Na-K-Cl cotransport activity in duck erythrocytes increases ~10-fold in response to osmotic cell shrinkage, norepinephrine, fluoride, or calyculin-A (an inhibitor of type-1 and -2a phosphatases). To test whether all four stimuli promote phosphorylation of the cotransport protein and whether this phosphorylation is catalyzed by the same kinase, the cotransporter was isolated from erythrocytes by immunoprecipitation and its pattern of phosphorylation was evaluated. Each stimulus evoked proportionate increases in cotransporter activity and phosphorylation. No two stimuli in combination evoked greater activation and phosphorylation than did the more potent of the two stimuli acting alone. Phosphoamino acid analysis of the cotransport protein indicated that phosphorylation occurs at serine and threonine residues. Phosphopeptide mapping revealed a distinctive pattern of eight major tryptic phosphopeptides, none of which were significantly phosphorylated in the unstimulated state. Maps of cotransporters activated by the four different stimuli were indistinguishable. Measurements of phosphorylation stoichiometry indicated that each cotransporter acquires ~5 phosphates on going from an inactive state in swollen cells to an active state in shrunken cells. Staurosporine, a kinase inhibitor with broad selectivity, inhibited each stimulus equally (IC50 ~ 0.7 μM). Staurosporine promptly reversed cotransporter activity and phosphorylation when added to shrinkage-stimulated but not to calyculin-stimulated cells, indicating that it enters the cell rapidly and blocks phosphorylation. These results suggest that cell shrinkage, cAMP, fluoride, and calyculin-A promote the phosphorylation of the Na-K-Cl cotransport protein at a similar constellation of serine and threonine residues. It is proposed that all modes of stimulation ultimately involve the same protein kinase.

Na-K-Cl cotransport is regulated by numerous first and second messengers through a complex and cell-specific interplay of stimulatory and inhibitory signals. The molecular mechanisms by which cell surface receptors, cell volume, cytosolic chloride, cytoskeletal architecture, and proliferative status modulate cotransport activity remain unknown. Early recognition that ion movement by the Na-K-Cl cotransporter, although energetically passive (2, 3), requires cytosolic ATP and Mg2+ (2, 4–7) prompted speculation that acute regulation might involve reversible phosphorylation of the cotransport protein, regulatory subunits, or upstream signal transducers. Circumstantial support came from demonstrations that cotransport activity is increased by agents that inhibit protein phosphatases (10, 11) and decreased by agents that inhibit protein kinases (11, 12). Recent studies have established that the Na-K-Cl cotransporter itself is a phosphoprotein (11, 13, 14) whose phosphorylation state parallels its activation state (13–18). While it is generally assumed that cotransporter phosphorylation is both necessary and sufficient for transport activity, recent research suggests that additional factors, including affinity-labeled proteins (19), cytoskeletal interactions (16, 20–22), and mechanical changes in the cell membrane (23) might influence cotransport activity.

Duck erythrocytes have long served as a premier model of electroneutral ion transport by virtue of their simplicity, uniformity, and ease of experimental manipulation. These cells manifest robust Na-K-Cl cotransport in response to four types of stimuli: osmotic cell shrinkage, elevated cytosolic cAMP, Ser-Thr phosphatase inhibitors (calyculin-A, okadaic acid, endothall thioanhydride, and deoxyxegenation), and norepinephrine. Unlike other modes of stimulation, the norepinephrine response is associated with increases in cytoplasmic cAMP (24) and cAMP-dependent protein kinase activity (8, 11), and can be blocked by kinase inhibitors like K-252a and H-9 at doses that disable cAMP-dependent protein kinase in intact avian erythrocytes (11). The same kinase inhibitors also block activation of cotransport by cell shrinkage, fluoride, and okadaic acid, but only at concentrations an order of magnitude higher (11). These observations suggest that the avian erythrocyte Na-K-Cl cotransporter is regulated by at least two kinases, one of which is cAMP-dependent protein kinase. The fact that K252a blocks activation by cell shrinkage, fluoride, and okadaic acid at a similar high dose raises the possibility that non-cAMP-dependent stimuli are transduced by the same kinase (25). While cotransport activity appears to be determined by a dynamic competition between ongoing protein kinase and phosphatase activities, a key question is whether all modes of stimulation involve phosphorylation of the cotransport protein itself and whether different stimuli involve different kinases.

The purpose of the present study was to test the hypothesis that four different modes of stimulation (cell shrinkage, cAMP, fluoride, and calyculin-A) involve phosphorylation of the cotransport protein at common sites. The recent advent of monoclonal antibodies capable of immunoprecipitating the Na-K-Cl cotransport protein from detergent extracts of 32P-labeled duck erythrocytes with high efficiency (26) now makes it possible to quantitatively compare the phosphorylation induced by different stimuli and to assess the physical disposition of phosphorylation sites. The results of this analysis suggest that all forms of activation promote phosphorylation of the cotransport protein at a common set of Ser/Thr sites.
Regulatory Phosphorylation of the Na-K-Cl Cotransporter

EXPERIMENTAL PROCEDURES

Materials—^86RbCl was obtained from DuPont NEN; staurosporine, calyculin-A, 5-(4-chlorophenylthio)-α-CMP were from Biomol; ML-7 was from LC Laboratories; protease inhibitors were from Boehringer Mannheim; silica gel plates (5748-7) and polyethylene-cellulose F TLC plates (5504) were from EM Reagents; thin layer cellulose sheets were from Eastman Kodak Co. (12355); PVDF membrane was from Millipore; chloroform, n-butyric acid, CHAPS, N-chlorosuccinimide, t-1-tyosylamido-2-phenylthyl chloromethyl ketone-treated trypsin, osin, and reagent grade chemicals were from Sigma.

A monoclonal antibody (T14) directed against the carboxyl-terminal 310 amino acids of the human colon Na-K-Cl cotransporter (hNKCC1) was developed as described previously (26). The T14 antibody detects 0.5% of duck erythrocyte cotransporter on Western blot and is inhibited by unlabeled cotransporter (2.5% hematocrit) for 45 min in fresh DFS at 41 °C to achieve a steady state with respect to ion and water contents. The portion of ^86Rb influx attributable to cotransport was determined (2.5% hematocrit) for 45 min in fresh DFS containing ^86RbCl to assess the effect of each activator becomes maximal and invariant. ^86Rb influx was initiated by the addition of isotope and terminated 1–4 min later by washing the cells with ice-cold AP buffer containing 1% Triton X-100, followed by one rinse with PBS. The immunoprecipitate was extracted into 70 μl of SDS-sample buffer (4% SDS, 50 mM dithiothreitol, 50 mM Tris-HCl, pH 6.8, 12% glycerol, and 0.01% Serva Blue G) and electrophoretically separated on 7.5% Tricine-SDS-polyacrylamide gels (28). Autoradiography was performed using x-ray film or a storage phosphor screen (PhosphorImager, Molecular Dynamics). Western blot analysis indicated that the efficiency of immunoprecipitation from extracts of both resting and stimulated cells exceeded 80%.

**Specific Activity of γ[^32P]ATP—** The γ[^32P] content of ATP in KCO,-neutralized perchloric acid extracts of ^2P-labeled cells was determined by ascending thin layer chromatography on polyethylene-cellulose plates in 0.85 M KH2PO4 at pH 3.4 (29). To quantitate γ[^32P]ATP, discrete spots of radioactivity were scraped from the plate and analyzed by liquid scintillation spectroscopy, correcting for a counting efficiency of 70% for γ[^32P]ATP bound to cell (8).

One-dimensional Phosphopeptide Analysis—γ[^32P]ATP was measured as described by Mayer and Krebs (30). A neutralized perchloric acid extract of labeled cells (20 mg) was applied to a column (5 × 40 mm) of Dowex 1-formate. The column was washed with 5 ml of 4 N formic acid, and ATP was eluted with 0.4 M ammonium formate in 4 N formic acid. The solution was lyophilized, dissolved in 300 μl of water, and lyophilized again. The dried material was dissolved in 450 μl of 100 mM imidazole (pH 7.4) containing 1 μg of phenol red to confirm neutral pH. ATP was converted to glucose-6-phosphate by adding 10 μM glucose, 0.05% bovine serum albumin, 50 μM cold ATP, and 5 units of yeast hexokinase, and incubated at 30 °C for 1 h. The solution was then applied to a Dowex 1-formate column, and the column was washed with 5 ml of water. Glucose-6-phosphate was eluted with 2 N formic acid, and after which residual [^32P]ATP was calculated from the [@#^32P]ATP bound to cell (85%) and for the efficiency of enzymatic formation and recovery of [^32P]glucose-6-phosphate (77%). The ratio of γ[^32P]ATP to total γ[^32P]ATP, i.e. the fraction of cellular γ[^32P]ATP converted into [^32P]glucose-6-phosphate by hexokinase, was determined to be 0.82 ± 0.02 (mean ± S.D., n = 3).

Two-dimensional Phosphopeptide Analysis—γ[^32P]ATP was calculated from the radioactivity of ATP in the extract, the γ[^32P]ATP:[^32P]ATP ratio (0.82), the number of cells extracted (1.4 × 10^8), the water content of the duck erythrocyte (99.4 fl; Ref. 31), and the concentration of ATP within the duck erythrocyte (3.3 mM; Ref. 7).

**Phosphoamino Acid Analysis—** Cotransporter protein was isolated from ^2P-labeled cells by immunoprecipitation, separated by SDS-PAGE, and electrophoretically transferred to PVDF membrane. Regions of PVDF containing ^2P-cotransporter were located by autoradiography, excised, and hydrolyzed in 6 N hydrochloric acid at 100 °C for 3 h. The hydrolysate was lyophilized, reconstituted in 20 μl of water containing 10 μg of unlabeled phosphoamino acids, and separated by one-dimensional thin layer electrophoresis (600 V for 5 h at 4 °C) on a silicone gel plate (Whatman 410–221) in a buffer consisting of formic acid, acetic acid, and water (25:78:89). Phosphoamino acids were visualized by ninhydrin staining and autoradiography using a storage phosphor screen.

**One-dimensional Phosphoprotein Analysis—** Gel slices containing ^2P-cotransporter were rinsed thoroughly with water, then treated with 15 μM N-chlorosuccinimide for 20 min to selectively cleave thymolpheryl peptide bonds (32). Proteolytic fragments were separated on a 7.5% Tricine SDS-polyacrylamide gel, and those containing γ[^32P] were detected by autoradiography using a storage phosphor screen.

**Two-dimensional Phosphoprotein Analysis—** Gel slices containing ^2P-cotransporter were rinsed thoroughly with water and equilibrated with 200 mM NH4HCO3. The gel pieces were then rotated with 500 μl of
The radioactivity in cellular $^{32}$P]ATP could be transferred by incubation of duck erythrocytes with $^{32}$Porthophosphate resulted in a slow equilibration of $^{32}$P into cellular ATP (Fig. 1). Half-maximal incorporation of $^{32}$P into the cotransport protein (Fig. 3) was measured as described under "Experimental Procedures." Data represent mean values ± S.D. from triplicate samples.

200 mM NH$_4$HCO$_3$ containing 100 µg of l-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin for 24 h at 37 °C, with the addition of 50 µg of freshly prepared trypsin after 17 h. The digest was then subjected to three cycles of lyophilization and reconstitution in water (500 µl). The final residue was dissolved in 20 µl of electrophoresis buffer (10% acetic acid, 1% pyridine, pH 3.5) and spotted onto thin layer cellulose sheets along with marker dyes (xylene cyanol FF and phenol red, 2 µg each). Phosphopeptide maps were generated by electrophoresis at 500 V for ~3 h followed by crossed ascending chromatography in 1-butanol:pyridine:acetic acid:water (60:40:12:48). To ensure uniformity between different samples, electrophoresis and chromatography were allowed to continue until each marker dye had migrated a fixed distance. Phosphopeptides were detected by autoradiography using a storage phosphor screen.

RESULTS

Time Course of $^{32}$P Incorporation into Cellular ATP—Incubation of duck erythrocytes with $^{32}$Porthophosphate resulted in a slow equilibration of $^{32}$P into cellular ATP (Fig. 1). Half-maximal incorporation required ~3 h, consistent with previous data on turkey erythrocytes (8). Analysis of extracts of cells labeled for 3 h revealed that 82 ± 2% (mean ± S.D., n = 3) of the radioactivity in cellular $^{32}$PATP could be transferred in vitro to glucose by hexokinase, indicating that the predominant form of radioactive ATP at this point in the labeling process is $\gamma$-$^{32}$PATP. All experiments described hereafter were performed on erythrocytes that had been prelabeled with $^{32}$P for 3 h and then exposed for 9–12 min to an activator of cotransport and ouabain (to block the Na/K pump). During the activation period, changes in cellular $^{32}$PATP (measured chromatographically), water content (measured gravimetrically), and pH were negligible, in agreement with previous studies (7, 8, 31).

Activators of Cotransport Promote Cotransport Protein Phosphorylation—Four factors known to stimulate cotransport activity (hypertonicity, norepinephrine, fluoride, and calyculin-A) promoted incorporation of $^{32}$P into the cotransport protein (Fig. 2). Optimal phosphorylation was obtained with doses that evoke maximal cotransport activity: 10 µM norepinephrine, 10 mM fluoride, 100 nM calyculin-A, and 100 mM sucrose (25). When added with the stimulus, 100 µM bumetanide abolished cotransporter activity but did not alter the rate or the extent of phosphorylation (data not shown); this observation supports the idea that cotransporter phosphorylation is the cause rather than the consequence of cotransport activity and excludes the possibility that bumetanide inhibits by blocking phosphorylation. The stimulatory effect of fluoride was not enhanced by the addition of 10 µM Al$^{3+}$ nor diminished by the chelation of Al$^{3+}$ with 1 mM deferoxamine mesylate (data not shown), discounting the possibility that it is due to aluminofluoride (AlF$	ext{}_4$) as proposed for rat parotid acinar cells (33).

Cotransporter Activity and Phosphorylation Co-vary with Changes in Cell Volume—The effect of osmotically induced changes in cell volume on cotransporter activity and phosphorylation was measured on paired suspensions of duck erythrocytes. The conditions used for $^{32}$P labeling (preincubation for 3 h at 10% hematocrit) had no significant effect on cell volume or on the cotransporter's subsequent responsiveness to various stimuli. In unstimulated cells, cotransporter activity remained low, averaging only 3–7% of maximal levels. Slight osmotic cell swelling, from 1.5 to 1.63 liters/kg of cell solid, caused a virtual cessation of cotransport activity and a slight reduction in cotransport protein phosphorylation (Figs. 3–5). Greater degrees of swelling, even to extreme prelytic dimensions, caused no further decrease in cotransporter phosphorylation. This residual phosphorylation persisted when the swollen erythrocytes were exposed for 12 min to 30 µM staurosporine, a broad spectrum protein kinase inhibitor that rapidly abolishes cotransport activity (e.g., Figs. 6–8). Together these results suggest that the cotransport protein possesses a minor subset of non-regulatory or inhibitory phosphorylation sites whose turnover is volume-independent and either staurosporine-insensitive or relatively slow.

Cell shrinkage evoked parallel increases in cotransporter activity and phosphorylation (Fig. 3). A 33% reduction in cell water, from 1.5 to 1.0 liter/kg of cell solid, evoked near-maximal transport and $^{32}$P incorporation. The effect of cell shrinkage on phosphorylation was somewhat selective for the cotransporter
as none of the major membrane phosphoproteins, of which 28 could be resolved on a 7.5% Tricine-SDS gel, were significantly affected by osmotic perturbation (data not shown).

Stoichiometry of Phosphorylation—If the phosphorylation observed here is the direct cause of transport function, each cotransport unit recruited into activity should acquire at least one phosphate group. To estimate the stoichiometry of phosphorylation, aliquots of 32P-labeled erythrocytes were analyzed for cell number, [γ-32P]ATP specific activity, and cotransport protein phosphorylation (i.e., the 32P content of the cotransport protein after isolation by immunoprecipitation and gel electrophoresis). The number of cotransporters in the immunoprecipitate was estimated from the average number of specific [3H]bumetanide binding sites on a maximally stimulated duck erythrocyte (3750), the actual number of erythrocytes subjected to immunoprecipitation (1.4 × 10^8), and the average efficiency of immunoprecipitation (80%). The number of phosphate groups associated with the cotransporter was calculated from the radioactivity of the 146-kDa cotransporter band and the measured specific activity of the phosphate source, i.e., [γ-32P]ATP. The results of four experiments in which the relationship between cell water content and phosphorylation stoichiometry was measured is shown in Fig. 4. In cells of normal volume, the nominally active cotransporter contained 2.3 ± 0.9 phosphate groups. Osmotic swelling reduced this ratio to 1.0 ± 0.4, whereas shrinkage increased it to 5.8 ± 1.2 (mean ± S.E., n = 4). The analysis therefore indicates that each cotransporter acquires 4.8 ± 0.9 phosphates on going from an inactive state in swollen cells to an active state in shrunken cells.2

2 C. Lytle, unpublished results.

3 This analysis assumes that each cotransporter binds a single molecule of [H]bumetanide avidly upon activation (42), and therefore ignores transporters that fail to respond to a given stimulus. The existence of “reserve cotransporters” is not unprecedented. In the shark

FIG. 3. Changes in cell volume coordinately affect Na-K-Cl cotransport activity and Na-K-Cl cotransport protein phosphorylation. 32P-Labeled erythrocytes were exposed for 12 min to a range of osmolality (220–420 mosM). Cotransport protein was isolated by consecutive immunoprecipitation and SDS-PAGE, and its 32P content (right ordinate) was determined by Cerenkov analysis. Cell volume and cotransport activity were measured as cell water content (abscissa) and bumetanide-sensitive 86Rb influx rate (left ordinate) on companion cells lacking 32P over the interval between 11 and 13 min. The water content of unstimulated 32P-labeled erythrocytes (1.55 liters/kg of cell solid) is indicated by the shaded bar. Similar results were obtained in three additional experiments.

FIG. 4. Stoichiometry of Na-K-Cl cotransport protein phosphorylation as a function of cell volume. Erythrocytes were labeled with 32P for 3 h, then exposed to an isotonic (323 mosM), hypotonic (223 mosM), or hypertonic (410 mosM) medium. After 9 min, samples from each cell suspension were obtained for analysis of wet cell mass, dry cell mass, [32P]ATP, and [32P]cotransport protein as described under “Experimental Procedures.” Data are expressed as moles of 32P incorporated into each mole of cotransport protein versus cell water content (volume). Shaded bar denotes normal (isosmotic) cell water content. Different symbols represent results from four independent experiments.

FIG. 5. Coordinate effects of four stimuli on Na-K-Cl cotransporter activity and phosphorylation: lack of additivity. Aliquots of duck erythrocytes (one labeled with 32P) were exposed for 12 min to stimuli, either separately or in combination, at their maximally effective doses: 10 μM norepinephrine (NE), 10 mM sodium fluoride (F^−), 100 mM sucrose (hypert), or 0.2 μM calyculin-A (cal). hypot, hypotonic Cotransport activity (open bars) was measured as bumetanide-sensitive uptake of 86Rb between 11 and 13 min. Phosphorylation (shaded bars) was measured as 32P content of cotransport protein at 12 min. Values were normalized to those with calyculin-A (designated as 100% maximal). Each bar represents the mean ± S.E. obtained in four to six experiments.
course of activation and phosphorylation of Na-K-Cl cotransporter after cell shrinkage and reversal by staurosporine. Bumetanide-sensitive $^{86}$Rb influx in duck erythrocytes was measured at successive 30-s intervals and plotted against the midpoint of the interval (open circles, solid line). In a separate experiment, cotransport protein phosphorylation was measured over the same time course (filled circles, dashed line). At 4 min, the medium was rendered hypertonic by the addition of 2 M sucrose to yield 100 mM. At 18 min, staurosporine was added to yield 30 $\mu$M staur, staurosporine.

Magnitude and Additivity of Stimuli—Paired measurements of cotransporter activity and phosphorylation indicated that norepinephrine, sucrose, fluoride, and calyculin-A, when applied individually at optimally effective concentrations, evoked similar levels of cotransport activity and cotransport protein phosphorylation (Fig. 5). With norepinephrine, activity increased 13-fold, and this was associated with a 5.7-fold increase in phosphorylation (Fig. 5). With norepinephrine, activity increased 13-fold, and this was associated with a 5.7-fold increase in phosphorylation.

Regulatory Phosphorylation of the Na-K-Cl Cotransporter

Cell shrinkage increased cotransporter activity and phosphorylation over the same time interval ($t_{1/2} = 0.8$ min) than that by cell shrinkage ($t_{1/2} = 5$ min). When applied to resting cells, staurosporine (15 $\mu$M) rendered the cotransporter refractory to cell shrinkage and calyculin-A (Fig. 7A) and to fluoride and norepinephrine (data not shown). When added to cells already stimulated by osmotic shrinkage, staurosporine caused cotransporter activity and phosphorylation to subside rapidly (Figs. 6 and 7B). This reversal was not observed in cells prestimulated with calyculin-A (Fig. 7B), indicating that staurosporine deactivates the cotransporter by inhibiting its phosphorylation rather than by stimulating its dephosphorylation. Thus, each of the four stimuli appears to be transduced by a kinase that is inhibited, either directly or indirectly, by staurosporine.

Staurosporine Blocks Each Stimulus Equipotently—If all modes of activation involve the same staurosporine-sensitive step, each stimulus should be inhibited by staurosporine equipotently. To test this hypothesis, erythrocytes were preincubated for 10 min with various doses of staurosporine before stimulation by norepinephrine, fluoride, or hypertonicity. As shown in Fig. 8, all three stimuli were inhibited to a half-maximal extent by a similar concentration of staurosporine ($0.7$ $\mu$M).

One-dimensional Phosphopeptide Analysis—To assess the distribution of phosphorylation sites, the cotransport protein was chemically fragmented with N-chlorosuccinimide, an agent that selectively cleaves tryptophanyl peptide bonds (32). After treatment of $^{32}$P-labeled cells with various stimuli, the cotransporter was isolated by immunoprecipitation and SDS-gel electrophoresis. Gel bands containing the 147-kDa cotransporter were then treated with N-chlorosuccinimide, and $^{32}$P-labeled cleavage products were analyzed by SDS-gel electrophoresis and autoradiography. Chemical cleavage for 20 min produced two major $^{32}$P-labeled fragments of 82 and 41 kDa (Fig. 9). The fragments appear to be different domains since (i) fragments of identical size were obtained with cleavage times half and twice as long, (ii) treatment with fresh N-chlorosuccinimide for an additional 20 min failed to convert the isolated 82-kDa fragment into the 41-kDa fragment, and (iii) a monoclonal antibody that recognizes the carboxyl terminus of the cotransport protein (26) recognized only the 41-kDa fragment on Western blots of N-chlorosuccinimide-treated protein (data not shown). With all four stimuli, each of the two fragments was phosphorylated to roughly similar extents. Fragments obtained from calyculin-stimulated cotransporters exhibited greater phosphorylation and slower electrophoretic mobility (88 and 45 kDa), which may reflect changes in the folding, net charge, or SDS-binding properties associated with the higher degree of phosphorylation.

These results indicate each stimulus promotes phosphorylation of two large domains, one of which comprises part of the carboxyl terminus.

Regulatory Phosphoacceptors—Phosphoamino acid analysis of the cotransport protein (Fig. 10) indicated that each of the four stimuli promote phosphorylation at serine and threonine residues. Phosphotyrosine was not detected in either resting or stimulated cotransporters.

Different Stimuli Promote Phosphorylation of Common Sites—Two-dimensional phosphopeptide maps of cotransporters isolated from stimulated erythrocytes revealed a distinctive pattern of eight prominent tryptic phosphopeptides (designated 1–8 in Figs. 11 and 12). Maps of cotransporters phosphorylated by
ated in response to cell shrinkage, fluoride, and norepinephrine were qualitatively indistinguishable (Fig. 11). None of the eight spots were detected in maps of unstimulated cotransporters (Fig. 11, control).

Because calyculin-A evoked more cotransporter phosphorylation and activity than did other stimuli (Figs. 2, 5, and 9), it was important to determine whether the phosphatase inhibitor promotes phosphorylation of different sites or more complete phosphorylation of the same sites. To distinguish between these possibilities, phosphopeptide maps of cotransporters stimulated by cell shrinkage and calyculin-A were compared. The patterns of phosphopeptides obtained with cell shrinkage and calyculin-A were similar (Fig. 12) and resembled those obtained with norepinephrine and fluoride (Fig. 11). No additional phosphopeptide spots were observed in maps of cotransporters stimulated by cell shrinkage and calyculin-A si-
exposure to 10 mM norepinephrine, hypertonicity (+100 mM sucrose), 10 mM fluoride, or 0.2 mM calyculin-A. Cotransport protein was immunoprecipitated, separated by SDS-PAGE, and electrophoretically transferred to PVDF membrane. Acid hydrolysates containing equivalent amounts of $^{32}$P were separated by thin layer electrophoresis and $^{32}$P-labeled amino acids were detected by autoradiography. Migration of phosphoserine ($P$-$\text{Ser}$), phosphotyrosine ($P$-$\text{Tyr}$), and phosphothreonine ($P$-$\text{Thr}$) standards from the origin is indicated on the left. One of two similar experiments is shown.

multaneously (Fig. 12). Hence, the greater degree of cotransporter phosphorylation and activity observed with calyculin-A appears to reflect a more complete phosphorylation of the same sites phosphorylated with other stimuli.

Whether each spot represents a unique phosphorylation site is uncertain. Some spots might represent precursors of others, or contain mixtures of different peptides, or contain a single peptide with multiple phosphorylation sites. Incomplete trypsinolysis is unlikely, however, since labeled cotransporters were digested overnight twice with fresh trypsin in great excess, and since different digests yielded consistent phosphopeptide patterns. Given the uniformity of each major spot, inadequate separation of peptide mixtures in two dimensions is also unlikely. It is apparent that upon activation a heterogeneous array of tryptic peptides are phosphorylated and that a similar array is observed with each stimulus.

**DISCUSSION**

The experiments described here suggest that activators of Na-K-Cl cotransporter in the avian erythrocyte (cell shrinkage, cAMP, fluoride, and calyculin-A) promote phosphorylation of the cotransport protein at a common constellation of serine and threonine residues. These results substantiate the concept that the cotransport protein is regulated by direct phosphorylation (11, 13, 14) and suggest that different stimuli act through the same kinase. The single kinase theory is supported by three lines of evidence. First, application of any two stimuli in combination evokes no greater cotransport activity or phosphorylation than does the more potent stimulus alone (Fig. 5). If the phosphorylation evoked by the four stimuli was catalyzed by different kinases acting on separate sites, application of the stimuli in pairs should yield the sum of the phosphorylation produced by the stimuli individually. The data indicate, rather, that phosphorylation by one stimulus precludes further phosphorylation by a different stimulus, and therefore suggest that the four stimuli act on common sites. Second, phosphopeptide maps of cotransporters activated by the four different stimuli are qualitatively indistinguishable (Figs. 11 and 12). Third, staurosporine equipotently blocks activation of the cotransporter by cell shrinkage, cAMP, and fluoride (Fig. 8), consistent with the hypothesis that each signal is transduced to the same sites by the same kinase.

The molecular switch on the cotransport protein that controls ion translocation appears to involve several serine and threonine residues. Estimates of phosphorylation stoichiometry indicate that the cotransport protein acquires ~5 phosphates on going from an inactive state in swollen cells to an active state in shrunken cells, and phosphopeptide maps show incorporation into a heterogeneous array of tryptic peptides. Chemical cleavage studies using N-chlorosuccinimide suggested that the incorporated phosphate is distributed evenly between amino and carboxyl segments of the cotransport protein. This agent splits the cotransporter into two immunologically distinct domains (82 and 41 kDa) whose combined mass (121 kDa) approaches that of the intact protein (145 kDa). With all four stimuli, comparable quantities of phosphate are incorporated into the different domains. Although the molecular structure of the avian cotransporter is undefined, known mem-
tein kinase A via inhibitor-1

Since PP-1 is known to be inhibited by cAMP-dependent procollin-A than to okadaic acid (7), it appears to be type-1 (PP-1). The rapidity at which resting state converts to active-phosphorylated state in unstimulated cells (7, 11). The finding of major phosphorylation sites in putative NH2- and COOH-terminal domains corroborates previous work on the chloride-secreting cells of the shark rectal gland. In these cells, as in duck erythrocytes, the Na-K-Cl cotransporter is stimulated and phosphorylated in response to cell shrinkage, unlike those activated by cAMP, are resistant to general kinase inhibition (by addition of K252a or by depletion of cellular ATP or Mg2+) and surmised that cell shrinkage might suppress dephosphorylation. However, this hypothesis was not borne out by the present study with staurosporine. When this kinase inhibitor was added to shrunken cells, cotransport activity and cotransport protein phosphorylation subsided rapidly (Fig. 6), suggesting that the deactivating phosphatase remains highly active after cell shrinkage.

Although still obscure, the transmission of the volume signal does not appear to require protein kinase C, cGMP-dependent protein kinase activity, or Ca2+/calmodulin-dependent protein kinase II, since modulators of these kinases (phorbol esters, dibutyryl cGMP, and cytosolic free Ca2+) have negligible effects on the phosphorylation state, activity, or volume responsiveness of the cotransport protein in duck erythrocytes.2 The recent identification of Ca2+-calmodulin-dependent myosin light chain kinase (MLCK) as a shrinkage-stimulated kinase (16, 39) prompted Klein and O’Neill to suggest that MLCK conveys the volume signal to the cotransport protein. This concept was based on two observations. First, volume changes in endothelial cells evoke parallel alterations in Na-K-Cl cotransport activity and myosin light chain phosphorylation; and second, both responses were inhibited equipotently by the MLCK inhibitor ML-7 (16). However, ML-7 did not block phosphorylation of the cotransport protein in response to cell shrinkage, suggesting that the effect of MLCK on the cotransporter is indirect, possibly through alterations in cytoskeletal structure (16). Further evidence that volume signal transduction does not require MLCK is that duck erythrocytes depleted of calcium (by preincubation in EGTA plus ionophore A23187) respond normally to cell shrinkage.2 A direct role is also unlikely for PKA, since (i) stimulation of cotransport by cell shrinkage and fluoride occurs without a significant increase in cytosolic cAMP (11, 24), (ii) stimulation by phosphatase inhibition with okadaic acid is not associated with increased PKA activity (11), (iii) stimulation by cell shrinkage persists in the presence of the kinase inhibitor K252a at concentrations that abolishes PKA activity (11), and (iv) the Na-K-Cl cotransporter of the shark rectal gland can be activated and phosphorylated in response to cAMP (13) although it lacks a consensus motif for PKA (40).

Although calycin-A evokes greater cotransporter phosphorylation and activity than the other stimuli, this appears to...
reflect a more complete phosphorylation of the same sites that are phosphorylated with the other stimuli. This conclusion is based on the fact that no additional phosphorylation occurs when calcineurin-stimulated cells are exposed to another stimulus, and that phosphopeptide maps of cotransporters phosphorylated by the various stimuli are similar. From these data it can also be concluded that each phosphorylated site is dephosphorylated by a calcineurin-sensitive phosphatase, presumably PP-1. In support of this notion, calyculin-A prevents dephosphorylation completely in vivo as well as in vitro. It is therefore not surprising that, after addition of calcineurin-A, the kinase, now unopposed by the phosphatase, drives cotransporters into the fully phosphorylated state.

An unresolved question is whether phosphorylation of the cotransport protein is both necessary and sufficient for activity. The finding that staurosporine blocks cotransport protein phosphorylation and activation supports the notion that phosphorylation is in fact necessary. Nevertheless, other modes of regulation that do not involve transporter phosphorylation cannot be excluded. Indeed, a recent investigation of the rat parotid gland revealed that some stimulators of cotransport activity (cAMP, AlF4-, and carbamylcholine) evoke prominent increases in cotransport protein phosphorylation, whereas others (cell shrinkage, thapsigargin, and calcineurin-A) do not (41). If such modes of regulation exist, they are not apparent in shark rectal gland cells (13, 18), human colonic T84 cells, or duck erythrocytes (this study) where osmotic shrinkage and calcineurin-A do promote cotransport protein phosphorylation.

In summary, these results suggest that the cotransport protein is phosphorylated at a common set of serine and threonine residues in response to cell shrinkage, cAMP, fluoride, and calcineurin-A. If confirmed by phosphopeptide sequence analysis, these results would obviate the need for multiple kinases acting on disparate sites of the cotransport protein and allow for a more simple model in which cotransport activity depends on the relative rates of one kinase and one phosphatase.

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