[Cl⁻]-dependent Phosphorylation of the Na-K-Cl Cotransport Protein of Dog Tracheal Epithelial Cells*

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Basolateral Na-K-Cl cotransport activity in primary cultures of dog tracheal epithelial cells is stimulated by β-adrenergic agents, such as isoproterenol, and by apical UTP, which acts through an apical P₂-purinergic receptor. While at least part of the stimulatory effect of isoproterenol appears to involve direct activation of the cotransporter via cAMP-dependent protein kinase, cotransport stimulation by apical UTP is entirely secondary to apical Cl⁻ efflux and a resultant decrease in intracellular [Cl⁻] (1,2) and/or cell shrinkage (Haas, M., and McBrayer, D. G. (1994) Am. J. Physiol. 266, C1440–C1452). In the secretory epithelia of the shark rectal gland and avian salt gland, Na-K-Cl cotransport activation by both cAMP-dependent and cAMP-independent secretagogues has been shown to be accompanied by phosphorylation of the cotransport protein itself (Lytle, C., and Forbush, B., III (1992) J. Biol. Chem. 267, 25438–25443); Torchia, J., Lytle, C., Pon, D. J., Forbush, B., III, and Sen, A. K. (1992) J. Biol. Chem. 267, 25444–25450). In the present study, we immunoprecipitate the 170-kDa Na-K-Cl cotransport protein of dog tracheal epithelial cells with a monoclonal antibody against the cotransporter of the intestinal cell line T84. Incubation of confluent primary cultures of tracheal epithelial cells with isoproterenol and apical UTP increases basolateral-to-apical 36Cl⁻ flux 3.4- and 2.6-fold, respectively, and produces similar increases (3.2- and 2.8-fold, respectively) in 32P incorporation into the 170-kDa cotransport protein. Decreasing [Cl⁻], (without concomitant cell shrinkage) by incubating cultures with apical nystatin and reduced apical [Cl⁻] [Cl⁻]⁻ Likewise increases both cotransport activity and cotransport protein phosphorylation. These effects become more pronounced with greater reductions in [Cl⁻]⁻; after 20 min of incubation with nystatin and 32 mM [Cl⁻]⁻, cotransport activity and 32P incorporation into the cotransport protein are increased 2.8- and 2.7-fold, respectively, similar to increases seen with apical UTP. 2-3-fold increases in cotransporter activity and phosphorylation are also seen in nystatin-treated cells under hypertonic conditions (50 mM sucrose added apically and basolaterally). These findings suggest a close correlation between Na-K-Cl cotransport activity and phosphorylation of the 170-kDa cotransport protein. The latter is phosphorylated in response to both reduced [Cl⁻], and cell shrinkage, either or both of which are likely to be involved in secondary cotransport activation in response to apical UTP.

In secretory epithelia, both cAMP-dependent and cAMP-independent secretagogues augment ion transport through multiple transport pathways to produce net salt and fluid secretion; these pathways include apical Cl⁻ channels and basolateral K⁺ channels as well as the basolateral Na-K-Cl cotransporter and other basolateral salt influx pathways (1–6). Na-K-CI cotransport is the primary basolateral influx pathway for Na⁺ and Cl⁻ in dog tracheal epithelium (1–3, 7), and cotransport activity is increased both by isoproterenol and cAMP analogues, as well as by apical triphosphate nucleotides such as UTP, which binds to an apical P₂-purinergic receptor and subsequently stimulates net salt and fluid secretion in a cAMP-independent manner (8–10). Previous studies employing primary cultures of dog tracheal epithelial cells have provided evidence that cAMP-dependent cotransport activation involves, at least in part, direct cotransport activation via cAMP-dependent protein kinase. By contrast, cotransport activation in response to apical UTP is entirely secondary to apical Cl⁻ efflux via activated channels (8, 9). The cellular signal for this secondary cotransport activation in dog tracheal cells is likely to be a decrease in intracellular [Cl⁻]⁻ ([Cl⁻]⁻) and/or in cell volume; both reduced [Cl⁻] and cell shrinkage can produce a level of cotransport activation similar to that seen with apical UTP (9). Furthermore, both reduced [Cl⁻] and cell shrinkage are known to activate Na-K-CI cotransport in a variety of tissues (11–13), including several secretory epithelia (14–17). In rat parotid acini, it was recently shown that reduced [Cl⁻] and cell shrinkage, is required for basolateral cotransport activation in response to carbachol (4), though this has not yet been resolved for other secretory epithelia including those of mammalian airways.

Studies of Lytle and Forbush (14, 15) in shark rectal gland epithelium and of Torchia and co-workers (18, 19) in avian salt gland epithelium have demonstrated phosphorylation of the Na-K-CI cotransport protein itself in response to cAMP-dependent and cAMP-independent secretagogues. Increased phosphorylation of the cotransport protein was also observed following incubation of intact shark rectal gland tubules in hypertonic and in Cl⁻-free media to reduce cell volume and [Cl⁻], respectively (14, 15). Furthermore, in the shark rectal gland it was shown that increasing degrees of hypertonic cell shrinkage produce increasing and approximately proportional stimulation of cotransport protein phosphorylation and [3H]benzmetanide binding (14), the latter having previously been shown to correlate closely with Na-K-CI cotransport activity (16, 20). Thus, in these secretory epithelia it appears that Na-K-CI cotransport activation involves phosphorylation of the cotransport protein itself. These results are consistent with

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findings in a variety of epithelial and nonepithelial tissues demonstrating that stimulation of Na-K-Cl cotransport by hormones, secondary messengers, and cell shrinkage can be blocked by protein kinase inhibitors and that protein phosphatase inhibitors augment cotransport activity both in the presence and absence of cotransport stimuli (14, 21–25).

In this study, we examine the phosphorylation of the Na-K-Cl cotransporter protein of dog tracheal epithelial cells in response to isoproterenol, apical UTP, cell shrinkage, and reduced [Cl\(^{-}\)]. Confluent primary cell cultures incubated under each of these conditions known to stimulate Na-K-Cl cotransport exhibit increased phosphorylation of the \(-170\)kDa Na-K-Cl cotransport protein, which is subsequently isolated by immunoprecipitation using a monoclonal antibody directed against the Na-K-Cl cotransport protein of T84 cells (26, 27). Of particular note, the degrees of cotransporter activation and phosphorylation in response to graded decreases in [Cl\(^{-}\]), (without concomitant cell shrinkage) are found to be closely correlated, suggesting the possibility of a Cl\(^{-}\}-sensitive protein kinase that phosphorylates, and thus activates, the Na-K-Cl cotransporter. A preliminary report has been presented in abstract form (28).

**MATERIALS AND METHODS**

Cell Culture—Dog tracheal epithelial cells were grown in primary culture on Transwell-COL supports (24.5-mm diameter, 0.4 \(\mu\)m pore size; Costar, Cambridge, MA) as described previously (29). The culture medium (F12–4x) was Ham's F-12 medium (Life Technologies, Inc.) containing 10.0 \(\mu\)g/ml insulin, 7.5 \(\mu\)g/ml endothelial cell growth supplement, 0.2 \(\mathrm{ng}\)/ml 3,3',5'-triodothyronine, and 0.36 \(\mu\)g/ml hydrocortisone (all from Collaborative Biomedical, Bedford, MA). The cells were cultured in an atmosphere of 5% CO\(_2\), 95% ambient air at 37°C. Confluence was detected by phase-contrast microscopy; this generally occurred after initial seeding for 124 mM [Cl\(^{-}\)]. Establishment of confluence, the apical and basolateral media were replaced with F12–4x containing 1 mM added CaCl\(_2\) (1.3 mM total [Ca\(^{2+}\)]). Airway cells grown in F12–4x on collagen-coated supports form tight monolayers of cuboidal to columnar cells with some basal cells present, resembling the native epithelium (29, 30).

As in previous studies (8, 9, 29), we measured the potential difference and resistance across each Transwell culture daily following optical detection of confluence, using an epithelial voltmeter with “chopstick” electrodes (World Precision Instruments, Sarasota, FL). Maximal potential difference and resistance values were attained 2 days after optical detection of confluence. All experiments were thus performed on day 2 following detection of confluence (day 5–6 in culture).

Transepithelial Na-K-Cl Flux Determinations—These determinations were performed as described previously (9). To summarize, the flux period was started by placing a Transwell culture in a well of a 6-well tissue culture dish containing 2 ml of F12–4x + 1 mM CaCl\(_2\) (37°C) to which 1 Ci of \(^{36}\)Cl\(^{-}\) had been added. 0.7 ml of the appropriate apical medium (F12–4x + 1 mM CaCl\(_2\) or one of the synthetic media described below) was added, and the dish containing the Transwell was then covered and placed on a gently rotating orbital shaker contained within a 37°C tissue culture incubator. At 2.5-min intervals, the apical medium was completely removed for counting and replaced with 0.7 ml of identical medium. For some incubations, immediately after removal of the 10-min sample, isoproterenol (Sigma; final concentration, 5 \(\mu\)M) was added to the basolateral medium, or UTP (Pharmacia Biotech Inc.; final concentration, 10 \(\mu\)M) was added to the apical medium. For the latter incubations, UTP was also present in the apical medium used to replace all subsequent samples taken.

In experiments where [Cl\(^{-}\]) or medium tonicity was varied, nystatin (Sigma) was added to the apical medium to increase apical membrane Ca\(^{2+}\) permeability (9). In our previous studies, nystatin was preincubated for 10 min with apical nystatin (final concentration, 350 units/ml, added from a 200,000-unit/ml stock solution in Me2SO), and nystatin was present in the apical medium throughout the flux period. Apical media used in these experiments were synthetic solutions containing 124 mM (NaCl + NaNO\(_3\)), 4 mM KCl, 10 mM sucrose, 10 mM glucose, 1.5 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 14 mM NaHCO\(_3\), 1 mM NaHPO\(_4\), and 10 mM Na-HEPES (Research Organics, Cleveland, OH), pH 7.4, at 37°C. The basolateral medium in these experiments was F12–4x + 1 mM CaCl\(_2\). During the initial 10 min of the flux period, the apical medium was isotonic and contained 124 mM Cl\(^{-}\). For experiments in which [Cl\(^{-}\]) was varied, following removal of the 10-min sample the apical medium was changed to one containing 66, 49, or 32 mM [Cl\(^{-}\)] (equimolar substitution with NO\(_3\)), and this apical medium was then used for the remainder of the flux period. Where toxicity was varied, after removal of the 10-min sample the Transwell was transferred to a well containing F12–4x containing 1 mM CaCl\(_2\) or 50 mM sucrose, with the same concentration of \(^{36}\)Cl\(^{-}\) as present during the initial 10 min. The apical medium then used for the remainder of the flux period was the 124 mM Cl\(^{-}\) synthetic medium described above, to which 50 mM sucrose was added.

Flux data are calculated as ratios of stimulated flux rate/control flux rate. The control rate is determined as the average of the counts in the first 20 min of the flux period, after apical and basolateral media collected during the first 10 min of each incubation, over which time flux rates remain linear (9). For incubations to which isoproterenol or UTP is added, the incubation proceeds for 10 additional minutes after addition of agonist, and the stimulated flux rate is determined as the average of the counts in the final two samples of the incubation (i.e. 17.5- and 20-min samples). For incubations in which [Cl\(^{-}\)] or medium tonicity is varied, the incubation proceeds for 20 min after the medium change, and the stimulated flux rate is again taken to be the average of the counts in the final two samples of the incubation (i.e. 27.5- and 30-min samples).

Phosphorylation Experiments—Transwell cultures were incubated for 90 min in a 37°C tissue culture incubator with apical and basolateral media identical to the 124 mM Cl\(^{-}\) synthetic medium described above. Except for the absence of Na2HPO\(_4\) and the addition of 0.022–0.056 mM LiCl (DuPont NEN). Apical medium volume was 1 ml, and basolateral medium was 2 ml. After the 90-min loading incubation, the cultures were washed once at room temperature, apically and basolaterally, with the media to be used in the final incubations. Final incubations were performed within a 37°C tissue culture incubator, with the samples gently rotated on an orbital shaker. For experiments testing effects of isoproterenol and apical UTP, cultures were incubated with or without these agonists for 10 min; apical (1 ml) and basolateral (2 ml) media were both F12–4x + 1 mM CaCl\(_2\). For experiments testing effects of reduced [Cl\(^{-}\)], or hypertonic shrinkage, cultures were incubated for a total of 40 min. For the initial 20 min, the apical medium (1 ml) was the 124 mM [Cl\(^{-}\)] described above for the flux studies, to which nystatin (350 units/ml) was added. The basolateral medium (2 ml) was F12–4x + 1 mM CaCl\(_2\). After 20 min, the apical medium was changed to one of the different synthetic media (32, 49, 66, or 124 mM [Cl\(^{-}\)]) described above, or both apical and basolateral media were changed to identical media with 50 mM added sucrose (hypertonic incubations) in the continued presence of apical nystatin.

Following the incubations, the cultures were washed rapidly by immersion in ice-cold, isotonic HEPES-buffered saline (pH 7.4). The filters were then cut from the Transwell supports and added to microcentrifuge tubes containing 0.4 ml of ice-cold lysing buffer containing 140 mM NaCl, 20 mM Na-HEPES (pH 7.4 at 2°C), and the following mixture of protease inhibitors: 5 mM Na-EDTA, 50 mM Na-pyrophosphate, 0.1 mM Na\(_2\) orthovanadate, 300 \(\mu\)M phenylmethylsulfonyl fluoride, 100 \(\mu\)M N-tosyl-\(\lambda\)-phenylalanyl chloromethyl ketone, 1.5 \(\mu\)M pepstatin, and 1.5 \(\mu\)M leupeptin (Sigma or Boehringer Mannheim). To ensure an adequate [32P] signal, 4–6 similarly incubated cultures comprised a single sample, and 2-3 filters from a given sample were added to a single microcentrifuge tube. The contents of each tube were then sonicated for 20 s with a probe sonifier (model 50 Sonic Disembrator, Fisher, setting 2) with the tube immersed in ice. Each tube was then tightly capped and vortexed vigorously. The contents of tubes containing filters from the same sample were then combined in 30-ml Corex tubes, 3 ml of additional lysing buffer was added to each tube, and the tube was sonicated for an additional 15 s with the tube immersed in ice. The contents of each tube were then transferred to 50-ml centrifuge tubes to which 20 ml of ice-cold lysing buffer was added; the tubes were then vortexed, and the filters were removed with forceps. The samples were then centrifuged successively for 5 min at 200 \(x\) g and for 15 min at 2,500 \(x\) g, both at 0°C. The pellets from these centrifugations, and for 45 min at 50,000 \(x\) g and 0°C. The pellet from this latter centrifugation, containing crude plasma membranes, was then suspended in lysing buffer containing 1% SDS (Sigma). These SDS-containing samples were then vortexed and incubated on ice for 10 min. To each sample, an equal volume of lysing buffer containing 9% Triton X-100 (Pierce) and a second, equal volume of lysing buffer without added detergent were then added. This mixture was incubated on ice for 50 min and then centrifuged in a microcentrifuge to remove insoluble debris. The supernatant was then mixed with a mouse monoclonal antibody developed by
Lytte et al. (26) against a ~38 kDa peptide encoding the carboxy-terminal 310 residues of the Na-K-Cl cotransporter of the T84 intestinal epithelial cell line (antibody T4, Refs. 26 and 27) (35 μg of T4 added per Transwell in the sample). Antibody T4, which preferentially reacts with the SDS-denatured form of the Na-K-Cl cotransport protein (26, 27), was purified from hybridoma supernatants by affinity chromatography using protein G-Sepharose (Sigma) prior to use in these experiments. Immediately following T4 addition, sufficient lysing buffer was then added to each sample to reduce its concentration of Triton X-100 to 2%, and the samples were incubated overnight at 4 °C on a rotating shaker.

The next morning, protein G-Sepharose (1 μl of packed beads per μg of T4) was added to each sample, and the samples were incubated an additional 2 h at 4 °C on the rotating shaker. The beads were then washed five times with ice-cold lysing buffer containing 1% Triton X-100, once with HEPES-buffered saline without added detergent, mixed with SDS-containing Laemmli sample buffer (31), and incubated at 50 °C for 1 h. The samples were then centrifuged; the supernatants as well as prestained protein standards (molecular mass range 27–180 kDa, Sigma) were loaded onto a 6% Laemmli SDS-polyacrylamide gel (31).

The proteins separated on the gel were then transferred electro-phasically to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA), which were subjected to autoradiography to determine 32P incorporation. Following this, the PVDF transfers were subjected to Western blotting with T4 (1.65 μg/ml) to determine the amount of ~170 kDa protein in each sample. Western blotting was done using the ECL method and reagents (Amer sham, Arlington Heights, IL), except for the peroxidase-conjugated secondary antibody (anti-mouse IgG) which was from Sigma. Films from autoradiography and Western blots were then analyzed by densitometry to determine the relative amounts (stimulated/control) of 32P and protein present in the ~170 kDa region; from these data ratios (stimulated/control) of 32P incorporated/unit protein were calculated for each sample. Densitometry peaks were corrected for background which was determined from regions of the gel lane in which there were no distinct band(s) identified; this was typically the top of the gel and/or the region between the 36.5 kDa standard and the tracking dye. For studies with isoproterenol and apical UTP, control levels of 32P incorporation were determined in the absence of added agonist; for studies of effects of reduced [Cl−], and cell shrinkage control levels were determined from isonicotic incubations with 124 mM apical [Cl−]. At least one appropriate control incubation was included in all experiments.

Photoaffinity Labeling Experiments—Labeling of intact dog tracheal epithelial cells with the photosensitive bumetanide analogue [3H]BSTBA was done using modifications of previously described procedures (32). Confluent Transwell cultures were incubated for 30 min within a 37 °C tissue culture incubator with 0.3 μM [3H]BSTBA added to the basolateral medium, with or without 20 μM unlabeled bumetanide. Apical volume was 1 ml, and basolateral volume was 2 ml; both media were F12. Six Transwell cultures were used for each condition to ensure detectable labeling. After the 30-min incubation, the filters were washed and cut from the Transwell supports and sonicated as described above for phosphorylation experiments. The filters were manually removed, and the remaining suspension was photolyzed for 20 min on ice as described previously (32). Plasma membranes were then isolated by centrifugation, solubilized, and run on 7% Laemmli (31) SDS-polyacrylamide gels. Gels were stained and cut into 4-mm slices that were digested and counted as described previously (32). All gel slices were counted for 20 min.

RESULTS

The Western blot in Fig. 1 shows that the monoclonal antibody T4, raised against the carboxy-terminal domain of the T84 cell Na-K-Cl cotransport protein (26, 27), reacts primarily with one protein from SDS-solubilized dog tracheal epithelial cell membranes of molecular mass ~170 kDa (lane 1). Our immunoprecipitation procedure using T4 (see "Materials and Methods") effectively removes all of this ~170-kDa protein from a supernatant containing SDS-solubilized dog tracheal epithelial membrane proteins (lane 2). The immunoprecipitated material (lane 3) contains primarily the ~170-kDa protein, though a ~45-kDa band is also noted on this and most

1 The abbreviation used is: [3H]BSTBA, [3H]-labeled 4-benzoyl-5-sulfamoyl-3-(3-thenyloxy)benzoic acid.

2 C. Lytle, unpublished experiments.
cotransport protein is also stimulated by reduced [Cl] to 32 mM in the presence of apical nystatin, which we find to decrease initial flux of \(2.8 \text{-fold, similar to the 2.7-fold increase seen in cotransport across the basolateral membrane and is } \text{metanide-sensitive (9), indicating that it represents primarily Na-K-Cl cotransport protein by an average of 3-fold over four experiments.}

The degree of stimulation of cotransport protein phosphorylation in response to each of these secretagogues correlates well with the stimulation of basolateral-to-apical \(2^{12}\text{Cl}^-\) flux by each compound, which over a large number of experiments averages 3.4-fold for isoproterenol and 2.6-fold for apical UTP (Table I). In Fig. 3 we also note a second band in the \(-45\text{-kDa region, the phosphorylation of which is increased in response to isoproterenol and UTP. As noted above (lane 3 of Fig. 1), we also observe this band on most Western blots of membrane protein immunoprecipitated using T4. These findings suggest that the \(-45\text{-kDa band may represent a proteolytic fragment of the } \sim 170\text{-kDa cotransport protein, which contains a regulatory phosphorylation site, although we have not studied this further.}

Fig. 4 shows that phosphorylation of the \(-170\text{-kDa Na-K-Cl cotransport protein is also stimulated by reduced [Cl] } (\text{lane 2}) \) and by hypertonic cell shrinkage (lane 3) in the absence of secretagogues. In this experiment, [Cl] was reduced by incubating the cells with apical nystatin and an apical medium containing reduced (32 m\text{M}) [Cl]. Nitrate substitutes for Cl to maintain isotonicity. We have previously shown (9) that this substitution does not produce cell shrinkage; thus, reducing [Cl] in the absence of concomitant cell shrinkage or hormonal stimulation stimulates cotransport protein phosphorylation. In eight separate experiments, we found that reducing apical [Cl] to 32 m\text{M} in the presence of apical nystatin stimulated \(32^\text{P} \) incorporation into the \(-170\text{-kDa cotransport protein by an average of 2.7-fold (Table I) compared with the level of incorporation seen with 124 m\text{M} apical [Cl] under otherwise identical conditions. We have done a large number of similar experiments (including those presented in Ref. 9) determining the effect of reducing [Cl] on basolateral-to-apical \(3^{6}\text{Cl}^-\) flux. In the presence of apical nystatin, this flux is limited by Cl transport across the basolateral membrane and is \(-90\% \text{ bu-}

metanide-sensitive (9), indicating that it represents primarily Na-K-Cl cotransport. After a similar period of exposure to apical nystatin and 32 m\text{M} apical [Cl] as used for the experiment in Fig. 4 (lane 2), we find an average stimulation of \(3^{6}\text{Cl}^-\) flux of 2.8-fold, similar to the 2.7-fold increase seen in cotransport protein phosphorylation. Addition of 50 m\text{M} sucrose to both apical and basolateral media (with 124 m\text{M} apical [Cl] in the presence of apical nystatin), which we find to decrease initial cell water content by \(-20\%\) (9), also stimulates cotransport protein phosphorylation by an average of 3-fold over four experiments (Table I, Fig. 4). This degree of stimulation is similar to the degree of \(3^{6}\text{Cl}^-\) flux stimulation seen in response to hypertonic shrinkage under similar conditions (2.4-fold; Table I).
The dependence of cotransport protein phosphorylation on the degree of \([\text{Cl}^-]\), reduction is examined in Fig. 5. When cells are exposed for 20 min to apical nystatin and 66 mM apical \([\text{Cl}^-]\), a relatively modest increase in phosphorylation of the \(\sim 170-kDa\) protein is observed compared with the level seen in cells incubated with nystatin and 124 mM apical \([\text{Cl}^-]\) (lanes 1 and 2 of Fig. 5; also see Table I). Progressively greater increases in phosphorylation are seen when apical \([\text{Cl}^-]\) is further reduced to 49 mM (lane 3 of Fig. 5) and 32 mM (lane 4). Table I again shows a strong correlation between cotransport protein phosphorylation and basolateral-to-apical \({}^{36}\text{Cl}^-\) flux stimulated by different degrees of \([\text{Cl}^-]\) reduction.

**DISCUSSION**

The results of this study demonstrate that the stimulation of basolateral Na-K-Cl cotransport in dog tracheal epithelial cells by cAMP-dependent and cAMP-independent secretagogues, as well as by hypertonic cell shrinkage, is accompanied by an increase in phosphorylation of the Na-K-Cl cotransport protein itself. Furthermore, the fractional increase in phosphorylation of the \(\sim 170-kDa\) cotransport protein seen in response to each of these cotransport stimuli is similar to the fractional increase in basolateral-to-apical \({}^{36}\text{Cl}^-\) flux promoted by each stimulus. These results are consistent with the hypothesis that activation of Na-K-Cl cotransport is achieved primarily through phosphorylation of the cotransport protein and are similar to results obtained by Lytle and Forbush (14, 15) in intact tubules from shark rectal gland.

We also find that reducing \([\text{Cl}^-]\), in the absence of secretagogue and without concomitant cell shrinkage, increases phosphorylation of the \(\sim 170-kDa\) Na-K-Cl cotransport protein. Furthermore, an excellent correlation is observed between this phosphorylation and the stimulation of cotransport activity in response to graded decreases in \([\text{Cl}^-]\). Thus, it appears that dog tracheal epithelial cells may contain a protein kinase and/or phosphatase that is sensitive to the level of \([\text{Cl}^-]\), with phosphorylation and activation of the Na-K-Cl cotransport protein resulting when \([\text{Cl}^-]\) becomes sufficiently reduced. Alternatively, levels of \([\text{Cl}^-]\), may somehow modulate the activity of other protein kinases(s) that phosphorylate the cotransporter, such as the putative volume-sensitive "V kinase" activated in response to cell shrinkage (14, 22, 23). Treharn et al. (35) recently described two protein kinases in human nasal epithelial plasma membranes that show peak activity at 40–50 mM \([\text{Cl}^-]\) and progressive, marked inhibition when \([\text{Cl}^-]\) is raised in the range from 50 to 150 mM. However, the only substrates of these kinases identified were proteins of 37 and 45 kDa.

Furthermore, one of these kinases was found to be inhibited by nitrate as well as by \([\text{Cl}^-]\) and was also inhibited by DIDS, which we find to have no significant effect on Na-K-Cl cotransport or its activation in dog tracheal epithelial cells or other cells (36,3).

The stimulatory effect of reducing \([\text{Cl}^-]\) on Na-K-Cl cotransport activation appears to be primarily a regulatory effect, as opposed to a thermodynamic one. We previously noted that changes in the thermodynamic driving force for basolateral cotransport produced by varying apical \([\text{Na}^+]\) and \([\text{K}^+]\) in the presence of apical nystatin had only small effects on basolateral-to-apical \({}^{36}\text{Cl}^-\) fluxes in dog tracheal cell cultures (9).

Furthermore, reducing \([\text{Cl}^-]\), augments saturable \([\text{H}]\)butemomabide binding in dog tracheal and shark rectal gland epithelial cells, respectively (9, 15); such binding reflects the activation state of the cotransporter rather than ion gradients (37). While we do not know the level of \([\text{Cl}^-]\) present at each apical \([\text{Cl}^-]\) with nystatin present, \([\text{Cl}^-]\) in the presence of 124 mM apical \([\text{Cl}^-]\) is most likely close to, but not greater than, \([\text{Cl}^-]\) in resting cells. In five separate experiments, we found the cell water content (9) of cultures incubated for 40 min with apical nystatin, and the 124 mM \([\text{Cl}^-]\) apical medium was slightly (mean of 7%) but not significantly lower than that of cultures incubated under identical conditions without nystatin. The presence of intracellular anions that do not permeate nystatin pores thus appears to maintain \([\text{Cl}^-]\) in nystatin-treated cells well below the level of apical \([\text{Cl}^-]\) (38). Notably, reducing apical \([\text{Cl}^-]\) to 66 mM appears to markedly reduce \([\text{Cl}^-]\); cultures exposed for 40 min to an apical medium with nystatin, 66 mM \([\text{Cl}^-]\) and guanocline which, unlike nitrate, does not permeate nystatin pores) as the substitute anion undergo a \(\sim 30\%\) loss of cell water (9).

Na-K-Cl cotransport activation in response to apical UTP is entirely secondary to apical \([\text{Cl}^-]\) efflux; inhibition of such efflux blocks cotransport activation by UTP (8, 9). The cellular signal for this secondary cotransport activation is not yet known, though two prime candidates would appear to be cell shrinkage that reduced \([\text{Cl}^-]\), both of which occur during secretagogue stimulation in dog tracheal and other secretory epithelia (3, 4, 39). In rat parotid acini, Robertson and Foskett (4) recently demonstrated that reduced \([\text{Cl}^-]\), but not cell shrinkage is required for carbachol stimulation of basolateral \([\text{Na}^+]\) entry, which in this tissue is mediated both by Na-K-Cl cotransport and by Na/H exchange. In shark rectal gland tubules, activation and phosphorylation of the Na-K-Cl cotransporter by

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3 M. Haas and D. G. McBrayer, unpublished experiments.
secretagogues can be mimicked by a variety of maneuvers that decrease [Cl\(^{-}\)], (15). Reduced [Cl\(^{-}\)] is also necessary for \(\beta_2\) agonist activation of Na\(^{+}\) influx via a nonselective cation channel in rat fetal distal lung epithelium (40) and for antidiuretic hormone and cAMP activation of nonselective cation channels in the distal renal tubule cell line A6 (41). Our findings in dog tracheal epithelial cells are also consistent with reduced [Cl\(^{-}\)], as an intracellular signal for secondary activation of Na-K-Cl cotransport. The degrees of stimulation of both basolateral-to-apical \(^{36}\)Cl\(^{-}\) flux and of cotransport protein phosphorylation seen when [Cl\(^{-}\)] is reduced by incubation with apical nystatin and 32 mM apical [Cl\(^{-}\)] are approximately equivalent to those produced by a dose of UTP (10 \(\mu\)M) that maximally stimulates transepithelial \(^{36}\)Cl\(^{-}\) transport and basolateral \([^{38}\)H]bumetanide binding in primary cultures of tracheal epithelial cells (8, 9). In addition, we observe a very strong correlation between cotransporter activation and phosphorylation in response to graded decreases in [Cl\(^{-}\)]. However, our findings also cannot exclude cell shrinkage as either a primary or adjunct signal for secondary cotransport activation in dog tracheal cells. A degree of hypertonicity that produces an initial decline in cell water content of \(-20\%\) (9) was found to stimulate both cotransporter activation and phosphorylation to approximately the same extent as that observed with 10 \(\mu\)M UTP. Like reduced [Cl\(^{-}\)], cell shrinkage appears to stimulate Na-K-Cl cotransport via activation of a protein kinase (and/or inhibition of a protein phosphatase). This appears not only to be the case in epithelial cells from dog trachea and shark rectal gland (14, 15) in which shrinkage has been shown to cause phosphorylation of the...
cotransporter itself, but also in several other cell types in which cotransport activation by cell shrinkage has been found to be blocked by protein kinase inhibitors (22, 23). The identity of the putative "V kinase" activated by cell shrinkage is also not known, though it does not appear to be cAMP-dependent protein kinase or protein kinase C (14, 22, 23). Studies aimed at the characterization and possible differentiation of putative [Cl\(^-\)]-sensitive and volume-sensitive kinase(s) responsible for Na-K-Cl cotransport activation in tracheal epithelial cells are presently being initiated.

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