Na-K-2Cl Cotransport in Intestinal Epithelial Cells

INFLUENCE OF CHLORIDE EFFLUX AND F-ACTIN ON REGULATION OF COTRANSPORTER ACTIVITY AND BUMETANIDE BINDING*

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Although cAMP-dependent epithelial chloride secretion is largely regulated via apical membrane chloride channels, cAMP also remodels basolateral F-actin and activates basolateral Na-K-2Cl cotransport. Whether activation of cotransport is a primary event or secondary to activation of chloride efflux is not established, and the basis for the cytoskeletal dependence is unknown. We studied cotransport in the intestinal line HT29 (which lacks cAMP-regulated chloride efflux) and in its subclone CL19A (in which this pathway is present). Cotransporter activity was enhanced by forskolin in both lines but to a considerably greater extent in subclone CL19A, in which the number of bumetanide binding sites was also observed to increase. The F-actin stabilizer phalloidin markedly attenuated CAMP-stimulated cotransport in CL19A monolayers, but the increase in bumetanide binding was preserved. These studies identify two mechanisms for activation of Na-K-2Cl cotransport by cAMP: components independent and dependent of cAMP-elicted chloride efflux. Additional Na-K-2Cl cotransporters become accessible to the cell surface coincident with the salt efflux-dependent activation of cotransport. While F-actin rearrangements influence salt efflux-dependent up-regulation of the cotransporter, this influence occurs independently of increases in bumetanide-accessible cotransporters.

Electrogenic chloride secretion is the fundamental means by which mucosal surfaces are hydrated; defective regulation of this process underlies a number of diseases of considerable importance, including cystic fibrosis and secretory diarrhea (1). Current models of cAMP-regulated chloride secretion emphasize apical membrane chloride channels as the primary site of regulation; the cystic fibrosis transmembrane conductance regulator (CFTR) is thought to represent this cAMP-regulated chloride channel (2). In order to maintain cellular composition during high rates of secretion, however, the rate of basolateral chloride entry must increase to balance the rate of apical chloride exit; the mechanism by which such coordination of apical and basolateral transport events is accomplished is incompletely understood (3, 4).

Internalization of chloride across the basolateral membrane of chloride-secreting epithelia occurs primarily via bumetanide-inhibitable Na-K-2Cl cotransport (5). Although in order to meet the demands of sustained secretion, Na-K-2Cl cotransport could increase simply on the basis of altered chemical gradients, evidence from a number of epithelial and non-epithelial cell types argues for complex and possibly multiple regulatory mechanisms. In shark rectal gland and avian erythrocytes, cotransporter activity appears to correlate with the phosphorylation state of the cotransporter protein (3, 4), suggesting that cAMP may directly activate the cotransporter via a cAMP-dependent protein kinase cascade. Furthermore, in several cell types, cotransport can be activated by hypertonic cell shrinkage (6), implying that, in the case of a secretory epithelium, Na-K-2Cl cotransport could increase as a secondary response to cAMP-elicted salt and water loss engendered by the activation of apical membrane chloride channels (3, 4, 7, 8). Finally, an additional level of regulation has been identified in several cell types whereby cAMP elicits an increase in the number of specific bumetanide binding sites, an event that may reflect an increase in the number of functional cotransporters in the plasma membrane (3, 4, 7–13).

We have shown that, in the chloride-secreting human intestinal epithelial cell line T84, cAMP not only activates apical membrane chloride efflux, but also concurrently elicits basolateral microfilament remodeling and enhances basolateral salt uptake via Na-K-2Cl cotransport. Furthermore, these additional basolateral events (but not cAMP-stimulated apical chloride exit) could be largely attenuated by the F-actin stabilizing agent phalloidin, with resultant inhibition of net chloride secretion (14, 15). Whether the observed cAMP-elicted activation of cotransport is a primary event or a secondary response to the activation of cAMP-regulated chloride efflux has not been elucidated, and the basis for the microfilament dependence of regulation is unknown.

One possible way to address the importance of cAMP-elicted chloride efflux to the regulation of cotransport by cAMP would be to eliminate the cAMP-regulated chloride efflux pathway. Unfortunately, of the available pharmacologic blockers of chloride channels, none is specific in its action or completely inhibitory (16). Alternatively, one could compare cotransporter regulation in cell lines with variable expression of the preamed cAMP-regulated chloride efflux pathway (CFTR) (17). In this regard, the pluripotent human intestinal epithelial cell line HT29 presents a unique model system in which to study the regulation of cotransport. Parent HT29 cells are unpolarized.
Monolayers were incubated in HPBR with or without 100 nM forskolin. Uptakes were expressed as nmol of $^{86}$Rb taken measured in the absence of bumetanide from the mean value of standard HPBR. As reported by others (24), standard HPBR was developed by Dr. Mark Haas (University of Chicago) to yield a 10 mM sodium gluconate, 25 mM potassium gluconate, 5 mM KC1, and 10 mM Tris-HCl, pH 7.4; binding experiments in the present study utilized this modified HPBR. Binding was terminated by rapid washing the dishes four times with an ice-cold solution containing 100 mM MgCl₂ and 10 mM Tris, pH 7.4. Radioactivity was extracted and measured in the presence of excess unlabeled bumetanide. In some experiments, bumetanide-sensitive uptake was expressed as a percent increase over control values.

Parent HT29 cells and subclone C1.19A cells grown on 35 mm Petri dishes were loaded with $^{125}$I, and serial one-min efflux measurements were performed as described under "Materials and Methods." After three 1-min baseline flux periods, 10 μM forskolin was added to the replacement buffer and four further 1-min flux values were obtained. Data are mean ± S.E. of n = 16 monolayers in each group.

**FIG. 1.** Effect of forskolin on anion efflux in parent HT29 cells and subclone C1.19A. Parent HT29 cells and subclone C1.19A cells grown on 35 mm Petri dishes were loaded with $^{125}$I, and serial one-min efflux measurements were performed as described under "Materials and Methods." After three 1-min baseline flux periods, 10 μM forskolin was added to the replacement buffer and four further 1-min flux values were obtained. Data are mean ± S.E. of n = 16 monolayers in each group.

**RESULTS**

Activation of Anion Efflux by cAMP—As shown in Fig. 1, forskolin (10 μM, a cAMP agonist) evoked a rapid increase in the rate constant of $^{125}$I efflux in 4-5-day-old monolayers of C1.19A cells but not in parent HT29 cells, thus confirming the presence of a cAMP-regulated chloride efflux pathway in the subclone expressing apical membrane components (C1.19A) and its absence in the parent HT29 line, consistent with the findings of...
others (17). The cAMP-regulated chloride efflux pathway remained present in post-confluent Cl.19A monolayers; in some HT29 monolayers older than 4-5 days, a small cAMP-elicited increase in 32P efflux that was delayed in onset could occasionally be detected (data not shown), as also noted by others (17).

Na-K-2Cl Cotransporter: Basal and cAMP-regulated—Under unstimulated conditions, bumetanide-sensitive 86Rb uptake was similar in 4-5-day-old monolayers of subclone Cl.19A and parent HT29 cells (13.1 ± 1.6 versus 10.6 ± 1.5 pmol of K \times mg of protein\(^{-1}\) \times min\(^{-1}\) for Cl.19A versus parent HT29, respectively, each \(n = 32\), n.s.). Uptake was linear over the first 10 min of exposure to tracer in both cell lines (data not shown). In response to 10 \(\mu\)M forskolin, bumetanide-sensitive 86Rb uptake (both in the presence or absence of ouabain) increased in time-dependent fashion in both cell lines (Fig. 2). In the absence of ouabain, peak stimulation was seen between 5 and 15 min, with decreased but still considerable stimulation evident after a 30-min exposure to forskolin. Dose-response curves in both cell lines measured after 10 min of forskolin treatment indicated maximal stimulation of bumetanide-sensitive 86Rb uptake in both lines at 10 \(\mu\)M forskolin (Fig. 3). Other investigators have demonstrated that 10 \(\mu\)M forskolin increases intracellular cAMP to equivalent levels in both parental and Cl.19A cells (17). For all subsequent experiments discussed below, 10 \(\mu\)M forskolin was used.

In parallel experiments performed on the same day with monolayers of equivalent age (4-5 days), bumetanide-sensitive 86Rb uptake was stimulated approximately 2-fold by 10 \(\mu\)M forskolin in parent HT29 cells but increased more than 3-fold in subclone Cl.19A (Fig. 4). Thus, despite the absence of a cAMP-activated chloride efflux pathway in parent HT29 cells, Na-K-2Cl cotransport was stimulated by forskolin in this cell line. Interestingly, the degree of stimulation appeared to be considerably greater in the polarized subclone Cl.19A (\(p < 0.001\) compared to parent HT29, \(n = 32\) for each group), which possesses the cAMP-regulated chloride efflux pathway. This difference between parent HT29 cells and the clonal line was also clearly apparent in the time-course experiments depicted in Fig. 2.

\[ [\text{H}] \text{Bumetanide Binding and Response to cAMP—Specific and nonspecific bumetanide binding characteristics for the two cell lines were similar; a representative experiment for Cl.19A cells is shown in Fig. 5. Binding of [\text{H}]bumetanide reached equilibrium within approximately 30-40 min (data not shown), consistent with the experience of others (4, 24). Under unstimulated conditions, the number of specific [\text{H}]bumetanide binding sites and the affinity for bumetanide were similar in parent HT29 and subclone Cl.19A cells (Fig. 6). By Scatchard analysis, \(B_{\text{max}}\) was 1.28 ± 0.17 versus 1.26 ± 0.17 pmol \times mg of protein\(^{-1}\) and \(K_d = 163 ± 21\) versus 182 ± 44 \(\mu\)M for HT29 versus Cl.19A, respectively, for four separate experiments. Thus, consistent with the functional data presented above, the number of functional membrane cotransporters as indicated by the number of available binding sites for bumetanide is similar in unstimulated parent HT29 cells and the Cl.19A subclone.

We next sought to determine whether the number of specific bumetanide binding sites increases in response to a cAMP stimulus in these cell lines (Fig. 7). In response to 10 \(\mu\)M forskolin, specific [\text{H}]bumetanide binding was observed to increase markedly (approximately 2-fold) in subclone Cl.19A. Interestingly, under the same binding conditions, and in parallel with experiments demonstrating functional activation of the cotransporter (by bumetanide-sensitive 86Rb uptake), no such increase in specific binding of [\text{H}]bumetanide was observed in HT29 cells. By Scatchard analysis, the increase in bumetanide binding in Cl.19A cells largely represented an increase in \(B_{\text{max}}\) (1.26 ± 0.17 versus 3.04 ± 0.13 pmol \times mg of protein\(^{-1}\) for unstimulated versus forskolin-stimulated, respectively; \(n = 4\); \(p < 0.0001\) with no significant change in \(K_d\) (182 ± 44 versus 191 ± 28 \(\mu\)M, respectively, for unstimulated versus forskolin-stimulated, respectively; \(n = 4\); not significant). There was no significant change in either \(B_{\text{max}}\) or \(K_d\) for parent HT29 cells after forskolin stimulation. Thus, in response to a cAMP stimulus, the number of accessible specific bumetanide binding sites was observed to increase only in subclone Cl.19A, which possesses the cAMP-regulated chloride efflux pathway.

Phalloidin Inhibits Activation of Cotransport by cAMP but...
this solution and replacing it with an identical buffer containing 1.5 pCi

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in HPBR with or without 10 μM forskolin for 10 min. 86Rb uptake was initiated by aspirating this solution and replacing it with an identical buffer containing 1.5 μCi x ml−1 asRb. After 3 min, uptakes were terminated and radioactivity extracted as described in Fig. 2 legend. Data are expressed as percent control, measured in HT29 and C1.19A cells bathed in buffer alone. Data are mean ± S.E. from a single experiment performed in triplicate and repeated on one additional occasion with similar results. Curves are drawn by hand.

FIG. 3. Effect of forskolin on Na-K-2Cl cotransporter activity in parent HT29 cells and subclone C1.19A: dose-response curves. Parent HT29 cells and subclone C1.19A cells were grown in 24-well dishes and 86Rb uptake studies were performed as described under "Materials and Methods." Monolayers were bathed in HPBR with or without 10 μM bumetanide for 15 min and then exposed to 0.033–10 μM forskolin for 10 min. 86Rb uptake was initiated by aspirating this solution and replacing it with an identical buffer containing 1.5 μCi x ml−1 asRb. After 3 min, uptake were terminated and radioactivity extracted as described in Fig. 2 legend. Data are expressed as percent control, measured in HT29 and C1.19A cells bathed in buffer alone. Data are mean ± S.E. from a single experiment performed in triplicate and repeated on one additional occasion with similar results. Curves are drawn by hand.

FIG. 4. Comparison of Na-K-2Cl cotransporter activity between parent HT29 cells and subclone C1.19A: basal and forskolin-stimulated. Parent HT29 cells and subclone C1.19A cells were grown on 35-mm Petri dishes and 86Rb uptake studies were performed as described under "Materials and Methods." Monolayers were bathed in HPBR with or without 10 μM forskolin in the presence or absence of 10 μM bumetanide for 10 min. 86Rb uptake was initiated by aspirating this solution and replacing it with an identical buffer containing 1.5 μCi x ml−1 asRb. After 3 min, uptakes were terminated and radioactivity extracted. Protein content of representative monolayers treated identically were determined, and data were then converted to nmol of K+ x mg of protein x min−1, as described under "Materials and Methods." Bars represent mean ± S.E. of n = 32 monolayers in each group.

Does Not Affect [3H]Bumetanide Binding—We reported previously that in the intestinal epithelial cell line T84, cAMP-mediated increases in Na-K-2Cl cotransport (assessed by bumetanide-sensitive 86Rb uptake) were largely attenuated in monolayers loaded with the actin stabilizer phalloidin (14). We utilized similar conditions to examine cotransporter function and [3H]bumetanide binding in the HT29 and C1.19A cell lines.

HT29 and C1.19A monolayers were loaded with phalloidin by overnight co-incubation in fresh media containing 33–100 μM phalloidin. 86Rb uptake was then measured under unstimulated and forskolin-stimulated conditions as above. The bumetanide-insensitive component of 86Rb uptake was examined as an estimate of Na+/K+-ATPase activity and an indicator of cell viability. Under both unstimulated and forskolin-stimulated conditions, and in both cell lines, the bumetanide-insensitive component of uptake (the ouabain-insensitive fraction) was not affected by phalloidin loading (data not shown), thus demonstrating the absence of nonspecific toxic effects of phalloidin on membrane transport. Further evidence of the absence of toxicity was obtained by measuring lactate dehydrogenase release (7.0 ± 2.5% release versus 6.2 ± 2.4% release for 100 μM phal-
The Na-K-2Cl cotransporter is an integral component of the transport apparatus of numerous secretory and absorptive epithelia (5, 27). In addition, activation of Na-K-2Cl cotransport is an important means by which many epithelial and non-epithelial cells restore normal cell volume after cell shrinkage (6). The interrelationship between the roles of the cotransporter in epithelial chloride secretion and cell volume regulation is not clearly understood. It has been speculated that vectorial transport by polarized epithelial cells could reflect a specialized adaptation of volume-regulatory transport processes present in non-polarized cells (1). Simplicistically viewed, a chloride-transporting epithelium accomplishes net secretion by restricting a salt-dumping pathway (chloride channels) to the apical membrane and a salt-loading pathway (the cotransporter) to the basolateral membrane. However, it remains to be established whether regulation of basolateral chloride uptake through the Na-K-2Cl cotransporter and chloride exit through apical chlo-
ride channels are linked via changes in cell volume or changes in intracellular ionic activities or, indeed, whether these transport events are independently regulated.

We have shown that cAMP activates Na-K-2Cl cotransport in the chloride-secreting T84 cell line and that both cAMP-mediated activation of Na-K-2Cl cotransport and net chloride secretion could be largely attenuated by phalloidin (14). This suggested that the basolateral Na-K-2Cl cotransporter could be an important regulatory site for net chloride secretion and that cotransporter activity was functionally modified by dynamic changes in F-actin. Because cell volume perturbations may be associated with actin remodeling (28), one possible explanation for these findings is that the cytoskeleton provides a means for transducing volume- regulatory signals to membrane-bound transport proteins such as the cotransporter.

We attempted to further elucidate the role of the apical chloride efflux pathway and the influence of F-actin in the cAMP-mediated regulation of Na-K-2Cl cotransport by examining the parent and a clonal derivative of a human intestinal epithelial cell line, in which the clone possesses a regulated chloride efflux pathway while the parent does not. We observed that, under unstimulated conditions, parent HT29 cells and subclone C1.19A display similar functionally defined Na-K-2Cl cotransporter activity and similar bumetanide binding characteristics, and others have shown the parent and subclone generate comparable cAMP signals (17). In response to maximal agonist-elicited activation of adenylate cyclase, rapid stimulation of ion translocation by the Na-K-2Cl cotransporter (bumetanide-sensitive \( { }^{3} \text{H} \) bumetanide uptake) was evident in both parent HT29 cells and subclone C1.19A. Thus, despite the absence of a cAMP-regulated chloride efflux pathway in parent HT29 cells, Na-K-2Cl cotransport doubled, implying that the presence of a regulated anion efflux pathway is not required for cotransporter activation. This finding is consistent with earlier work by Turner and co-workers (18, 20), who observed that 30-90 min exposure of HT29 cells to forskolin activated bumetanide-sensitive potassium influx in HT29 cells. We also establish that activation of cotransport by forskolin is rapid, apparent within 2 min, evidence consistent with a direct role of cAMP in cotransporter regulation. However, activation of cotransport by cAMP in subclone C1.19A was considerably greater than in the parent HT29 cell line, suggesting that the presence of the regulated chloride efflux pathway may augment the response of the cotransporter to a cAMP stimulus.

This difference between parent HT29 cells and subclone C1.19A is consistent with the two general mechanisms for cotransporter regulation by cAMP proposed by Haas et al. (3): a direct mechanism in which cotransport is activated by cAMP-dependent phosphorylation and an indirect mechanism in which cotransport is activated as a secondary response to salt loss through cAMP-regulated chloride channels. The exact mechanism by which cAMP is capable of directly activating cotransport is unknown, and it is interesting in this regard to recognize that in some systems, cAMP has been found, in fact, to inhibit cotransport (5). Recently reported biochemical characterization of the cotransporter protein of shark rectal gland and avian erythrocytes indicate that activation of cotransport indeed correlates with the state of phosphorylation of the putative cotransporter protein, although whether the cotransporter itself is the substrate for the action of cAMP-dependent protein kinase remains to be established (10, 13).

Whether the apparent augmentation of cAMP-stimulated Na-K-2Cl cotransport in cells possessing a regulated chloride efflux pathway is due to cell shrinkage or to changes in intracellular ion activities is not clear. Little is known about the cell volume regulatory mechanisms of either HT29 or C1.19A cells. However, it is interesting to note that bumetanide-sensitive potassium uptake in HT29 cells does not increase in response to hypertonicity (20), suggesting that the smaller cAMP elicited increase in cotransporter activity observed in parent HT29 cells cannot be attributed solely to the absence of cell shrinkage that would result from cAMP-dependent salt efflux. There is some evidence that cotransporter activity may be modulated by cytoplasmic chloride activity (8), possibly through allosteric inhibition at an internal modifier site; it therefore seems possible that some of the apparent volume- regulatory responsiveness of the cotransporter may, in fact, be due to changes in intracellular chloride activity rather than cell shrinkage per se (29).

One possible explanation for the greater cAMP-elicited increase of cotransport in subclone C1.19A is suggested by the [\( { }^{3} \text{H} \)bumetanide-binding data. In response to cAMP, the numbers of specific binding sites increases markedly in the differentiated subclone, whereas parent HT29 cells showed only a minimal change in bumetanide binding sites. Thus, one could speculate that the number of functional cotransporters in the plasma membrane of C1.19A cells is up-regulated in response to cAMP, with the increase in numbers of cotransporters being associated with the chloride efflux-dependent (indirect) component of cAMP-mediated volume regulation. The lesser degree of activation of cotransport in parent HT29 cells may thus be due to the absence of this recruitment phenomenon. There may, however, be other explanations. For example, although it is likely that bumetanide binds to the cotransporter itself (probably at a low affinity chloride site; Refs. 5 and 27), it does not necessarily follow that specific bumetanide binding is, in fact, a reflection of the total number of membrane cotransporters; one could speculate that the appearance of new bumetanide binding sites reflects not the recruitment of new cotransporters into the membrane but instead an activation of dormant cotransporters already present, the "active" conformation of the cotransporter being capable of binding bumetanide. If true, however, it does not explain why phalloidin loading would affect cotransporter activity but not the increase in bumetanide binding. One difficulty inherent in bumetanide binding studies is that equilibrium binding requires a 30-40 min incubation with this ligand, making it difficult to correlate precisely the rapid increases in cotransporter activity evident by ion tracer methodology with enhanced specific bumetanide binding. However, the absence of cAMP-induced increases in bumetanide binding in parent HT29 cells despite a 2-fold increase in specific bumetanide uptake suggests that cotransporter activity and number are not necessarily tightly correlated. Slotki (30) recently reported that long term stimulation of HT29 cells by forskolin did not result in an increase in specific bumetanide binding, although cotransporter activity remained increased; the short term effects of forskolin on binding were not addressed in that study. It has been speculated that the actin-based cytoskeleton may serve to transduce regulatory signals for the Na-K-2Cl cotransporter (5); however, until recently, direct evidence for this proposal has been lacking. In addition to our recent demonstration of the microfilament dependence of Na-K-2Cl cotransporter activity in the T84 and now in the C1.19A cell lines, Jessen and Hoffman (31) reported that disruption of cellular actin with cytochalasin B activates the cotransporter in Ehrlich ascites cells. More recently, activation of Na-K-2Cl cotransport in endothelial cells has been shown to correlate with phosphorylation of myosin light chain (32). Because of the paradigm that correlates activation of cotransport with numbers of bumetanide binding sites, initially we favored a mechanism in which additional cotransporter units were recruited into the plasma membrane by a microfilament-dependent event, analogous to the insertion of the vasopressin-regulated water channel in toad bladder and mammalian collecting duct (a process that has been shown to be augmented by cytochalasins and inhibi-
ited by phallotoxins) (33-36). However, the present study indicates that phalloidin does not affect the cAMP-mediated increase in bumetanide binding in Cl.19A cells despite inhibiting bumetanide-sensitive Rb uptake. Therefore, phalloidin appears to inhibit ion translocation by the cotransporter but not the increase in bumetanide binding in C1.19A cells despite inhibiting CAMP-mediated regulation.

Whether actin filaments directly associate with the cotransporter and influence its function cannot be determined from the present study. The cytoplasmic aspect of the plasma membrane of all eukaryotic cells is lined by a cortical meshwork consisting not only of actin filaments but also a number of actin-associated proteins. Dynamic changes in submembranous actin polymerization may thus alter the kinetics and equilibria of a number of biochemical reactions involving actin-associated proteins and various membrane proteins linked to the cytoskeleton. Several examples of ion transport proteins that are linked to the actin-based cytoskeleton have now been identified. For example, the Na-K ATPase of renal epithelia has been shown to bind ankyrin and fodrin, thereby forming a metabolically stable complex with actin (37). This association appears to be of importance not only in the maintenance of the polarized distribution of the Na-K ATPase but also in modulation of pump function (38, 39). A direct regulatory effect of short actin filaments on the open probability of a sodium channel in the A6 renal epithelial cell line has recently been demonstrated (40, 41), and melanoma cells devoid of actin-binding protein have been observed to be unable to volume-regulate or activate potassium channels in response to a hypoosmotic stimulus (42). While the present study provides additional evidence that the cortical cytoskeleton can exert a regulatory influence over the function of the Na-K-2Cl cotransporter, further investigation is needed to define the structural basis for this interaction.

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