The Regulation of Na/K/2Cl Cotransport and Bumetanide Binding in Avian Erythrocytes by Protein Phosphorylation and Dephosphorylation

EFFECTS OF KINASE INHIBITORS AND OKADAIC ACID

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The Na/K/2Cl cotransport system in the avian erythrocyte can be activated by agents that raise intracellular cAMP suggesting the involvement of cAMP-dependent protein kinase (cAMP-PK) in its regulation. Another group of stimuli including fluoride and hypertonicity stimulate cotransport via cAMP-independent means. To further investigate the role of phosphorylation in these processes, we examined the effects of protein kinase inhibitors of 8-(p-Cl-phenylthio)-cAMP (cpt-cAMP), fluoride and hypertonic activation of cotransport in duck red cells, and [3H]bumetanide binding to isolated membranes. Preincubation of cells with the kinase inhibitors K-252a (K. ~ 1.6 μM) and H-9 (K. ~ 100 μM) blocked cpt-cAMP activation of bumetanide-sensitive 86Rb influx and bumetanide binding. These inhibitors also led to a rapid deactivation of cotransport and decrease in bumetanide binding when added to cells maximally stimulated by cpt-cAMP. K-252a and H-9 inhibited cotransport activation by cAMP-independent stimuli, but 10-fold higher concentrations were required, implying the involvement of a cAMP-independent phosphorylation process in the mechanism of action of these agents. Removal of stimuli that elevate cAMP leads to a rapid reversal of cotransport indicating the presence of active protein phosphatases in these cells. The protein phosphatase inhibitor okadaic acid (OA, EC50: 630 nM) stimulated both Na/K/2Cl cotransport and bumetanide binding to membranes. As with fluoride and hypertonic stimulation, the OA effect was inhibited only at relatively high concentrations of K-252a. Phosphorylation of the membrane skeletal protein goblin (M. 230,000) at specific cAMP-dependent sites was used as an in situ marker for the state of activation of cAMP-PK. Goblin phosphorylation at these sites was increased by norepinephrine and cpt-cAMP and rapidly reversed by K-252a and H-9, confirming that both inhibitors do block cAMP-PK activity. While OA markedly increased overall phosphorylation of many erythrocyte membrane proteins, including goblin, it did not affect goblin phosphorylation at specific cAMP-dependent sites. These results implicate a cAMP-independent protein kinase in the mediation of the OA effect on cotransport and bumetanide binding. The bumetanide-binding component of the avian erythrocyte cotransporter, an M ~ 150,000 protein that can be photolabeled with the bumetanide analog [3H]4-benzoyl-5-sulfamoyl-3-(3-thenyloxy)-benzoic acid was found to be a phosphoprotein. These results strongly support the hypothesis that phosphorylation and dephosphorylation, possibly of the Na/K/2Cl cotransporter itself, regulates the activity of the system and suggest that both cAMP-dependent and -independent protein kinases may be involved.

The regulation of Na/K/2Cl cotransport is a tissue-specific phenomenon, and a wide variety of agents affect the activity of this system (for reviews see Palfrey and Rao, 1983; O'Grady et al., 1987, Haas, 1989). The situation is complicated by the fact that the same agent may have opposite effects in different tissues. For example, cyclic nucleotides stimulate cotransport in some tissues (cAMP: duck and turkey erythrocyte and cultured chick heart cells; cGMP: vascular smooth muscle cells) while they inhibit in others (cAMP: vascular endothelial cells, fibroblasts, human and ferret erythrocytes; cGMP: flounder intestine). These observations raise important questions as to the role of protein phosphorylation and dephosphorylation in the control of Na/K/2Cl cotransport. In several types of avian erythrocytes the system is activated by agents that raise intracellular cAMP (e.g. norepinephrine, adenosine, cholera toxin, forskolin, and cAMP analogs; Palfrey and Greengard, 1981). The major receptors for cAMP in avian erythrocytes, as in other mammalian cells, are the regulatory subunits (R1 and R2) of cAMP-dependent protein kinase (cAMP-PK) (Alper et al., 1980b). Occupation of cAMP sites on R1 as well as stimulation of membrane protein phosphorylation have been found to correlate well with α2-adrenergic activation of cotransport in turkey erythrocytes (Alper et al., 1980a, 1980b; Palfrey et al., 1980; Palfrey and Greengard, 1981). Thus, a role for phosphorylation in the regulation of Na/K/2Cl cotransport in these cells, either by direct phos-
phorylation of the cotransport protein or of an associated regulatory protein, was hypothesized (Alper et al., 1980a, 1980b; Palfrey et al., 1980). Cotransport can also be activated by hypertonicity, fluoride, and deoxygenation without an accompanying rise in cellular cAMP levels, suggesting that (a) separate mechanism(s) that are cAMP-independent activate the system (Palfrey and Greenberg, 1981). The role of protein phosphorylation in regulatory phenomena can be probed in intact cells with the use of low molecular weight inhibitors that interact with the nucleotide substrate-binding sites in a number of kinases (Hidaka et al., 1984; Kase et al. 1987). In this report we analyze the effect of these inhibitors on regulation of Na/K/2Cl cotransport by both cAMP-dependent and -independent stimuli.

In comparison with the processes involved in cotransport stimulation little is known about the presumptive dephosphorylation events that may deactivate the system. Blockade of the β-receptor in NE-stimulated cells, or simple removal of cAMP analogs, leads to a reversal of cotransport activation, the rapidity of which differs between avian species (Alper et al., 1980a). The recent discovery of permeant protein phosphatase inhibitors such as okadaic acid (OA, for review see Cohen et al., 1987) provides an important tool for the investigation of dephosphorylative processes in the control of physiological events. In the present report we show that OA by itself is able to activate cotransport in the absence of an elevation in cAMP, thus providing evidence for alternative phosphorylation pathways in the regulation of this system.

The simplest hypothesis to explain the effects of intracellular messengers on Na/K/2Cl cotransport is that components of the transport system itself are subject to reversible phosphorylation. Until recently little evidence was available as to the protein composition of the Na/K/2Cl cotransporter. The introduction of a photoaffinity label based on the potent cotransport inhibitor bumetanide has yielded important clues as to the identity of the diuretic-binding component of the system (Haas and Forbush, 1986, 1988; Haas, 1989). Studies in the avian erythrocyte have shown that this compound specifically labels a protein of M., ~150,000 (Haas and Forbush, 1988). Analogous results have been obtained in other tissues such as mammalian kidney and shark rectal gland (Haas, 1989). The apparent molecular mass of this moiety is compatible with our recent estimates of the size of the mRNA coding for the rat kidney Na/K/2Cl cotransporter based on expression in the Xenopus oocyte system (Burnham et al., 1990) and analysis of the sedimentation behavior of bumetanide-binding proteins in sucrose gradients (Turner and George, 1990). Here, we have taken advantage of the photolabeling technique to directly address whether the bumetanide-binding component of the avian erythrocyte membrane is a phosphoprotein.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Bumetanide (specific activity 27 or 66 Ci/mmol) were the generous gifts of Drs. B. Forbush III (Yale University) and R. James Turner (National Institutes of Health). BSTBA was the gift of Prof. L. F. Feit (Lederle Laboratories, Pearl River, N.Y.). Okadaic acid (OA, for review see Cohen et al., 1987) was the generous gift of Dr. P. de Lanerolle (University of Illinois) or Dr. A. C. Nairn (Rockefeller University) or was obtained from Moana Biochemicals (Honolulu, Hawaii); it was also used as a 500 x stock solution in dimethyl sulfoxide. Wheat germ agglutinin (WGA) and WGA-Sepharose were obtained from Sigma. 8-[3-Chlorophenylthio]-cAMP (cpt-CAMP) was from Boehringer Mannheim. Biotinylated WGA and streptavidin-horseradish peroxidase were from Molecular Probes. Gel electrophoresis reagents and cellulose thin layer chromatography plates were from Eastman Kodak.

**Preparation of Cells, Unidirectional Influx Measurements, Isolation of Membranes, and Determination of [3H]Bumetanide Binding to Isolated Membranes**—These were carried out exactly as described previously (Pewitt et al., 1989). Protein Phosphorylation—Cells were labeled with [32P]orthophosphate, membranes prepared, and analyzed by SDS-PAGE and autoradiography essentially as previously described (Alper et al., 1980a, 1980b). Counts in goblin were determined by liquid scintillation counting of dried gel pieces. One- and two-dimensional phosphopeptide maps of goblin were generated by standard methods (Alper et al., 1980a, 1980b).

**Solubilization of Bumetanide-Receptor Complex**—[3H]Bumetanide (250 nm) was bound to intact NE (0.01 mM)-stimulated duck erythrocytes (10% hematocrit) in “High K” buffer (in mM: 142.5 NaCl, 15 KCl, 10 HEPES, pH 7.4, and 0.2% glucose), and membranes were isolated. The cells were lysed in ice-cold lysis buffer (10 mM HEPES, pH 7.0 and 5 mmol MgCl₂) and membranes prepared as described previously (Pewitt et al., 1990). Subsequent steps were performed rapidly at 0–4°C to minimize dissociation of the [3H]bumetanide receptor complex. The isolated membranes (0.4 mg/ml) were incubated with two volumes of a solution yielding final concentrations of HEPES (20 mM, pH 7.4), KCl (1 mM) and glycerol (20%), Triton X-100 (0.06–1.5%), and solubilized material was isolated by centrifugation at 40 000 g for 30 min. The supernatant was produced by vortexing in lysis buffer and filtered through a GF-B glass fiber filter to determine residual bound [3H]bumetanide (Pewitt et al., 1990). The proteins in the supernatant were precipitated with 0.5 volumes of polyethylene glycol (50% PEG, final concentration 12%) and solubilized. This material was used as the source of the [3H]bumetanide receptor complex.

**RESULTS**

**Protein Kinase Inhibitors Block and Reverse cpt-CAMP Stimulation of Na/K/2Cl Cotransport**—The cAMP analog cpt-cAMP was used to stimulate Na/K/2Cl cotransport directly, thus bypassing receptor-mediated phenomena at the cell surface. The dose-response relationship of cpt-cAMP stimulation of bumetanide-sensitive 86Rb influx into duck red cells showed a half-maximal effect at 0.03 mM and was saturated at 0.5 mM cpt-cAMP (data not shown); the latter concentration was used in subsequent experiments.

The protein kinase inhibitors K-252a (40 μM) and H-9 (500 μM) rapidly reversed cpt-cAMP stimulation of 86Rb influx (Fig. 1). Complete reversal was obtained 5 and 10 min after addition of K-252a and H-9, respectively. cpt-cAMP stimulated 86Rb influx was then measured in erythrocytes preincubated with K-252a in the range of 0.3–30 μM. The data were fit by a rectangular hyperbola yielding an apparent Kₐ value.
on bumetanide-insensitive $^{86}$Rb influx in duck erythrocytes.

Specific effects were noted at concentrations of H-9 in excess of 200 pM, which is more potent than H-9, and some non-specific effects were found to block CAMP-stimulated cotransport with an apparent Ki of -100 pM. K-252a was found to block CAMP-stimulated cotransport with an apparent Ki of -100 pM. At a concentration of 40 pM, K-252a had no effect on bumetanide-insensitive $^{86}$Rb influx and passive K influx.

**Kinase Inhibitors Block the cAMP-mediated Stimulation of $[^{3}H]$Bumetanide Binding to Isolated Red Cell Membranes**—We previously found an excellent correlation between the activation state of Na/K/2Cl cotransport in intact cells and $[^{3}H]$bumetanide binding to isolated membranes from those cells (Pewitt et al., 1990). To examine whether this relationship held in cells treated with kinase inhibitors, measurements of both parameters were made in cells treated with K-252a. cAMP caused a 25-fold increase in $^{86}$Rb influx in these cells and an 11-fold increase in specific $[^{3}H]$bumetanide binding in the isolated membranes, similar to our previous observations made with NE-stimulated cells (Pewitt et al., 1990). Preincubation of cells with K-252a (40 pM) reduced the cAMP stimulation of $^{86}$Rb uptake by >90% and correspondingly reduced the stimulation of specific $[^{3}H]$bumetanide binding in isolated membranes by 83% (Fig. 3). To assess whether the effect of K-252a was at the level of cAMP activation of Na/K/2Cl cotransport rather than the function of the transporter after activation, we tested the ability of the inhibitor to directly modulate $[^{3}H]$bumetanide binding in isolated membranes from stimulated cells. No effect of the compound on this parameter was observed at concentrations up to 40 μM, suggesting that K-252a does not directly interact with the diuretic-binding component of the transport system.

**Kinase Inhibitors Also Block Na/K/2Cl Cotransport Stimulation by cAMP-independent Stimuli**—The effect of kinase inhibitors on two cAMP-independent stimuli of Na/K/2Cl cotransport was tested. In a direct comparison, cotransport was activated by three different agents: cAMP (0.5 mM), hypertonicity (398 mOsm), and NaF (10 mM) in the presence of increasing concentrations of K-252a (0.3–50 μM). The dose-response curves for K-252a inhibition of fluoride and hypertonic stimulation were clearly right-shifted from that obtained with cAMP (Fig. 4). The effect of the kinase inhibitor on either cAMP-independent stimulus was half-maximal at 20–30 μM and virtually complete at 100 μM. A similar differential effect was noted with H-9: at 0.5 mM this compound completely inhibited cAMP stimulation while fluoride and hypertonic stimulation were only slightly affected (data not shown). However, at this concentration of H-9, nonspecific effects (i.e. agglutination and discoloration of the erythrocytes) were noted with H-9.

**Kinase Inhibitors Inhibit Basolateral Sodium and Potassium Transporters**—To assess whether the effect of K-252a was at the level of cAMP activation of Na/K/2Cl cotransport rather than the function of the transporter after activation, we tested the ability of the inhibitor to directly modulate $[^{3}H]$bumetanide binding in isolated membranes from stimulated cells. No effect of the compound on this parameter was observed at concentrations up to 40 μM, suggesting that K-252a does not directly interact with the diuretic-binding component of the transport system.

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phenomenon (Fig. 5). During the course of these experiments, we were surprised to find that this agent was capable of stimulating Na/K/2Cl cotransport activation by cAMP-independent stimuli. Aliquots of erythrocytes (10% hematocrit) were preincubated with K-252a (1–100 μM) for 10 min and then diluted 1:1 into HEPES-buffered saline (Pewitt et al., 1990). 0.2% glucose containing one of the following activators: 0.5 mM cpt-cAMP; 10 mM NaF or hypertonicity (388 mM, i.e. addition of 60 mM sucrose to normal medium). After 10 min at 41 °C, 86Rb influx (1-min uptake) was determined for each condition. The results are expressed as percent maximal bumetanide-sensitive 86Rb influx for each stimulus. The maximum values of stimulated 86Rb influx for cpt-cAMP, fluoride, and hypertonic treatments in this experiment were 160, 135, and 116 nmol K equivalents liter−1 • h−1, respectively. The error bars indicate the standard deviation of triplicate samples.

To test the possibility of protein phosphatase activity in Na/K/2Cl cotransport regulation, the ability of OA to prevent the reversal of NE-stimulated cotransport by propranolol (Alper et al., 1979; Pewitt et al., 1990) was tested. As expected, OA pretreatment completely prevented the propranolol reversal phenomenon (Fig. 5). During the course of these experiments, however, it was noted that addition of OA to NE-stimulated cells always caused a further increase in cotransport rate. When the effect of OA (1 μM) alone on 86Rb influx was tested, we were surprised to find that this agent was capable of stimulating cotransport to a level even higher than that of NE or cpt-cAMP. The time course of OA stimulation of bumetanide-sensitive 86Rb influx is shown in Fig. 6A; after an initial lag of 1 min, a maximum rate was reached 4–5 min after OA addition, comparable to the time course of stimulation by other agents (cf. Pewitt et al., 1990). The dose-response relationship yielded half-maximal stimulation of cotransport at 0.25 μM (Fig. 6B). OA stimulation was completely inhibited by bumetanide (10 μM), and the compound had no effect on bumetanide-insensitive 86Rb influx at concentrations up to 5 μM.

If the effect of OA on cotransport is due to inhibition of protein phosphatase activity, it seems reasonable to assume that the relevant kinase might be susceptible to inhibition by K-252a and H-9. Hence, we tested the effect of preincubation with these agents on OA stimulation of 86Rb influx. Both compounds were able to block cotransport activation by OA. The dose-response curve for K-252a is shown in Fig. 7; half-maximal inhibition was seen at ~40 μM, slightly greater than that seen with fluoride or hypertonic stimulation. This puts OA nominally into that group of cotransport activators that are cAMP-independent, a classification supported by the phosphorylation data reported below.

The effect of OA on [3H]bumetanide binding to isolated membranes was also tested (not shown). In these experiments, membranes from OA-treated cells showed 0.42 pmol/mg protein specific [3H]bumetanide binding, while those isolated from cpt-cAMP-stimulated cells contained 0.89 pmol/mg protein. The lower specific binding in the case of OA, despite maximal activation of cotransport, appears to be due to increased lability of stimulated bumetanide binding in these cells during the membrane isolation procedure, and is currently being studied further.

Phosphorylation of Goblin in Intact Erythrocytes—To obtain a readout of the state of membrane phosphorylation under the different conditions used in this study, particularly with respect to the activation of cAMP-PK, we investigated the phosphorylation of the membrane skeletal protein goblin. This protein is immunologically related to human erythrocyte ankyrin (Moon and Lazarides, 1984) and is found in all avian species thus far studied.2 As described previously, avian erythrocyte goblin has several phosphorylation sites, many of which are cAMP-independent (Alper et al., 1986b). Thus, it is necessary to use various peptide mapping techniques to accurately determine the effects of various agents on phosphorylation at cAMP-PK-specific sites. In agreement with earlier findings (Alper et al., 1986b), when the phosphorylation state of intact goblin was quantitated in the present experiments only marginal effects of cAMP or kinase inhibitors was detected (Fig. 8A). In contrast, OA had a dramatic effect on the phosphorylation of goblin (~3-fold increase) as well as several other membrane proteins (Fig. 8A). When goblin was proteolyzed using Staphylococcus aureus V8 protease a prominent M, ~37,000 peptide whose phosphorylation was stimulated by cpt-cAMP and inhibited by K-252a and H-9 was found (Fig. 8B; cf. Alper et al., 1986a; Palfrey et al., 1980). OA also had a

2 H. C. Palfrey, unpublished observations.
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A large stimulatory effect on the phosphorylation of this peptide, but as previously reported (Palfrey and Greengard, 1981), NaF was without effect.

A more complete resolution of the CAMP-PK-specific sites of goblin can be achieved by tryptic phosphopeptide fingerprinting (Alper et al., 1980b). In agreement with results on turkey erythrocyte goblin, CAMP elevation in duck erythrocytes led to the phosphorylation of two major tryptic peptides in goblin (labeled a and b in Fig. 8C) that are well-resolved from other phosphorylated peptides. Phosphorylation of peptides a and b was rapidly reversed by treatment of cpt-CAMP-stimulated cells with either K-252a or H-9 (not shown). Fingerprinting revealed that while OA led to increased phosphorylation of several phosphopeptides it did not stimulate the phosphorylation of CAMP-PK sites in goblin (Fig. 8C). Therefore, the elevation in phosphorylation of the M, ~37,000 peptide (from V8 protease digestion) by OA must occur on CAMP-PK-independent sites.

Identification of a PHIBSTBA- and WGA-binding Phosphoprotein—WGA reduces bumetanide-sensitive 86Rb influx in the avian erythrocyte suggesting that this lectin reacts with some component associated with the Na/K/2Cl cotransporter. Moreover, the solubilized mouse kidney Na/K/2Cl cotransporter is retained by a WGA affinity column.3 To examine the possibility that the avian erythrocyte cotransporter binds directly to WGA, the diuretic-binding component was labeled in intact cells with [3H]bumetanide, solubilized, and applied to a WGA-Sepharose affinity column. NE-treated duck erythrocytes were incubated with [3H]bumetanide, and membranes were isolated, working quickly at 0 °C to minimize dissociation of the [3H]bumetanide-receptor complex. To ascertain optimal conditions for solubilization, membranes were treated with increasing concentrations of Triton X-100 (0–1.8%) in a high salt buffer and bumetan-
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FIG. 8. Phosphorylation of goblin in response to activators and inhibitors. Erythrocytes were prelabeled with [³²P]orthophosphate (0.5 mCi/ml) for 8 h, then incubated for 10 min in 0.5 mM cpt-cAMP, 10 mM NaF, or 2 μM OA. Aliquots of cpt-cAMP-treated cells were further treated for 10 min with K-252a (20 μM) or H-9 (200 μM). After rapidly washing cells at 4 °C to eliminate extracellular label, membranes were isolated as described (Pewitt et al., 1990). A, autoradiograph of 7.5% SDS PAGE of membrane proteins (20 μg) from each condition. ³²P incorporation into the goblin band in this experiment (as determined by scintillation counting) was (in cpm): control (2033); cpt-CAMP (2387); cpt-CAMP + K252a (1846); cpt-CAMP + H-9 (1542); OA (6284); NaF (2288). The experiment was repeated twice with similar results. B, goblin was excised from the gel shown in A and subjected to limited digestion with S. aureus V8 protease (0.5 μg/lane) and the peptides were separated by 15% SDS PAGE and visualized by autoradiography. The position of the prominent 37-kDa peptide is indicated. C, goblin was excised from gels similar to those shown in A and subjected to limit trypsin digestion (50 μg/ml for 24 h at 37 °C). The resultant peptides were separated on 20 × 20-cm cellulose plates in two dimensions by sequential electrophoresis at pH 3.5 (horizontal dimension) and chromatography (vertical dimension) (Alper et al., 1980b). The phosphorylation of the peptides marked α and β is stimulated by cAMP but not OA. However, cAMP-independent peptides show increased incorporation of ³²P with OA (e.g. arrows).

ide-protein complexes were identified in the 150,000 × g supernatant by PEG precipitation. 0.06% Triton X-100 proved to be the optimal concentration of this detergent for solubilization of the intact complex (Fig. 9A) and was used in subsequent experiments (cf. Turner and George, 1990). Once solubilized, the complex dissociated with a t₁/₂ ~4 h at 0 °C (not shown), allowing sufficient time to chromatograph the extract on WGA-Sepharose. The complex was specifically
eluted from such an affinity column by N-acetylglucosamine (Fig. 9B). These results indicate that the bumetanide-binding complex of the avian erythrocyte Na/K/2Cl cotransporter binds WGA and is most likely a glycoprotein.

To ascertain if the bumetanide-binding component itself interacted with the lectin, WGA-binding proteins present in duck erythrocyte membranes were first identified on nitrocellulose blots of SDS-PAGE-separated material. WGA was found to label two species in the duck erythrocyte membrane, an upper fainter band of $M_r \sim 152,000$ and a lower stronger band of $M_r \sim 140,000$ (Fig. 10A). To assess whether either band comigrated with a bumetanide-binding protein, $[^3H]$BSTBA-labeled membranes were separated by SDS-PAGE, transferred to nitrocellulose, and then stained with WGA as described above. The WGA-stained blot was cut into strips which were counted. Two regions were found to incorporate significant amounts of $[^3H]$BSTBA in agreement with the results of Haas and Forbus (1988). The lower $M_r$ material was previously found to be poorly competed by unlabeled bumetanide and may represent nonspecific labeling while labeling of the $M_r \sim 150,000$ protein had the typical characteristics of binding to the Na/K/2Cl cotransporter, including ion dependence. As shown in Fig. 104, the higher $M_r$ BSTBA-labeled material corresponded to the upper band of the WGA staining doublet.

![Graph](image)

**Fig. 9. Interaction of the solubilized $[^3H]$bumetanide-receptor complex with WGA-Sepharose.** A, optimal solubilization of the reversible bumetanide-protein complex achieved at 0.06% Triton X-100. $[^3H]$Bumetanide bound in the pellet and PEG-precipitable counts in the supernatant were determined following Triton X-100 solubilization and ultracentrifugation. B, after ultracentrifugation, the soluble bumetanide-protein complex was diluted 1:1 in buffer without Triton X-100 or salt and chromatographed over a WGA-Sepharose column (1 ml) at 4°C. The column was washed extensively then eluted with N-acetylglucosamine (NAGA, 0.2 M). Aliquots of each fraction (1 ml) were PEG-precipitated, filtered, and scintillation-counted.

We then sought to address whether the $M_r \sim 152,000$ WGA-binding protein was also a phosphoprotein. $[^32P]$-Labeled membrane proteins were separated by two-dimensional electrophoresis, followed by blotting to nitrocellulose, staining with WGA and autoradiography (Fig. 10B). A phosphorylated protein located on the autoradiogram was found to exactly overlay the higher $M_r$, more acidic PI of the two WGA-stained proteins, corresponding to the lighter staining band in the one-dimensional gel that incorporated $[^3H]$BSTBA. This constitutes strong preliminary evidence that the WGA- and bumetanide-binding component of the Na/K/2Cl cotransporter is a phosphoprotein.

**DISCUSSION**

The modulatory role of protein phosphorylation in the regulation of many membrane transport systems, particularly ion channels, has become increasingly apparent over the last several years (e.g. Levitan, 1988). In only very few cases have the mechanisms and sites of action of kinases on transporters been elucidated. With less well-characterized systems such as the Na/K/2Cl cotransporter, although the occurrence of regulatory phosphorylation events is suspected, the molecular mechanisms are poorly defined. In particular, the ability of a variety of hormones and growth factors to both positively and negatively affect cotransport is not understood. The present work employing permeant kinase and phosphatase inhibitors lends support to the contention that protein phosphorylation and dephosphorylation is crucial to the activation/deactivation processes governing cotransport activity in avian erythrocytes. The fact that both of the kinase inhibitors used here could block ctp-cAMP activation of Na/K/2Cl cotransport confirms the notion that cAMP activation of Na/K/2Cl cotransport is mediated through cAMP-dependent phosphorylation (Alper et al., 1980a, 1980b; Palfrey et al., 1980; Palfrey and Greengard, 1981). K-252a inhibits cyclic nucleotide-dependent protein kinases, protein kinase C, and myosin light chain kinase with $K_m$ values of between 18 and 25 nM in vitro at an [ATP] of 5 μM (Kase et al., 1987). Subsequent studies of myosin light chain showed that this enzyme was half-maximally inhibited by 3 μM K-252a in the presence of 2 mM ATP (Nakano et al., 1988), reflecting the competitive interaction of this inhibitor with ATP. As the [ATP] in the avian erythrocyte is reported to be 2–5 mM (Allen, 1967; Palfrey and Rao, 1983) it is not surprising that 1–5 μM K-252a was needed to half-maximally inhibit cAMP-stimulated cotransport. Similar considerations apply to the unrelated compound H-9; it also inhibits several kinases by competing with ATP, although the $K_m$ values found with various enzymes in vitro are one to two orders of magnitude higher than those of K-252a (Hidaka et al., 1984). Consequently, high micromolar concentrations of H-9 were needed to block or reverse cotransport stimulation. A related compound, H-8, was previously used to block cyclic nucleotide effects on Na/K/2Cl cotransport in flounder intestine (O'Grady et al., 1988).

Activation of Na/K/2Cl cotransport by the cAMP-dependent agents fluoride and hypertonicity was also blocked by both kinase inhibitors. However, 10-fold higher concentrations of K-252a were needed than that required to inhibit ctp-cAMP stimulation. Several lines of evidence indicate that fluoride and hypertonicity are not acting via a cAMP-dependent protein phosphorylation process, the most important of which are that they neither raise cAMP nor stimulate cAMP-dependent protein phosphorylation (Palfrey and Greengard, 1981). Previous results with the cross-linking reagent diamide also showed a clear distinction between inhibition of cotransport activated by cAMP-elevating agents and cAMP-independent stimuli (Palfrey et al., 1980). As yet, no explanation for the effects of the cAMP-independent class of stimuli has emerged; indeed, members of this class might activate cotransport by different mechanisms. On the basis of the fact that K-252a inhibited both hypertonic- and fluoride-stimulated cotransport with approximately the same $K_m$ value, it is possible to speculate that some non-cAMP-mediated protein phosphorylation event is involved in the activation mecha-
Fig. 10. Identification of a bumetanide- and BSTBA-labeled species as a WGA-binding protein and a phosphoprotein in duck erythrocyte membranes. A, [3H]BSTBA and WGA double-labeling of duck erythrocyte membranes. Duck erythrocytes were incubated with NE (10^{-5} M) in the presence of 0.3 pM [3H]BSTBA for 15 min. The cells were then lysed, centrifuged, and the resultant ghosts photolyzed at 0°C for 20 min (Haas and Forbush, 1988). Membranes were then prepared and proteins separated by 5.5% SDS PAGE. Following transfer to nitrocellulose paper, the blot was developed with WGA-biotin/streptavidin-horseradish peroxidase, xeroxed, and cut into 4-mm strips which were then counted (upper panel). Slice 14 corresponds to a peak of [3H]BSTBA incorporation and to the upper band (M, 152,000) of a WGA-staining doublet. A photograph of a WGA-stained blot of membranes run on 7.5% SDS-PAGE is included for comparison with the xeroxed strip (lower panel). B, WGA and 32P double-labeling of duck erythrocyte membranes. Duck erythrocytes were prelabeled with [32P]orthophosphate (1 mCi/ml), stimulated with NE (10 min), and membranes isolated as described under "Experimental Procedures." The membrane proteins were separated by two-dimensional isoelectric focusing, 7.5% SDS-PAGE and transferred to nitrocellulose. The blot was developed with WGA-biotin/streptavidin-horseradish peroxidase and autoradiographed. The portion of the WGA blot corresponding to the boxed portion of the autoradiogram is shown in the inset. The faint upper M, 152,000 WGA-stained spot (p1 - 5) corresponds to a minor phosphoprotein (downward arrows), the M, 140,000 WGA-binding protein incorporates negligible 32P under these conditions (sideways arrows); a slightly lower M, prominent phosphoprotein was not stained by WGA (upward arrows). The M, 152,000 and 140,000 WGA-stained proteins were less well-separated on this gel due to heavier loading. The most heavily 32P-labeled proteins above and below the boxed area are goblin (M, = 230,000) and band 3 (M, = 100,000).

Changes in cell volume are known to produce alterations in membrane transport systems in a variety of cell types, but little progress has been made in deciphering transduction events in response to tonicity. Recently, a proposal that the mechanism by which hypertonic cell shrinkage activates Na/K/2Cl cotransport is involved changes in intracellular free [Mg^{2+}] has been made (Starke and McManus, 1990). In duck red cells, both hypertonic shrinkage and hypoxia (another cotransport stimulus) increased cytosolic [Mg^{2+}] and elevation in cellular [Mg^{2+}] using A23187 was found to stimulate cotransport. Moreover, raised cytosolic [Mg^{2+}] appears to decrease the degree of cell shrinkage required for cotransport activation, perhaps by shifting the volume "set point" for such activation (Starke and McManus, 1990). It is possible that these effects of Mg^{2+} are mediated through protein phosphorylation, as Mg^{2+} is essential for kinase activity and variable effects of the cation on the activities of different protein kinases (e.g. Hallenbeck and Walsh, 1983) and phosphatases (e.g. Ballou and Fischer, 1986) have been reported. Another
observation of relevance to the possible involvement of protein kinase in the mechanism of hypertonic activation in the present case are the results of Grinstein et al. (1986) in shrunken lymphocytes. There, a non-protein kinase C-dependent phosphorylation process was implicated in the volume recovery mechanism exhibited by those cells.

The effect of protein kinase antagonists on cotransport was paralleled by their inhibition of \( ^{3}H \)bumetanide binding to membranes isolated from cpt-cAMP-stimulated cells. We have previously shown that there is an excellent correlation between activation and deactivation of cotransport in intact duck erythrocytes and diuretic binding to isolated membranes from these cells (Pewitt et al., 1990). This relationship was found to hold true with the kinase inhibitors studied here. K-252a had no effect on bumetanide binding in isolated membranes, indicating that it does not interact with the cotransporter itself. These data are consistent with the idea that kinase inhibitors block the activation of the Na/K/2Cl cotransporter and do not affect its transport function.

The observation that OA alone activates Na/K/2Cl cotransport in avian erythrocytes indicates that a dynamic equilibrium exists in the quiescent cell between a phosphorylated and dephosphorylated state of a phosphoprotein associated with Na/K/2Cl cotransport activity. The rapidity of OA stimulation suggests that in the unstimulated erythrocyte there is a high turnover rate of the protein-bound phosphate groups relevant to cotransport. Such an interpretation is supported by the rapid effect of kinase inhibitors to reverse cotransport in stimulated cells, as discussed above. The effect of OA is not mediated by blocking dephosphorylation of protein(s) normally phosphorylated by cAMP-PK. One possible scenario, that a low level of free cAMP-PK catalytic subunit exists in the cell whose influence on cotransport in the absence of hormonal stimulation is normally insignificant but can be amplified by prevention of dephosphorylation, does not seem tenable. First, the effects of OA on cotransport were as rapid as those of NE or cpt-cAMP and the maximal effect actually exceeded those elicited by cAMP-dependent agents. If a low level of catalytic subunit were present then build-up of phosphate on the relevant protein should be slow. Second, inhibition of the OA response by K-252a occurred at higher concentrations of the kinase inhibitor than those affecting cAMP-PK catalytic activity. Third, direct examination of cAMP-specific phosphorylation sites on goblin by tryptic fingerprinting revealed no effect of OA, despite a large increase in the overall phosphorylation of this protein. Tentatively, we hypothesize that a cAMP-independent kinase may phosphorylate the cotransporter and/or an associated regulatory protein and that it is prevention of dephosphorylation of these sites by OA that is responsible for its effects on cotransport. Whether this could be the same kinase proposed to mediate the effects of NaF and hypertonicity is not clear, but the similarity in the K-252a dose-inhibition curves suggests that this may be so. The nature of this putative kinase is unknown; however, it is unlikely to be a calmodulin-dependent enzyme or protein kinase C, both of which are present in duck erythrocytes, as neither Ca ionophores nor phorbol esters stimulate Na/K/2Cl cotransport in these cells.

OA is a polyether fatty acid that selectively inhibits the catalytic activity of protein phosphatases-1 and -2A while having little effect on other phosphates and no effect on several kinases (Bialojan and Takai, 1988; Haystead et al., 1989). When isolated hepatocytes or adipocytes are incubated with OA there is marked increase in \(^{32}P\)-labeling of cellular proteins, with a half-maximal effect at 200 nM and a maximal effect at 1 \( \mu \)M (Haystead et al., 1989; see Cohen et al., 1990, for review). Interestingly, addition of OA alone to adipocytes is capable of stimulating glucose transport to a level equivalent to that induced by insulin, paralleling observations in the present work. The varying IC\(_{50}\) values for different phosphatases allows OA to be used to distinguish between these activities in cell-free systems, but in whole cells complications can arise in differentiating between the effect of OA on protein phosphatases-1 and -2A. For example, the intracellular concentration of these phosphatases may approach micromolar levels in some tissues (Cohen, 1989). As the inhibition by OA is stoichiometric, the EC\(_{50}\) value of 630 nM found in the present work implicates protein phosphatase-1 and/or -2A as primary candidates in mediating the OA response. In preliminary experiments, we have attempted to further delineate the role of phosphatase activity in cotransport regulation by assessing the effects of exogenous catalytic subunit of protein phosphatase-2A on \(^{3}H\)bumetanide binding activity. Our preliminary results indicate that incubation of membranes possessing high binding activity with this enzyme results in a rapid decline in binding that is pyrophosphate-inhibitable. We are currently assessing the role of endogenous duck erythrocyte phosphatases in regulating the cotransporter.

Thus far it has not been possible to analyze the effects of agents that regulate Na/K/2Cl cotransport at the level of the cotransport protein itself. Purification and reconstitution of this cotransport system has yet to be achieved; but recent work using the photoaffinity probe BSTBA suggests that a \( M_\text{r} \sim 150,000 \) protein is the diuretic-binding component (Haas, 1989). In the present work we have found that a bumetanide-binding species of the duck erythrocyte membrane binds WGA and that a \( M_\text{r} \sim 152,000 \) WGA-binding protein of these membranes is labeled by BSTBA. The property of WGA binding was then utilized to show that this protein also incorporated \(^{32}P\) in NE-stimulated cells. The results suggest that the diuretic-binding component of the Na/K/2Cl cotransporter is a glycoprotein that is itself phosphorylated, and one may speculate that phosphorylation alters the conformation of the molecule to an active form. Because of the low abundance of this protein in the duck erythrocyte membrane (see also Haas and Forbush, 1988) it has not been possible as yet to quantitate its phosphorylation in response to stimulation, but the application of affinity chromatographic or antibody-based techniques should allow us to conduct such studies in the near future.

REFERENCES


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