Na\(_2\)K:2Cl Cotransporter (NKCC) of Intestinal Epithelial Cells

SURFACE EXPRESSION IN RESPONSE TO cAMP

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During intestinal chloride secretion, epithelial uptake of salts is accomplished largely by a bumetanide-sensitive Na\(_2\)K:2Cl cotransporter designated here as NKCC. Using monoclonal antibodies directed against NKCC from the human crypt epithelial cell line, T84, we define its surface localization as a function of cotransporter activation. Immunochemistry, confocal localization, and selective surface biotinylation studies revealed that the 195-kDa NKCC protein is polarized to the basolateral domain. Following immunoprecipitation, several polypeptides coprecipitated with the 195-kDa cotransporter including two prominent proteins of molecular mass 160 and 130 kDa. Immunoblotting with three distinct anti-NKCC monoclonal antibodies in conjunction with deglycosylation experiments suggested that the 160- and 130-kDa bands represented novel proteins unrelated to the cotransporter. Stimulation of T84 monolayers with cAMP agonists, a condition which elicits chloride secretion and leads to microfilament-dependent NKCC activation, did not significantly increase the number of bumetanide-binding sites and only marginally increased surface expression of the 195-kDa cotransporter available for surface biotinylation. In contrast, cAMP agonist stimulation increased the surface expression of the coprecipitating 160- and 130-kDa proteins ~6-fold. The increase in surface 160- and 130-kDa proteins was attenuated by phalloidin preloading the cells, a condition which also prevents activation of NKCC without influencing the activity of other membrane transporters participating in chloride secretion. These studies define the polarized distribution of the NKCC protein on intestinal epithelia, indicate that NKCC may be associated with two other previously unidentified membrane proteins and such association is influenced by the F-actin cytoskeleton.

Mucosal surfaces, such as the lining of the intestine, maintain hydration in part as a result of the ion transport process known as electrogenic Cl\(^-\) secretion. Important human diseases result when the ability to mount Cl\(^-\) secretion is either impaired (i.e. cystic fibrosis) or enhanced (i.e. secretory diarrhea). Cl\(^-\) secretion represents the orchestrated activities of four membrane transport events. Basolateral ion uptake is largely accounted for by the bumetanide-sensitive Na\(_2\)K:2Cl cotransporter, designated in this report as NKCC. Human intestinal NKCC relates to a recently cloned 195-kDa glycoprotein with 12 predicted transmembrane domains (1). When stimulated by hormones such as VIP or by cAMP agonists, NKCC activity is up-regulated allowing for the electroneutral influx of a 1:1:2 ratio of sodium, potassium, and chloride ions in an electroneutral fashion (for recent review, see Ref. 2). Relative homeostasis of intracellular Na\(^+\) and K\(^+\) is achieved by the presence on the basolateral membrane of both the Na-K-ATPase pump and a channel-like K\(^+\) efflux pathway. Regulated opening of Cl\(^-\) channels on the apical membrane in the presence of the electrochemical gradient favoring Cl\(^-\) exit permits vectorial secretory movement of this anion which is coupled to paracellular Na\(^+\) and water movement resulting in net secretion of isotonic fluid (3, 4).

Several recent studies of cAMP-mediated Cl\(^-\) secretion have focused on discerning mechanisms by which the apical Cl\(^-\) channel is regulated. However, in order to sustain Cl\(^-\) secretion, basolateral Cl\(^-\) uptake through NKCC must increase in parallel with Cl\(^-\) channel activation (5). NKCC is complexly regulated in that cotransport activity responds to additional influences other than chemical potential. For example, the activity of NKCC has been shown to positively correlate with serine/threonine phosphorylation of the corresponding protein in shark rectal gland (6). In many, but not all tissues, cAMP-mediated activation of NKCC has been shown to be accompanied by increased binding for the radiolabeled ligand bumetanide. It is not clear whether such bumetanide binding increments simply reflect activation of existing cell surface cotransporters or actual recruitment of additional cotransporter units to the cell surface.

Using T84 cells, a human intestinal epithelial model, we have previously shown that activation of NKCC, which parallels cAMP-mediated Cl\(^-\) secretion, also occurs in conjunction with reordering of basolateral F-actin microfilaments (7). Stabilization of F-actin with phalloidin prevented both microfilament reordering and activation of basolateral NKCC. This effect was shown to be specific for the cotransporter since phalloidin loading did not adversely affect either Na-K-ATPase activity or the regulated efflux of Cl\(^-\) and K\(^+\) through their respective channels (8). Studies in HT29 cells (a human intestinal epithelial cell line that lacks a cAMP-mediated chloride efflux pathway) and its subclone, Cl.19A cells (possessing a functional cAMP-regulated chloride efflux pathway), have yielded further insights into NKCC activation. These studies indicated that NKCC activity was up-regulated in both cell lines by cAMP; however, an increase in bumetanide-binding
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sites on the cell surface occurs only in the subclone, Cl.19A (9). Surprisingly, however, the cAMP-elicted increase in bumetanide binding observed in clone Cl.19A cells is unaffected when F-actin is stabilized even though such prevention of cytoskeletal reordering results in a ~60% attenuation of functional measures of NKCC activation. The studies conducted on the HT29 cell line further indicated that exposure to cAMP agonists increases the activity of NKCC by a mechanism which is independent of Cl efflux, only minimally reduced by phallolidin, and is not associated with an increase in bumetanide binding. Thus it appears that two regulatory responses may exist: (a) that associated with Cl efflux and dependent on F-actin rearrangement and (b) that which is independent of chloride efflux and not associated with changes in F-actin organization. The relationship of cAMP elicted changes in bumetanide binding to cAMP-elicted cytoskeletal remodeling in these studies remains unsettled, although such observations unmask the complexity of regulatory influences on this key salt uptake process, and in doing so highlight the need for studies aimed more directly at assessing the level of surface expression of the cotransporter during stimulated secretion.

Here we employ monoclonal antibodies raised against NKCC derived from the human intestinal epithelial cell line T84 for studies of the cell surface expression of the corresponding ~195-kDa protein under conditions in which the activity of the cotransporter is stimulated by cAMP agonists. By confocal and electron microscopic immunolocalization as well as selective surface biotinylation, the 195-kDa protein is shown to be distinctly polarized to the basolateral domain of T84 cells. Surface biotinylation experiments identified two prominent, previously unidentified proteins of ~160 and ~130 kDa that consistently coprecipitate with the cotransporter. The coprecipitating proteins also represent integral membrane proteins since they possess external residues available for surface biotinylation. Basolateral surface biotinylation experiments suggest that expression of the 160- and 130-kDa proteins is increased severalfold upon stimulation with the cAMP agonist forskolin while expression of the 195-kDa cotransporter protein, examined by both surface biotinylation and bumetanide binding, is only modestly influenced. Last, phallolidin loading which prevents both the cAMP-elicted activation of the cotransporter as well as the concurrent reordering of the basolateral cytoskeletal F-actin, also prevents the forskolin-elicted increase in surface expression of the 160- and 130-kDa co-precipitating proteins. These data suggest that the cotransporter may associate with two distinct integral membrane proteins and that such associated proteins may participate in the regulation of this key salt uptake pathway.

MATERIALS AND METHODS

Cell Culture

T84 cells, a human colonic carcinoma cell line that functionally and morphologically resembles crypt intestinal epithelium (10, 11) were grown as confluent monolayers in a 1:1 mixture of Dulbecco-Vogt modified Eagle’s media and Hank’s balanced salt solution (HBSS). Sigma, without phenol red or bicarbonate, plus 10 mM HEPES, pH 7.4) warmed to 37 °C, and transferred to a new 24-well tissue culture plate containing fresh HBSS. To determine currents, transepithelial potential, and resistance a commercial voltage clamp (Bioengineering Department, University of Iowa) was interfaced with an equilibrated pair of calomel electrodes submerged in saturated KCl and a pair of Ag-AgCl electrodes submerged in HBSS. For electrical determinations, one calomel and one Ag-AgCl electrode is placed on each side of the monolayer and a pulse of 25 μA of current is passed across the monolayer. Using Ohm’s Law (V = IR), the resultant voltage deflection and the transepithelial resistance (R) can be calculated. The short circuit current is (Isc), which represents the total amount of external current that is required to nullify the cell’s active ion transport across the epithelium, can also be determined by Ohm’s Law.

[3H]Bumetanide Binding

Binding of [3H]bumetanide was carried out as described previously (9). Briefly, [3H]bumetanide (Amersham Corp.) was purified by high performance liquid chromatography using a Microsorb C-18 semi-preparative column (Ranin, Woburn, MA) using a protocol developed by Dr. Mark Haas (University of Chicago) to yield a 10 μM stock in ethanol with a specific activity of 81 Ci/mmol. Such purification was found to be essential in order to reduce nonspecific binding. T84 cells were grown on 1-cm² permeable filters as described above and incubated with [3H]bumetanide for 60 min at 37 °C. Cells were then excised from the inserts and radioactivity measured by liquid scintillation.

86Rb Uptake

Functional activation of the basolateral NKCC cotransporter was assessed as bumetanide-inhibitable 86Rb uptake, with bumetanide (10 μM) serving as a specific inhibitor of cotransport and 86Rb as a tracer for K⁺ as described previously (8). Briefly, monolayers grown on 1-cm² supports were equilibrated in buffer for 20 min and then transferred to wells containing fresh buffer with or without bumetanide (10 μM) and with or without forskolin for 5 min. Uptakes were initiated by transferring monolayers to an uptake buffer identical to the preincubation buffer also containing 1.5 μM stock in ethanol. The filters were then sharply excised from the inserts and radioactivity measured by liquid scintillation. The bumetanide sensitive component of uptake, expressed as nmoL of K⁺ mg protein⁻¹ min⁻¹, was derived by subtracting individual values of uptake measured in the absence of bumetanide from the mean value of the uptakes measured in the presence of bumetanide. Proteins were determined from representative monolayers using a spectrophotometric bicinchoninic acid assay from Pierce (Rockford, IL).

Antibodies Against T84 Na⁺:K⁺:Cl⁻ Cotransporter

A battery of monoclonal antibodies were generated against the T84 cell NKCC cotransporter (12). In this study, we used monoclonal antibodies T9, T4, and T10 that were purified from ascites fluid by a 50% ammonium sulfate precipitation.

Biotinylation and Immunoprecipitation

Surface biotinylation of T84 membranes was carried out as described previously (13). Briefly, T84 cells grown on 5-cm² supports were equilibrated in buffer for 20 min and then transferred to wells containing fresh buffer with or without bumetanide (10 μM) and with or without forskolin for 5 min. Uptakes were initiated by transferring monolayers to an uptake buffer identical to the preincubation buffer also containing 1.5 μM stock in ethanol. The filters were then sharply excised from the inserts and radioactivity measured by liquid scintillation. The bumetanide sensitive component of uptake, expressed as nmoL of K⁺ mg protein⁻¹ min⁻¹, was derived by subtracting individual values of uptake measured in the absence of bumetanide from the mean value of the uptakes measured in the presence of bumetanide. Proteins were determined from representative monolayers using a spectrophotometric bicinchoninic acid assay from Pierce (Rockford, IL).

Electrophysiology

Electrophysiological studies were carried out at 37 °C on T84 cells grown to confluency on 24-well Costar inserts used 7–14 days post-plating. The inserts consist of an outer well or chamber that corresponds to the basolateral surface of the monolayer and an inner chamber that is in contact with the apical surface. Inserts are washed with Hank’s balanced salt solution (HBSS), Sigma, without phenol red or bicarbonate, plus 10 mM HEPES, pH 7.4) warmed to 37 °C, and transferred to a new 24-well tissue culture plate containing fresh HBSS. To determine currents, transepithelial potential, and resistance a commercial voltage clamp (Bioengineering Department, University of Iowa) was interfaced with an equilibrated pair of calomel electrodes submerged in saturated KCl and a pair of Ag-AgCl electrodes submerged in HBSS. For electrical determinations, one calomel and one Ag-AgCl electrode is placed on each side of the monolayer and a pulse of 25 μA of current is passed across the monolayer. Using Ohm’s Law (V = IR), the resultant voltage deflection and the transepithelial resistance (R) can be calculated. The short circuit current is (Isc), which represents the total amount of external current that is required to nullify the cell’s active ion transport across the epithelium, can also be determined by Ohm’s Law.

1 The abbreviations used are: HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline.
chymostatin, 10 μM leupeptin, 1 μM pepstatin, 1 μM bestatin. Cells from three 10 cm dishes were pelleted out by centrifugation at 1000 × g for 10 min. All subsequent steps were carried out at 4°C. Pellets were resuspended in 600 μL of the same buffer containing 2% Triton X-100 and rapidly vortexed. The extract (approximately 2.5 × 10^7 cell equivalents/ml) was then centrifuged at 1000 × g for 10 min to remove nuclei and large particulates. The resultant supernatant containing solubilized T84 cell biotinylated membranes was then incubated for 1 h with constant rocking in protein A-Sepharose (Sigma) to preclear the solution of any nonspecific binding. Protein A-Sepharose was pelleted out by centrifugation in a microcentrifuge for 10 s at 10,000 × g. The resultant supernatant was incubated overnight with 2–10 μg of T9 monoclonal antibody. Antibody-antigen complexes were isolated from the extract by incubation with protein A-Sepharose for 2.5 h. The antibody-antigen/protein A-Sepharose complex was microcentrifuged and repeatedly washed (5–6 times) with immunoprecipitation wash buffer (10 mM NaH2PO4, pH 7.4, 1% Nonidet P-40, 0.4 M NaCl, 2 mM EDTA, 0.1 M sodium fluoride, 1 mM benzamidine, 10 μM chymostatin, 10 μM leupeptin, 1 μM pepstatin, 1 μM bestatin). The antibody-antigen complex was separated from protein A-Sepharose by boiling for 5–6 min in reducing sample buffer (2.5% SDS, 5% β-mercaptoethanol, 25 mM Tris-HCl, pH 6.8, 20% glycerol, 0.01% bromphenol blue) followed by a 30-s microcentrifugation. The final supernatant containing the biotinylated, immunoprecipitated protein was run on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described below. Nitrocellulose filters were blocked overnight at 4°C in 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.4), 0.2% Triton X-100, 0.1% gelatin, followed by a 1-h incubation at room temperature in horseradish peroxidase-conjugated avidin (Pierce) diluted 1:10,000. The antibody-antigen complex was separated from protein A-Sepharose by boiling for 5–6 min in reducing sample buffer (2.5% SDS, 5% β-mercaptoethanol, 25 mM Tris-HCl, pH 6.8, 20% glycerol, 0.01% bromphenol blue) followed by a 30-s microcentrifugation. The final supernatant containing the biotinylated, immunoprecipitated protein was run on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described below. Nitrocellulose filters were blocked overnight at 4°C in 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.4), 0.2% Triton X-100, 0.1% gelatin, followed by a 1-h incubation at room temperature in horseradish peroxidase-conjugated avidin (Pierce) diluted 1:10,000 in the same buffer. Nitrocellulose filters were washed 10 ×, 10 min each with TTBS (20 mM Tris, pH 7.5, 150 mM NaCl, 0.2% Tween 20, 0.02% NaN3) followed by washing 5 times (10 min/wash) with TTBS and 5 times with TBS. The filters were incubated with peroxidase-conjugated goat anti-mouse IgG (Cell) at a 1:10,000 dilution for 2 h at room temperature. Following secondary antibody incubation, the filters are washed 5 times with TTBS followed by 5 times with TBS and developed by enhanced chemiluminescence (ECL).

Confocal and Electron Microscopy

Confocal Microscopy—T84 cells grown on permeable filters or glass coverslips were rinsed twice in PBS and fixed in methanol at −20°C for 25 min. Cells were rinsed 5 times for 3 min in PBS containing 0.08% saponin, 0.2% gelatin at room temperature. Fixed monolayers were then incubated in a 1:1000 dilution of the T9 monoclonal antibody for 1 h followed by 3–5 rinses in PBS. Monolayers were incubated at room temperature in a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody for 30 min followed by extensive washes in gelatin PBS. Monolayers were mounted in a phenylene diamine/glycerol/PBS medium on glass slides and viewed on a Zeiss Axiophot microscope equipped with epifluorescence and a Bio-Rad MRC 600 confocal imaging system.

Electron Microscopy—Ultrastructural localization of NKCC was performed using the T9 antibody and 10-nm colloidal gold-conjugated secondary antibodies. Filter-mounted monolayers were fixed using the periodate-lysine-paraformaldehyde technique, embedded in LR White, sectioned, and stained with antibody on grids as described previously (17). Sections, mounted on gold grids, were sequentially blocked for 5 min with 0.2% gelatin in PBS and then exposed for 2 h at room temperature to 5% non-fat dry milk in PBS with 0.1% gelatin. Sections were then exposed to primary antibody (diluted 1:10) for 2 h, washed four times with 0.2% Triton X-100 in PBS, and stained for 1 h with goat anti-mouse secondary antibody conjugated to 10-nm colloidal gold as described previously (17). Sections were evaluated at a magnification of 20,000 × for orientation and images of oriented sections were obtained for further quantitation. Morphometry was used to determine: the number of gold particles within ~30 nm (3 particle diameters) of the lateral membrane; the area of this membrane associated compartment, the number of gold particles ~30–150 nm from the lateral membrane; the area of this latter peribasal space compartment; the number of gold particles >150 nm from the lateral membrane, the area of this latter region. Since the distance between an epitope and a gold particle affixed to a secondary antibody is roughly 30 nm (5) the first distance was defined as lateral membrane associated, the second distance as submembraneous, and the third as background (all normalized to the respective areas).

RESULTS

Polarization of the NKCC Cotransporter on the T84 Cell Surface—The functionally defined NKCC cotransport process has been localized to apical membranes in some tissues (18), while to basolateral membranes in others including mammalian intestinal epithelia (19, 20). To assess whether the basolaterally defined functional polarity in the intestine reflects specific protein targeting or alternatively, a domain specific activation of a uniformly distributed protein, we used the approaches of laser confocal microscopy, electron microscopy, and selective surface biotinylation. As shown in Fig. 1, immunofluorescence confocal microscopy monolayers revealed that immunolabeling T84 monolayers using either T9 or T4 anti-NKCC antibodies specifically labeled basolateral membranes of T84 cells when grown as polarized monolayers on permeable supports (Fig. 1A). Reconstruction of confocal images to obtain xz axis sections revealed that such staining was restricted to the basolateral domain (Fig. 1B). Immunogold localization (Fig. 2) suggested that the vast majority of NKCC was associated with the lateral membrane. Quantitative analyses of 1,252 particles was performed (see “Materials and Methods”), 81% of specific staining was within the distance from the lateral membrane directly accounted for by the distance between an epitope and a gold particle affixed to a secondary antibody. The number of particles ~30–150 nm removed from the membrane accounted for 19% of specific staining. Thus, the vast majority of specific staining of NKCC by immun-EM localization was found to already exist on the lateral membrane in the unstimulated state.
lated bands migrating between 50 and 70 kDa represent im-
the 195-kDa NKCC protein (Figs. 3 and 5). Heavily biotiny-
described above resulted in several bands coprecipitating with 
NKCC Cotransporter—

lane 3 following selectivesbasolateralielation (Fig. 3, 
protein was consistently detected in the immunoprecipitate 
cotransporter antibody. In contrast, a biotinylated 195-kDa 
xpressed 195-kDa protein when precipitated with the T9 anti-
porter antibody along the basolateral membrane with no apparent 
staining on the apical surface. 

The surface biotinylation experiments 
level using selective apicalor basolateral biotinylation (Fig. 3). 

Surface distribution of NKCC was analyzed at the protein 
level using selective apical or basolateral biotinylation (Fig. 3). 
Monolayers biotinylated apically lacked the presence of surface 
expressed 195-kDa protein when precipitated with the T9 anti-
porter antibody. In contrast, a biotinylated 195-kDa 
protein was consistently detected in the immunoprecipitate 
following selective basolateral biotinylation (Fig. 3, basolateral 
lane).

Proteins of ~160 and ~130-kDa Coprecipitate with the 
NKCC Cotransporter—The surface biotinylation experiments 
described above resulted in several bands coprecipitating with 
the 195-kDa NKCC protein (Figs. 3 and 5). Heavily biotinyl-
ated bands migrating between 50 and 70 kDa represent im-
munoglobulin heavy chain bands that become biotinylated dur-
ing immunoprecipitation washes. Biotinylated antibody bands 
vary in intensity from preparation to preparation due to a 
nonspecific accumulation of free biotin during handling (data 
not shown). Two prominent bands of relative molecular mass 
~160 and ~130 kDa (Fig. 3, basolateral lane; Fig. 5, lane 2) are 
biotinylated by surface labeling and appear to represent mem-
brane proteins with ectodomains containing free amine groups. 
To determine whether the 160- and 130-kDa proteins contained 
the epitope recognized by T9, Western blotting with the T9 
antibody was performed. As shown in Fig. 4, T9 recognized only 
the 195-kDa cotransporter and not the 160- and 130-kDa 
films. Two additional monoclonal antibodies (T4 and T10) also 

FIG. 1. Laser confocal localization of the 195-kDa NaK2Cl 
cotransporter (NKCC) using an anti-T84 NKCC monoclonal an-
tibody (T9). A, confocal image of T84 cells taken ~6 μm from the apical 
surface (midway down the T84 cell). Fluorescence staining appeared 
uniformly distributed on the basolateral membrane. B, confocal image 
taken in the xz direction showing lateral staining of the anti-cotrans-
porter antibody along the basolateral membrane with no apparent 
staining on the apical surface.

FIG. 2. Transmission electron micrograph showing immuno-
gold localization of NKCC using the T9 monoclonal antibody. 
Colloidal gold particles shown here predominantly localize along the 
length of the lateral membrane (see “Results”). Arrowheads indicate 
apical surface microvilli; asterisks indicate the tight junction. Bar = 0.5 
μm.

There is no detection of NKCC present on the apical membrane. Baso-
lateral, biotin-avidin blot depicting immunoprecipitated protein from 
monolayers biotinylated on the basolateral surface. The 195-kDa NKCC 
band is selectively expressed on the basolateral membrane (arrow). In 
addition, several polypeptides coprecipitate with the 195-kDa NKCC 
protein, including two heavily biotinylated polypeptides of ~160 and 
~130 kDa (arrowheads). The 160/130-kDa coprecipitating proteins are 
predominately expressed on the basolateral membrane. Immunoglobu-
lin bands also detectable on the avidin blot since they become differen-
tially biotinylated during the biotinylation procedure. Molecular weight 
markers were, myosin (205 kDa), β-galactosidase (116 kDa), phospho-
rylase b (97.5 kDa), and ovalbumin (45 kDa).

These bands do not appear consistently from preparation to 
preparation and may represent degradation products of the 
160- and/or 130-kDa polypeptides (Fig. 5, lane 2). Distinctive 
antibodies raised against the 195-kDa cotransporter do not 
immunocross-react with the 160, 130, or lower molecular 
weight bands present in the immunoprecipitate. To test 
whether the 160- and 130-kDa proteins might represent deg-
lycosylated precursors which were simply not recognized by the 
T9 antibody on Western blots, we deglycosylated T84 extracts 
with N-glycosidase prior to performing immunoprecipitation 
with the T9 antibody. As shown in Fig. 6, the deglycosylated 
core peptide of the 195-kDa cotransporter of T84 cells runs, 
similar to the deglycosylated cotransporter of the shark (1), at 
~130 kDa. This deglycosylation product, unlike the coprecipi-
tating 160- and 130-kDa proteins, is readily blotted by the T9 
antibody. In aggregate, the above data indicate that NKCC 
immunoprecipitates as a complex with several other membrane 
proteins including two major proteins of 160 and 130 kDa 
which do not appear to be precursor forms of the cotransporter.

Surface Expression of NKCC and Coprecipitating Proteins in
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Forskolin-stimulated Monolayers—As shown in Fig. 7, forskolin stimulation of T84 monolayers results in rapid induction of Cl\(^-\) secretion as well as activation of the cotransporter as measured by bumetanide-sensitive \(^{86}\)Rb uptake. These events, as well as the associated remodeling of basolateral F-actin microfilaments (7) are prevented by phalloidin loading (Fig. 7). Under these conditions there is no evidence of cellular toxicity and in fact other regulated ion transport events are unaffected (Fig. 7B) (7). We first examined whether forskolin-stimulated activation of the cotransporter resulted in increased specific binding of a radiolabeled drug, bumetanide, which binds specifically to the 195-kDa cotransporter. In data not shown, \(^{3}H\)bumetanide binding was found to be near-saturated at 0.25–0.5 \(\mu\)M, consistent with tracheal epithelial cells (21) and HT 29 cells (9). Forskolin did not substantially increase specific binding or alter the apparent affinity for forskolin in preliminary experiments. This was also found to be true in binding studies performed using normal (135 mM) rather than low chloride (15 mM) buffer (data not shown). Thus, activation of cotransport by cAMP in T84 monolayers appears to occur largely by enhanced ion translocation per bumetanide-binding site rather than by an increase in the total number of binding sites. As shown in Fig. 7D, forskolin and/or phalloidin loading did not detectably increase specific binding of bumetanide to

FIG. 4. Western blot analysis of NKCC immunoprecipitated with the T9 antibody. Immuno-}

blots were probed with 20 \(\mu\)g/ml T9, T4, or T10 antibodies. Lanes were equivalently loaded with immunoprecipitated protein from \(2.5 \times 10^7\) cell equivalents/ml. Each of the three monoclonals recognize the 195-kDa band. Interestingly, the T10 monoclonal demonstrated a lower affinity for the 195-kDa polypeptide compared with T9 and T4. Immunoglobulin heavy chain bands (Antibody) present in the immunoprecipitate extract were readily recognized by the goat anti-mouse secondary antibody.

FIG. 5. Biotinylation and immunoprecipitation with the T4 antibody. Lane 1, immunoprecipitated NKCC from monolayers biotinylated apically. As observed with the T9 antibody, there is no detectable cotransporter protein on the apical surface. Lane 2, basolaterally biotinylated T84 monolayers, immunoprecipitated with the T4 antibody. T4 also immunoprecipitates the 195-kDa protein from the basolateral membrane along with several biotinylated polypeptides. The two prominent 160- and 130-kDa bands also coprecipitate with the 195-kDa NKCC band. Lane 3, Western blot of immunoprecipitated protein probed with the T4 antibody. Only the 195-kDa NKCC protein is readily identified by the antibody (dashed line). Molecular weight markers were myosin (205 kDa), \(\beta\)-galactosidase (116 kDa), phosphorylase b (97.5 kDa), and ovalbumin (45 kDa).

FIG. 6. T84 extracts were treated with N-glycanase for 90 min followed by immunoprecipitation with the T9 antibody. Immunoprecipitates were analyzed by Western blotting with the T9 antibody. Deglycosylation of the 195-kDa cotransporter, resulting in a band of \(\sim 130\) kDa, did not affect the immunocross-reactivity of the cotransporter with the T9 antibody.

FIG. 7. Effect of cytoskeletal stabilization on forskolin-stimulated transepithelial Cl\(^-\) secretion, NKCC function, and \(^{3}H\)bumetanide binding in T84 monolayers grown on permeable collagen-coated supports. A, forskolin (10 \(\mu\)M) induces rapid increase in short circuit current of T84 monolayers (open squares) which is profoundly attenuated in monolayers pre-loaded with 33 \(\mu\)M phalloidin (open triangles). Data points represent mean \(\pm\) S.E. for \(n = 4\) each group. B, absence of nonspecific cellular toxicity of phalloidin loading on T84 monolayers as evidenced by preservation of transepithelial Cl\(^-\) secretory response (short-circuit current) to 0.1 \(\mu\)M carbachol, a Ca\(^{2+}\)-mediated secretagogue, in monolayers prestimulated with 10 \(\mu\)M forskolin (left-side vertical axis). Additionally, transepithelial resistance (TER, right-side vertical axis), a sensitive index of junctional integrity, is not different between control monolayers and monolayers pre-loaded with 33 \(\mu\)M phalloidin for 16 h. All \(n = 5–4\). C, NKCC cotransport functionally defined as bumetanide-sensitive K\(^+\) (\(^{86}\)Rb) uptake, using \(^{86}\)Rb as a tracer for K\(^+\) and bumetanide (10 \(\mu\)M) as a specific inhibitor of NKCC cotransporter activity. Control monolayers (light bars) display a 5-fold increase in NKCC cotransporter activity in response to 10 \(\mu\)M forskolin. Both unstimulated and forskolin-stimulated cotransporter activity is markedly attenuated in monolayers pre-loaded with 33 \(\mu\)M phalloidin (dark bars) T84 monolayers. Monolayers were treated with or without forskolin (10 \(\mu\)M) for 40 min in buffer containing 0.25 \(\mu\)M \(^{3}H\)bumetanide. D, specific binding represents the difference between total binding and binding measured in the presence of 50 \(\mu\)M excess unlabeled bumetanide. Data points represent mean \(\pm\) S.E. for \(n = 7\) monolayers in each group.
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**Fig. 8.** Examination of the surface expression of NKCC and coprecipitating proteins after treatment with the cAMP agonist, forskolin. A, biotin-avidin blot of T84 monolayers treated with (+) and without (−) the cAMP agonist forskolin followed by selective cell surface biotinylation. Apical biotinylation shows no detection of the cotransporter protein in the presence of forskolin. Basolateral biotinylation after forskolin treatment shows an up-regulation of the 160- and 130-kDa coprecipitating proteins (arrowheads). B, histogram of scanning densitometry of the avidin blot in A (Basolateral only) depicting a ~6-fold increase in the 160- and 130-kDa bands with only a marginal increase in the 195-kDa cotransporter protein. Molecular weight markers used were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.5 kDa), and ovalbumin (45 kDa).

T84 cells. Selective surface biotinylation experiments (Fig. 8A) similarly suggested that movement of the 195-kDa cotransporter to the basolateral surface was modest at best following forskolin stimulation. Scanning densitometry of gels from seven independent experiments (Fig. 8B) showed that the average increase in density of the 195-kDa biotinylated band in response to forskolin stimulation was ~2.2-fold. In contrast, the major 160- and 130-kDa coprecipitating proteins were prominently up-regulated in forskolin-stimulated monolayers. Scanning densitometry of bands obtained from the seven experiments showed mean increases in biotinylation density of the 160- and 130-kDa bands of 5.8- and 6.1-fold, respectively (Fig. 8B), both $p < 0.06$ compared with the change in the 195-kDa band. Experiment to experiment variation in the forskolin-induced increase in the 160- and 130-kDa bands was examined in order to assess whether the proportionality of the increments in the 160- and 130-kDa bands relative to the amount of the 195-kDa band was fixed. Examined in this fashion, the correlation coefficient between increments in the 160- versus the 130-kDa bands was 0.86 suggesting that a proportional relationship in the forskolin-stimulated increase in surface expression of these proteins exists.

The apparent forskolin-stimulated surface expression of the 160- and 130-kDa proteins which coprecipitate with NKCC was examined in phalloidin loaded cells (Fig. 9), a condition which blocks functionally-defined activation of the cotransporter (see Fig. 7). Scanning densitometry of biotinylated immunoprecipitation experiments indicated approximately a 3–5-fold attenuation of the forskolin-induced up-regulation of the 160- and 130-kDa bands in cells preloaded with phalloidin (Fig. 9). The slight up-regulation in surface expression of the 195-kDa NKCC band was only marginally affected by phalloidin preloading conditions.

**DISCUSSION**

We show that the distribution of the Na,K,2Cl cotransport protein (NKCC) is basolaterally polarized in intestinal epithelia. We have identified two prominent membrane proteins, of molecular mass 160 and 130 kDa, that coimmunoprecipitate with the 195-kDa NKCC protein from T84 extracts. The 160- and 130-kDa coprecipitating polypeptides possess extracellular domains accessible to cell surface biotinylation thus indicating that they are transmembrane proteins. Based on Western blot analysis and deglycosylation with N-glycanase, the 160- and 130-kDa polypeptides appear to be distinct from the 195-kDa NKCC protein. Activation of NKCC, elicited by cAMP agonist stimulation, is accompanied by a modest increase in surface expression of the 195-kDa cotransporter protein (and no change in bumetanide binding) but is paralleled by an approximately 6-fold increase in the 160- and 130-kDa coprecipitating proteins. Phalloidin loading which prevents both the cAMP-elicited activation of NKCC as well as the basolateral F-actin rearrangement also diminishes the observed forskolin induced increase the 160- and 130-kDa polypeptides.

**Basolateral Polarization of NKCC in Intestinal Epithelia**—Mammalian respiratory, intestinal, and shark rectal gland epithelia possess a basolaterally polarized bumetanide-sensitive transport process in which movement of sodium, potassium, and chloride is coupled in an approximate 1:1:2 stoichiometry. Conversely, mammalian renal (thick ascending limb) epithelia and flounder intestinal epithelia possess a similar transport activity on the apical membrane (for recent review, see Refs. 19 and 21). The divergence in polarization of the NKCC transport process may reflect differences in apical/basolateral targeting due to tissue specific functionality of cotransporter activity. The question arises, however, whether the identical or highly homologous protein representing cotransporter activity is differentially targeted in a tissue-specific fashion, or whether the respective cotransporter protein is not specifically targeted per se but differentially activated within selected membrane domains. Here we find by confocal microscopy, quantitative electron microscopic morphometry, and surface biotinylation that the ~195-kDa protein recognized by antibodies raised against the cotransporter protein selectively appears on the basolateral membranes of intestinal epithelia. Such studies suggest selective basolateral targeting of the cotransporter oc-
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curs in this tissue. How then might the differential targeting between epithelia be explained? The first hint that NKCC activities between tissue types might represent differences in proteins or protein isoforms arose from the recognition that subtle site-determined functional differences exist in cotransporter activity. For example, as recently reviewed by Haas (19, 21), the affinity for bumetanide varies 10-fold between basolateral cotransporters of shark rectal gland, mammalian intestinal, and respiratory epithelial as compared to apical cotransporters from mammalian renal epithelia (thick ascending limb and medulla). It is of interest that the basolateral group are those in which the cotransporter participates in transepithelial salt and water secretion whereas the apical cotransporters participate in transepithelial absorption. Recent evidence suggests that different, although closely related, proteins may underlie such functional and membrane targeting specificities in cotransporters expressed in different epithelia. Using probes derived from the cloned shark rectal gland cotransporter, Payne and Forbush (2) have recently cloned a closely related but unique protein from renal cDNA libraries which corresponds to a smaller transcript which is seen in renal, but not intestinal tissues. Gamba et al. (22) have also recently cloned a cDNA which recognizes a similarly sized transcript, encoding a protein with functional characteristics of a Na,K,2Cl cotransporter, and, under high stringency conditions, blots with renal but not intestinal or respiratory tissues. In aggregate such data suggest that distinctive “secretory” and “absorptive” forms of the cotransporter may exist. If so it is likely that such differences include targeting sequences which ultimately determine the surface distribution of cotransporters between various epithelia.

The Intestinal Epithelial NKCC May Associate with Other Membrane Proteins—Immunoprecipitation of NKCC with

characterized and the expression of this protein in oocyte assays confers the transport characteristics of this SGLT transporter. Recently, however, Veyhl et al. (23) have cloned an unrelated 56-kDa protein (RS-1) from mammalian renal tissue which significantly modifies the activity of the SGLT-1 mediated Na-glucose cotransport when co-expressed. Of interest, the ability of RS-1 to enhance SGLT-1 “activity” is exceedingly sensitive to the stoichiometry of SGLT-1:RS-1 expression. Based on these and supplementary observations, it was proposed that RS-1 is a subunit of a complex in which the dominant function is defined by SGLT-1, however, the accompanying accessory proteins (RS-1) serve to fine tune the activity. Thus it is possible that individual transport proteins, whose function in expression assays satisfies stoichiometry of ion transport events and inhibitor recognition (such as has been shown for the Na,K:2Cl cotransporter; reviewed extensively in Ref. 19, might, when naturally expressed, display activities which are modified by associated proteins as well as by direct events such as phosphorylation or insertion of the primary protein into the membrane.

Up-regulation of NKCC Function and Expression of 160- and 130-kDa Proteins Are Dependent on Reorganization of the Microfilament Cytoskeleton—In the presence of cAMP agonist stimulation, there is an up-regulation of basolateral NKCC activity, a process attenuated by stabilization of the actin cytoskeleton (Fig. 6) (7, 8). We have observed under forskolin stimulated conditions a substantial (∼6-fold) increase in coprecipitation of surface expressed 160- and 130-kDa polypeptides with only a slight increase in surface expression of the 195-kDa polypeptide. [3H]Bumetanide binding studies also revealed no increase in bumetanide-binding sites upon forskolin stimulation, consistent with the view that surface expression of the cotransporter is not greatly altered in response to this agonist. These observations raise the possibility that regulation of NKCC may circuitously involve action on 195-kDa cotransporter protein by other proteins, such as the 160- and 130-kDa proteins or the cytoskeleton or both. It is thus possible, although speculative, that the mode of up-regulation of NKCC in T84 cells may involve recruitment of the 160- and 130-kDa polypeptides into the cotransporter complex, an event demonstrated to be blocked in the presence of phalloidin (see Fig. 9). Taken in concert, these data suggest an involvement of the actin cytoskeleton both in regulating functional cotransporter activity as well as the surface expression of the coprecipitating 160- and 130-kDa polypeptides. The causal link between the 160/130-kDa polypeptides and cotransporter activation is currently obscure. Further identification and analysis of the 160- and 130-kDa polypeptides may provide valuable information on the events which occur during cAMP induced cotransporter activation.

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