of ^{86}Rb uptake between forskolin-stimulated control or phalloidin-loaded monolayers (74.1±5.9 nmol $K^+ g^{-1} min^{-1}$ for control, n = 24, vs. 63.4±4.5 for phalloidin, n = 22, NS), implying an absence of cytoskeletal influence on the Na^+/K^+-ATPase under these conditions. Na^+ pump activity was also assessed by transepithelial transport studies. Forskolin-stimulated monolayers bathed in nominally Cl^--free buffer generate an I_{sc} (Na^+ absorptive current) in response to apical addition of nystatin, which reflects the activity of the Na^+/K^+-ATPase (17). There was no difference between the steady-state I_{sc} generated by control and phalloidin-loaded monolayers 8–10 min after exposure to nystatin (8.7±0.7 vs. 7.5±0.5 μA/cm^{-2}, respectively, each n = 18, NS). Therefore, neither assay indicated significant influence of cytoskeletal stabilization on Na^+ pump activity.

Effect of cytoskeletal stabilization on cAMP stimulation of $Na^+/K^+/2Cl^-$-cotransporter activity. Exposure of monolayers to forskolin resulted in a time-dependent increase in the bumetanide-sensitive component of ^{86}Rb uptake compared to unstimulated controls (Fig. 3). In contrast, no significant increase

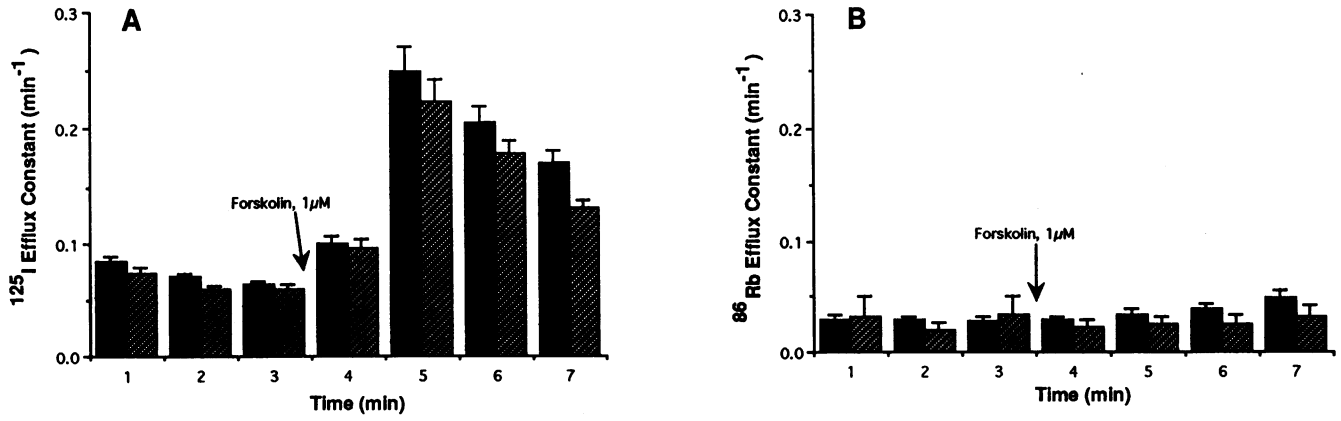

Figure 2. Unaltered activation of Cl^- and K^+ efflux by cAMP under phalloidin-loaded conditions. (*A*) Sequential 1-min rate constants measured in T84 cell monolayers preloaded with phalloidin (*hatched bars*, n = 22) demonstrate that the increase in the rate of efflux of ^{125}I elicited by forskolin (1 μM) is not significantly different from control monolayers (*solid bars*, n = 34). (*B*) Similarly, the forskolin-elicited increase in the rate constant of ^{86}Rb efflux is not different between control (*solid bars*, n = 24) and phalloidin-loaded monolayers (*hatched bars*, n = 10). ■, −Phalloidin; ▨, +Phalloidin.

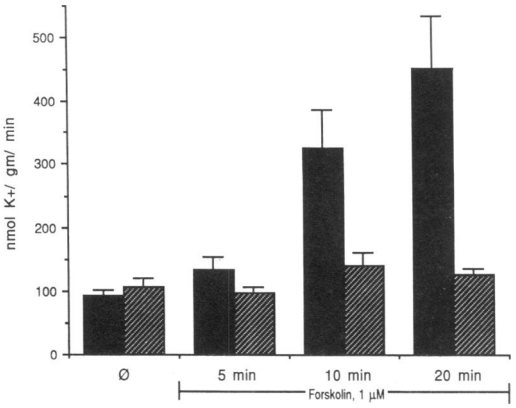

Figure 3. cAMP activates $Na^+/K^+/2Cl^-$ cotransport in T84 cells. Forskolin (1 μM) increases $Na^+/K^+/2Cl^-$ cotransporter activity (*solid bars*), measured by 1-min bumetanide-sensitive ^{86}Rb uptake using semiconfluent monolayers grown on 35-mm dishes 5 min (n = 57), 10 min (n = 14), or 20 min (n = 7) after addition of agonist compared to controls (n = 35) (by ANOVA F = 16.49, $P < 0.001$). In contrast, the bumetanide-insensitive component (*hatched bars*) shows no significant increase with forskolin stimulation. The vertical axis expresses ^{86}Rb as corrected for the specific activity of K^+, for which it acts as a tracer, calculated as in (28). ■, Sensitive; ▨, Insensitive.

in the bumetanide-insensitive component of uptake (Na^+/K^+-ATPase) was noted (Fig. 3). Such data indicate that cotransporter activity is upregulated in response to cAMP and that this upregulation occurs subsequent to the opening of the regulated apical Cl^- efflux pathway (compare with ^{125}I efflux time course, Fig. 2 *A*).

Fig. 4 shows the I_{sc} responses and the bumetanide-sensitive component of ^{86}Rb uptake ($Na^+/K^+/2Cl^-$ cotransporter activity) in forskolin-stimulated control and phalloidin-loaded monolayers. Cytoskeletal stabilization by phalloidin loading was found to reduce cAMP-induced cotransporter activity by ~ 70%. This reduction is comparable to the inhibition of the overall I_{sc} response and would appear to account for the observed inhibitory effect of phalloidin loading on cAMP-elicited Cl^- secretion. Thus, cytoskeletal stabilization by phalloidin prevents the up-regulation of $Na^+/K^+/2Cl^-$ cotransporter activity, which we show characterizes cAMP-mediated Cl^- secretion. In the absence of any agonist, bumetanide-sensitive ^{86}Rb uptake was also reduced by phalloidin loading (92±20 vs. 31±4 nmol-g^{-1}-min^{-1} for ^{86}Rb uptake for control and phalloidin-loaded monolayers, respectively, in the basal state, $P < 0.01$), but bumetanide-insensitive uptake was unaffected. Lastly, subsets of experiments performed on confluent monolayers grown on permeable supports revealed that basolateral bumetanide-sensitive ^{86}Rb uptake was stimulated by forskolin (data not shown) and was similarly inhibited by phalloidin loading (71±8% inhibition of uptake under forskolin-stimulated, phalloidin-loaded conditions compared to controls, n = 6), indicating that with either plastic- or collagen/filter-grown cells, microfilament-dependent regulation of this cotransporter by cAMP is evident.

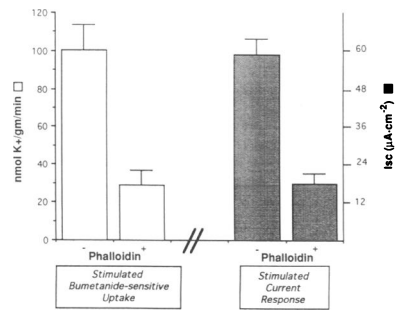

Figure 4. Phalloidin-loading inhibits cAMP-stimulation of $Na^+/K^+/2Cl^-$ cotransporter activity in T84 cells. The left hand side of the figure (*white bars*) indicates that $Na^+/K^+/2Cl^-$ cotransporter activity as measured by bumetanide-sensitive ^{86}Rb uptake under forskolin-stimulated conditions is inhibited under phalloidin-loaded conditions compared to controls (n = 12 for each, $P < 0.001$); the percent reduction in bumetanide-sensitive ^{86}Rb uptake is comparable to the inhibition of the forskolin-stimulated peak I_{sc} (*black bars*) under these conditions, shown in the right side of the figure, data taken from Fig. 1. The relative reduction of ^{86}Rb uptake was similar between cells grown on 35-mm dishes or permeable supports.

Discussion

The actin-based cytoskeleton, once thought to be responsible primarily for the maintenance of cell shape and surface polarity, has been shown to play an important and active role in the regulation of epithelial barrier function through modulation of tight junction integrity (18, 19). It now appears that the cytoskeleton may influence transcellular ion transport events as well. For example, patch clamp studies of a renal epithelial Na^+ channel have indicated that addition of the actin-severing protein gelsolin to the membrane patch can influence channel conductance (20). Additionally, we reported that cAMP-elicited Cl^- secretion in T84 cells is accompanied by significant cytoskeletal remodeling, and that both the secretory and cytoskeletal responses may be largely inhibited by NBD-phallicidin (5). In the present study, we show that electrogenic Cl^- secretion induced by cAMP is accompanied by activation of the $Na^+/K^+/2Cl^-$ cotransporter, and that while phalloidin loading has minimal or no influence on Cl^- and K^+ channels and the Na^+/K^+-ATPase, it effectively inhibits both the basal and cAMP-induced activity of the cotransporter.

A major site of regulation of cAMP-elicited Cl^- secretion is known to be the apical membrane Cl^- channels (8–10). However, the present experiments indicate that stabilization of F-actin by phalloidin does not affect the basal or cAMP-stimulated states of the apical Cl^- channels, excluding the possibility that the inhibitory effect of phalloidin on cAMP-elicited Cl^- secretion occurs at this site. This result is consistent with our previously reported morphologic data indicating that the cAMP-induced rearrangement of microfilaments was confined to the basolateral aspect of the cells, while apical and perijunctional F-actin was unaffected (5).

Stimulation of basolateral K^+ conductance occurs as part of the secretory response to both Ca^{++}-mediated and, to a lesser extent, cAMP-mediated agonists. Cytoskeletal stabilization did not influence the stimulation of ^{86}Rb efflux by forskolin or the much greater stimulation of ^{86}Rb efflux by carbachol. Basolateral K^+ channels, therefore, do not appear to be influenced by phalloidin loading in the basal, forskolin, or carbachol stimulated states. We also could not demonstrate a major inhibitory effect of phalloidin on the activity of the basolateral

Na^+/K^+-ATPase. This finding is particularly interesting because, in renal epithelia, the Na^+ pump has been shown to form a metabolically stable complex with the actin microfilament system, although the primary importance of this cytoskeletal association is thought to involve anchoring the Na^+ pump to the basolateral domain and not in functional modulation of pump activity (2).

We show that during cAMP-elicited electrogenic Cl^- secretion, $Na^+/K^+/2Cl^-$ cotransporter activity is increased in T84 cells. cAMP agonists have been shown by others to enhance bumetanide-sensitive, ouabain-resistant K^+ uptake in HT29 cells in their undifferentiated state, although little is known concerning ion transport in this intestinal stem cell line (21). Cotransporter activity has also been shown to be up-regulated by cAMP in respiratory epithelia (22), erythrocytes (23), and endothelial cells (24). The mechanism of regulation of $Na^+/K^+/2Cl^-$ cotransporter activity in the above divergent cell types is unknown. However, [^{3}H]bumetanide binding studies (22–24) have uniformly established that the number of functional cotransporters on the plasma membrane is increased in response to cAMP-mediated agonists.

Phalloidin loading was found to markedly inhibit cotransporter activity under forskolin-stimulated conditions. This basolateral Cl^- entry step may be, therefore, rate-limiting for net Cl^- secretion, explaining the marked attenuation of the cAMP-elicited I_{sc} despite apparently normal function of the other transport elements. A physiologic example in which cotransporter function may limit net Cl^- secretion has been provided by Haas et al., who reported greater up-regulation of cotransporter activity in response to cAMP in tracheal compared to bronchial epithelia and speculated that such differential regulation of cotransporter function could account for the observed difference in efficiency of Cl^- secretion between these two epithelia (22).

Our data suggest cytoskeletal involvement in cAMP-mediated up-regulation of the cotransporter. Regulated microfilament-dependent insertion of other membrane transport proteins, such as water channels (25) into plasma membranes, is well known. Cytoskeletal linkage to the cotransporter has been suggested previously (6), but direct evidence for such a relationship has been lacking. Haas and Forbush have identified a 150-kD protein with high affinity for a photoreactive bumetanide analog (26), which may represent the cotransporter itself, but whether this protein is associated with the cytoskeleton is as yet unknown. Our data raises the intriguing possibility that an intermediate phosphorylation step of a cytoskeletal protein is necessary for cotransporter up-regulation, since prevention of microfilament remodeling by phalloidin also blocked the cAMP-elicited increase in cotransporter activity.

The exact relationship by which a cAMP stimulus leads to both F-actin reorganization and enhanced cotransporter function in T84 cells is uncertain. Since up-regulation of cotransporter activity probably involves the appearance of additional functional cotransporters on the basolateral membrane, it is conceivable that cotransporter activity is directly influenced by an A-kinase dependent phosphorylation of a cytoskeletal element, leading to microfilament-dependent insertion or activation of cotransporters. It has been proposed that $Na^+/K^+/2Cl^-$ cotransporter up-regulation may be signaled by decreases in cell volume caused by the obligatory water loss accompanying efflux of cellular Cl^- and/or K^+ (22), or even by decreases in cytosolic Cl^- activity (27). While altered cell volume or intracellular ion activities could play a role in the cAMP-mediated up-regulation of T84 cell cotransporter function, our data imply that the microfilamentous cytoskeleton is crucial for the transduction of such a putative signal. We do not know whether phalloidin loading causes secondary cell shrinkage attributed to F-actin influence over basal cotransporter activity; however, since phalloidin loading does not interfere with cAMP-elicited salt efflux via Cl^- and K^+ channels, both cell shrinkage and decreased intracellular [Cl^-] must follow forskolin treatment, yet in our experiments cotransporter activity remained unchanged over baseline in the phalloidin-loaded state. Our data strongly support a key role for the cytoskeleton in the dynamic regulation of intestinal salt and water secretion.

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References

1. Bennett V. 1983. Proteins involved in membrane-cytoskeleton association in human erythrocytes: spectrin, ankyrin and band 3. *Methods Enzymol.* 96:313–324.

2. 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.

3. Molitoris, B. A., and W. J. Nelson. 1990. Alterations in the establishment and maintenance of epithelial cell polarity as a basis for disease processes. *J. Clin. Invest.* 85:3–9.

4. Dharmsathaphorn, 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:G204–208.

5. Shapiro, M., J. Matthews, G. Hecht, C. Delp, and J. L. Madara. 1991. Stabilization of F-actin prevents cAMP-elicited Cl^- secretion in T84 cells. *J. Clin. Invest.* 87:1903–1909.

6. 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.

7. Dawson, D. C., and N. W. Richards. 1990. Basolateral K conductance: role in regulation of NaCl absorption and secretion. *Am. J. Physiol.* 259:C181–C195.

8. Mandel, K. G., K. Dharmsathaphorn, and J. A. McRoberts. 1986. Characterization of a cyclic AMP-activated Cl^- transport pathway in the apical membrane of a human colonic epithelial cell line. *J. Biol. Chem.* 261:704–712.

9. Halm, D. R., G. R. Rechkemmer, R. A. Schoumacher, and R. A. Frizzell. 1988. Apical membrane chloride channels in a colonic cell line activated by secretory agonists. *Am. J. Physiol.* 254:C505–C511.

10. McRoberts, J. A., G. Beuerlein, and K. Dharmsathaphorn. 1985. Cyclic AMP and Ca^{2+}-activated K^+ transport in a human colonic epithelial cell line. *J. Biol. Chem.* 260:14163–14172.

11. Dharmsathaphorn, K., and J. L. Madara. 1990. Established intestinal cell lines as model systems for electrolyte transport studies. *Methods Enzymol.* 192:354–389.

12. Nash, S., C. Parkos, A. Nusrat, C. Delp, and J. L. Madara. 1991. In vitro model of intestinal crypt abscess. A novel neutrophil-derived secretagogue activity. *J. Clin. Invest.* 87:1474–1477.

13. Vlengarik, C. J., R. J. Bridges, and R. A. Frizzell. 1990. A simple assay for agonist-regulated Cl and K conductances in salt-secreting epithelial cells. *Am. J. Physiol.* 259:C358–C364.

14. McRoberts, J. A., S. Erlinger, M. J. Rindler, and M. H. Saier, Jr. 1982. Furosemide-sensitive salt transport in the Madin-Darby canine kidney cell line: evidence for the cotransport of Na^+, K^+, and Cl^-. *J. Biol. Chem.* 257:2260–2266.

15. O'Neill, W. C., and J. D. Klein. 1992. Regulation of vascular endothelial cell volume by Na-K-2Cl cotransport. *Am. J. Physiol.* 246:C436–444.

16. Paris, S., and J. Pouyssegur. 1986. Growth factors activate the bumetanide-sensitive Na+/K+/Cl− cotransport in hamster fibroblasts. *J. Biol. Chem.* 261:6177–6183.

17. Kirk, K. L., D. R. Halm, and D. C. Dawson. 1980. Active sodium transport by turtle colon via an electrogenic Na-K exchange pump. *Nature (Lond.).* 287:237–239.

18. 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.

19. Duffey, M. E., B. Hainau, S. Ho, and C. J. Bentzel. 1981. Regulation of epithelial tight junctional permeability by cyclic AMP. *Nature (Lond.).* 294:451–453.

20. Cantiello, H. F., J. L. Stow, A. G. Prat, and D. A. Ausiello. 1991. Actin filaments regulate Na^+ channel activity. *Am. J. Physiol.* 261:C882–C888.

21. Kim, H. D., Y. S. Tsai, C. C. Franklin, and J. T. Turner. 1988. Characterization of $Na^+/K^+/Cl^-$ cotransport in cultures HT29 human colonic adenocarcinoma cells. *Biochim. Biophys. Acta.* 946:397–404.

22. Haas, M., L. G. Johnson, and R. C. Boucher. 1990. Regulation of Na-K-Cl cotransport in cultured canine airway epithelia: a ^3H-bumetanide binding study. *Am. J. Physiol.* 259:C557–C569.

23. Haas, M., and B. Forbush III. 1986. [^3H]bumetanide binding to duck red cells: correlation with inhibition of (Na+K+2Cl) cotransport. *J. Biol. Chem.* 261:8434–8441.

24. O'Donnell, M. E. 1989. [^3H]bumetanide binding in vascular endothelial cells. Quantitation of Na-K-Cl cotransporters. *J. Biol. Chem.* 264:20326–20330.

25. Hardy, M. A., and D. R. DiBona. 1982. Microfilaments and the hydroosmotic action of vasopressin in toad urinary bladder. *Am. J. Physiol.* 243:C200–204.

26. Haas, M., and B. Forbush III. 1987. Photolabelling of a 150 kDa (Na+K+Cl) cotransport protein from dog kidney with a bumetanide analog. *Am. J. Physiol.* 253:C243–252.

27. Breitwasser, G. E., A. A. Altimirano, and J. M. Russell. 1990. Osmotic stimulation of Na^+-K^+-Cl^- cotransport in squid giant axon is $[Cl^-]_i$ dependent. *Am. J. Physiol.* 258:C749–753.

28. Owen, N. E., and M. L. Prastein. 1985. Na/K/Cl cotransport in cultured human fibroblasts. *J. Biol. Chem.* 260:1445–1451.