Selective basolateral localization of overexpressed Na-K-ATPase β1- and β2-subunits is disrupted by butyrate treatment of MDCK cells

Melissa D. Laughery, Rebecca J. Clifford, Yiqing Chi, and Jack H. Kaplan

Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois

Submitted 8 September 2006; accepted in final form 22 February 2007

Laughery MD, Clifford RJ, Chi Y, Kaplan JH. Selective basolateral localization of overexpressed Na-K-ATPase β1- and β2-subunits is disrupted by butyrate treatment of MDCK cells. Am J Physiol Renal Physiol 292: F1718–F1725, 2007. First published March 6, 2007; doi:10.1152/ajprenal.00360.2006.—The exclusive basolateral localization of the Na-K-ATPase in kidney epithelium is a critical aspect of nephron function. It has been suggested that mislocalized delivery of the Na-K-ATPase to the apical surface in autosomal dominant polycystic kidney disease (ADPKD) is due to the inappropriate expression of an alternative isoform of the β-subunit, the β2-isoform. It has been reported that heterologous expression of this β2-isoform in Madin-Darby canine kidney (MDCK) cells results in apical delivery of the Na-K-ATPase. We created a MDCK cell line containing a tetracycline-inducible promoter and expressed either myc-tagged β2- or flag-tagged β1-subunits to study the surface localization of these β-subunit isoforms in polarized monolayers. We find that the β2-isoform is targeted to the basolateral surface of the plasma membrane in a polarization pattern indistinguishable from the β1-isoform. However, inclusion of butyrate in the growth medium leads to upregulation of overexpressed β1- or β2-subunits and to their appearance at the apical surface. The β2-isoform expressed in MDCK cells does not assemble into α1β2 heterodimers with the endogenous α1. Our findings demonstrate that expression of the β2-isoform does not lead to apical localization of the Na-K-ATPase in MDCK cells and provides evidence for an unexpected effect of butyrate on the trafficking of Na pump subunits.

Madin-Darby canine kidney cells; apical localization; sodium pump subunits

THE Na-K-ATPase, OR THE SODIUM pump, plays a central role in ion regulation. At the cellular level, the functioning of the pump to transport Na⁺ out and K⁺ into the cell is essential for cell volume homeostasis and the maintenance of the inward Na⁺ gradient that is used to fuel the accumulation of a range of substrates, such as amino acids and sugars. At the tissue level, the Na-K-ATPase has a key role in kidney epithelia to drive the uptake of Na⁺ from the filtrate, thereby allowing the homeostatic control of fluid and electrolyte levels in the body. The directional uptake of Na⁺ across epithelia relies on the polarized localization of the Na-K-ATPase at the basolateral surface in the epithelial layer of cells lining the nephron.

The minimal functional unit of the Na-K-ATPase is comprised of a heterodimer of an α-subunit in complex with a β-subunit (9, 16). The α-subunit, which contains all the motifs essential for ATP hydrolysis and ion transport, is not trafficked out of the endoplasmic reticulum (ER) until it assembles with the β-subunit (8, 19). Hence, one of the roles attributed to the β-subunit is that of a molecular chaperone, involved in the folding, stabilization, and targeting of the α-subunit (for a review, see Ref. 9). Localization signals (3, 13) and cytoskeletal binding motifs (7) have been identified in the α-subunit, suggesting that the α-subunit has a role in cellular sorting of the functional Na-K-ATPase heterodimer; however, other evidence establishes a role for the β-subunit in polarized Na-K-ATPase targeting (34–36). Despite significant efforts to understand the mechanism of pump polarization, the signals and interactions that direct the localization are still not well established.

The most commonly expressed isoform of the β-subunit, β1, is present in most tissue types, including the kidney and is generally associated with basolateral targeting of the Na-K-ATPase in polarized epithelial cells. The β2-isoform, which was first identified as the adhesion molecule on glia (AMOG) (11), has restricted expression, limited primarily to particular regions in the brain (12, 20), epithelial cells of the eye (38), and inner ear (12), and is only normally found in the kidney during development before a strict basolateral polarization of the Na-K-ATPase is established (4). Abnormal apical localization of the Na-K-ATPase in kidney epithelia has been established in autosomal dominant polycystic kidney disease (ADPKD) (39, 40) and has been correlated with the inappropriate expression of the β2-subunit in ADPKD cyst cells.

In an earlier report, the β2-isoform, which is not normally expressed in MDCK cells, was observed at the apical surface (39) in stably transfected MDCK cells. This led to the suggestion that the apical delivery of the Na-K-ATPase in ADPKD is driven by the presence of the apically targeted β2-isoform. However, a direct interaction of the β2-isoform with the endogenous α1-subunit was not established and the molecular factors leading to apical targeting of the β2-isoform were not identified. In a more recent report, the β2-isoform fused with YFP was found localized in the basolateral membrane when expressed in MDCK cells (34). The different β2-subunit localization in MDCK cells observed in these two reports has not been accounted for.

We investigated the localization of the β1- and β2-subunit isoforms stably expressed in MDCK cells under tetracycline (tet) regulation. We find that the β2-isoform, like the β1-isoform, is exclusively at the basolateral surface of the cell membrane under normal growth conditions. This strict polarization is disrupted by the presence of butyrate in the growth medium. The presence of butyrate causes the overexpressed β1- or β2-subunits to appear at the apical as well as the basolateral surface in an isoform-independent fashion. We find...
that only the inappropriate localization of the β₁ isoform is associated with mistargeting of the α-subunit and that the β₂ isoform does not assemble with the endogenous α at a detectable level. Since butyrate was present in earlier work in which the β₂ isoform was observed apically in MDCK cells (39), our results provide an experimental basis for the observed apical delivery of β₂ and document an unexpected effect of butyrate on Na pump localization.

METHODS

Cell maintenance. MDCK cell lines were maintained at 37°C in a humidified incubator with 5% CO₂ in DMEM supplemented with 25 mM HEPES buffer, 10% fetal bovine serum (tet screened), 10 U/ml penicillin, 10 µg/ml streptomycin, 2.5 µg/ml fungizone, and 5 µg/ml plasmocin. Media were supplemented with 500 µg/ml zeocin, 400 µg/ml hygromycin B, and/or 6 µg/ml blasticidin as appropriate. For induction of the gene of interest, 1 µg/ml tet was added to growth media. Media were supplemented with 10 mM sodium butyrate as indicated in specific experiments. Cell lines were split every 2–3 days after trypsin-EDTA treatment to detach cells from tissue culture plates.

Tetracycline-inducible MDCK, MDCK βmyc, and MDCK βflag cells. The background cell line was a type I, high-resistance MDCK cell line (6, 7), which was converted into a MDCK/FlpIn cell line by using the Flp-In system (Invitrogen). This system creates an isotopic cell line with single Flp recombination target (FRT) sites (8). This MDCK/FlpIn line was treated with a kind gift from us from the late Dr. R. B. Gunn (Emory University School of Medicine at Atlanta). To generate a tet-inducible line (MDCK/FlpIn/T-Rex), MDCK/FlpIn cells were stably transfected with pcDNA5/TR ( Gibco/Invitrogen) that constitutively expresses the tet repressor under the control of the human CMV promoter. Colonies of cells were selected, screened, expanded, and harvested according to manufacturer’s protocol. The selected MDCK/FlpIn/T-Rex host cell line was cotransfected with pOG44 and pcDNA5/FRT/TO expression vector harboring either the rat β₁ cDNA fused to a COOH-terminal myc tag (β₁myc) or the sheep β₁ cDNA with a COOH-terminal flag tag (β₁flag) within the FRT site for homologous recombination. Integration of the gene of interest was selected with 800 µg/ml hygromycin B resistance to create the MDCK/FlpIn/T-Rex/β₁myc and the MDCK/FlpIn/T-Rex/β₁flag cell lines. Expression of the gene of interest in this system is repressed by the tet repressor and repression is relieved by the addition of 1 µg/ml tet to the growth media. For ease of communication, in this work the MDCK/FlpIn/T-Rex cells are simply referred to as MDCK, the MDCK/FlpIn/T-Rex/β₁myc as MDCK β₁myc, and the MDCK/FlpIn/T-Rex/β₁flag as MDCK β₁flag.

MDCK cell membrane preparations. Cells were grown to confluence on 10-cm plates, washed twice with PBS, scraped, pelleted (1,000 g for 10 min), and stored at −20°C. Pellets were thawed and resuspended in ice-cold homogenizing buffer (HB: 10 mM Tris-HCl, 2 mM EDTA, 250 mM sucrose, pH 7.4) and broken open by dounce homogenization and/or passing through a low gauge needle. Unbroken cells were removed by centrifugation at 1,000 g for 10 min. To obtain total membrane preparations, the supernatant was spun in a TLA-55 rotor for 30 min at 55,000 rpm. Alternatively, the supernatant was loaded into a 5-step sucrose gradient and membrane-enriched fractions were separated by ultracentrifugation. Pellets were suspended in HB supplemented with 1X protease inhibitor cocktail (Roche 1836153), and protein concentrations were determined by the Lowry method as previously described (15).

Na-K-ATPase activity. The difference in phosphate (P_i) liberated by 50 µg of total membrane protein in the presence or absence of 150 mM ouabain was determined as previously described (15) and is reported here in nanomoles of liberated P_i per milligram of protein per hour.
Samples were separated by SDS-PAGE and subjected to Western blot analysis.

**Communoprecipitation.** Thirty micrograms of plasma membrane-enriched fractions were solubilized in 1% NP-40, 150 mM NaCl, 10 mM Tris, 2 mM EDTA, pH 7.5, for 1 h at 4°C with rotation. Samples were spun at 55,000 rpm in TLA-55 for 30 min, and supernatant was incubated with 20 μl of anti-Loop or 5 μl of anti-myc antibody for 1 h at 4°C. One hundred microliters of protein G-Sepharose beads were added, and samples were incubated overnight at 4°C with rotation. Samples were spun at 8,000 g for 30 s, supernatant was aspirated, and washed three times with solubilization buffer, once with 0.5 M NaCl, 10 mM Tris, 2 mM EDTA, pH 7.5, and once with water. Precipitate was eluted from beads with SB, separated by SDS-PAGE, and transferred to PVDF membranes. Blots were blocked with 5% milk in PBS for at least 1 h before immunoprobing in PBS with 0.5% milk, 0.1% Tween for 1 h to overnight followed by 3 × 10-min PBS-Tween washes. Antibodies were used for Western blot at 37°C with a 1:1,000 dilution for 4 h before immunoprobing in PBS with 0.5% milk, 0.1% Tween for 1 h to overnight followed by 3 × 10-min PBS-Tween washes. Antibodies were used for Western blot at the following dilutions: 1:400,000 of anti-α1 (ABR MA 3-929), 1:400,000 of anti-β1 (ABR MA 3-930), 1:500 of anti-β2 (BD Transduction Laboratories no. 610915), 1:1,000 of anti-KETY (gift from Dr. J. Kyte, University of California, San Diego), 1:1,000 of anti-myc (Cell Signaling no. 2276), 1:1,000 of anti-FLAG M2 (Sigma F-3165), 1:500 of anti-SERCA, 1:500 of anti-E-cadherin (AbCam ab8996), 1:5,000 to 25,000 of horseradish peroxidase (HRP)-conjugated goat anti-mouse or antirabbit IgG. HRP-conjugated secondary antibodies and chemiluminescent reagents were used for signal detection.

**RESULTS**

Tetracycline-regulated overexpression of β-subunits in MDCK cells. To study the polarized localization of β-isoforms of the Na-K-ATPase, we created a system in which a protein can be selectively expressed in MDCK cells under the control of a tet-inducible promoter. The cDNA for either the rat β2-isoform with a COOH-terminal myc tag or the sheep β1-isoform with a COOH-terminal flag tag was introduced into the tet-controlled site and stable lines were isolated. Western blot analysis shown in Fig. 1A demonstrates tet-induced expression of β2myc, which is detectable with either the anti-myc (panel 1) or anti-β2 (panel 2) antibodies, in MDCK β2myc. The expression of the β2myc subunit does not greatly alter the levels of the endogenous β1- or α-subunits (Fig. 1A, panels 3 and 4). Western blot signal for the sarcoplasmic reticulum Ca pump (SERCA), a homolog of the Na-K-ATPase α-subunit, which functions independent of any β-subunit, served as a loading control (Fig. 1, A and B, bottom).

The β1flag is only expressed in the presence of tet in the MDCK β1flag cells, as revealed by Western blot with the anti-flag antibody (Fig. 1B, panel 1). Tet-induction of β1flag in MDCK β1flag cells markedly increases the level of β1 subunits (Fig. 1B, panel 2). Although an associated increase in translation of α has been observed on expression of β1 in MSV-MDCK cells (25), we find that overexpression of β1flag in the tet-inducible MDCK cell does not significantly increase the cellular level of the endogenous α-subunit (Fig. 1B, panel 3). The Na-K-ATPase activity measurements presented in Fig. 1C confirm that overexpression of β1flag or β2myc subunits by tet induction does not increase the cellular level of the ouabain-sensitive Na-K-ATPase activity, consistent with a stable cellular level of α-subunits observed by Western blot analysis in Fig. 1, A and B.

Cellular localization of β2myc in tet-induced cells. Confocal microscopy was performed to compare the cellular localization of the β2myc with the endogenous Na-K-ATPase in tet-induced MDCK β2myc cells. Figure 2A, top left, shows the basolateral localization of the endogenous α-subunit of the Na-K-ATPase in a focal plane located in the center of the cell monolayer, as detected with the α-subunit COOH-terminal antibody, anti-KETY. This basolateral localization of the endogenous Na-K-ATPase has been well established in MDCK cells. Unlike the endogenous pump, the β2myc subunit was detected primarily in intracellular compartments by the
anti-myc antibody (Fig. 2A, top right). DAPI staining of the nuclei is presented in the bottom left and a merged image in the bottom right of Fig. 2A. These images are consistent with previous observations (17) that the majority of the overexpressed β2myc subunits are located in intracellular compartments.

To further characterize the localization of the β2myc and to determine whether any of the expressed β2myc was targeted to the cell surface, we performed fractionation of cell membranes isolated from tet-induced MDCK β2myc cells. Results presented in Fig. 2B establish that the β2myc subunit is present at significant levels in all three fractions (Fig. 2B, top). Whereas the endogenous α- and β1-subunits are primarily located in the plasma membrane-enriched fraction (Fig. 2B, middle and bottom), β2myc in the plasma membrane-enriched fraction (P) migrates at a higher molecular weight than the predominant form in the endoplasmic reticulum-enriched fraction (E). Deglycosylation experiments using Endo H and PNGase F confirmed that the β2myc in the ER is a core N-linked glycosylated form (β2mycC) and the shift in molecular weight in the plasma membrane-enriched fraction is due to modification of the core glycosylation chains to a higher-order form (β2mycH) (data not shown).

Polarized basolateral localization of β1 and β2myc detected by surface labeling of polarized monolayers. To characterize the polarized distribution of surface β-isoforms in MDCK cells, surface proteins of an MDCK β2myc cell monolayer grown on a permeable support were biotinylated from either the apical (A) or basolateral (B) compartment. The Western blot in Fig. 3A detects endogenous β1-subunit only at the basolateral surface and not the apical surface. This result is consistent with the well-established selective targeting of the endogenous Na-K-ATPase to the basolateral surface of MDCK cells (5, 14). Figure 3B demonstrates that the β2myc subunit at the cell surface is also localized predominantly at the basolateral and not the apical membrane domain. This result establishes that the heterologous β2-isofom at the cell surface exhibits the same basolateral polarization as the endogenous β1-isofom and is in contrast to the β2myc apical localization report by Wilson et al. (39).

Butyrate alters surface polarity of β2myc. When the β2-subunit was detected at the apical surface of MDCK cells in previous work, butyrate was present in the growth media (39). However, butyrate was not present in Fig. 3 or in previous work in which the β2-subunit fused to GFP was found exclusively in the basolateral surface of MDCK cells (34). We therefore examined the role of butyrate in the expression and targeting of Na-K-ATPase subunits. We found that butyrate treatment of tet-induced MDCK β2myc cells increases the tet-induced expression of the β2myc subunit in these cells (Fig. 4A, bottom). We also observed a decrease in the cellular level of endogenous β1-subunits in butyrate-treated MDCK β2myc cells (Fig. 4A, middle), although the cellular level of α-subunits remained fairly consistent after butyrate treatment (Fig. 4A, top).

Fig. 2. Cellular localization of the β2myc isoform. A: tet-induced MDCK β2myc cells were grown in polarized monolayers and subject to confocal imaging in which anti-KETYY, an antibody recognizing α-subunit of the Na-K-ATPase, was detected with a Cy 3-conjugated secondary (top left) and anti-myc was detected with an Alexa 488 secondary (top right). DAPI stain indicates nuclei (bottom left) and a merged image is presented in bottom right. B: alternatively, total membranes were obtained and separated into organelle-enriched fractions by ultracentrifugation in a step sucrose gradient. Equal amounts of protein from each organelle-enriched fraction were separated by SDS-PAGE and Western blot was performed with anti-β2, anti-β1, or anti-α antibodies. Subunit carrying core (c) or higher-order modified (h) N-linked glycosylation chains are indicated.

Fig. 3. Polarity determination of β-isoforms by cell surface biotinylation. Monolayers of MDCK β2myc cells were grown on permeable supports until tight junctions had formed and cells were polarized. β2myc Expression was induced by addition of 1 mg/ml tet for 2 days in the (+) conditions. An amine-directed biotin reagent was added to either the apical (A) or basolateral (B) compartment to label surface proteins. Cells were lysed in detergent buffer, and the labeled surface proteins were precipitated with streptavidin beads, eluted, and separated by SDS-PAGE. Western blot analysis was performed with anti-β1 (A) or anti-β2 (B) as detecting antibodies.
To determine whether butyrate influences the polarization of the β2-subunit in MDCK cells, we biotinylated surface proteins from tet-induced or uninduced MDCK β2-myc cells grown in the presence or absence of butyrate. In the absence of butyrate, the β2-myc is only present on the basolateral surface (Figs. 3B and 4B, top). However, in the presence of butyrate, the β2-myc subunit can be labeled from both the apical and basolateral compartments (Fig. 4B, top). Western blot analysis of the same samples detecting the Na-K-ATPase α-subunit with the anti-KETYY antibody reveals that the endogenous α-subunit remains polarized to the basolateral surface during butyrate treatment, regardless of β2-myc induction (Fig. 4B, bottom). This demonstrates that the presence of butyrate does not allow the biotin label to cross the monolayer.

β2-myc subunit does not associate with endogenous Na-K-ATPase α-subunits in MDCK cells. Our observation that endogenous pump α-subunit basolateral localization is not affected by altered distribution of the surface-targeted β2-myc subunit in the presence of butyrate (Fig. 4B, bottom) suggests that the β2-myc does not assemble with the Na-K-ATPase α-subunit in MDCK cells to form an α1β2 heterodimer. We therefore performed coimmunoprecipitations from the plasma membrane-enriched fraction of tet-induced MDCK β2-myc cells with antibodies directed at either the myc epitope or the endogenous α-subunit to establish whether α1β2 assembly occurs. Figure 5A, top, shows that the myc antibody is capable of immunoprecipitating the β2-myc subunit from a plasma membrane-enriched sample. However, the myc antibody does not coimmunoprecipitate the α-subunit (Fig. 5A, bottom). Likewise, an antibody directed at the major cytoplasmic loop of the α-subunit which precipitates the α-subunit (Fig. 5B, middle) does not coimmunoprecipitate the expressed β2-myc (Fig. 5B, top), although the endogenous β1-isoform is coimmunoprecipitated under the same conditions (Fig. 5B, bottom). Butyrate treatment for 2 days at 10 mM enhances the expression of the tet-induced β2-myc (see Fig. 4A) but does not alter coimmunoprecipitation results (Fig. 5C). Control precipitations lacking protein input or antibody provided no evidence to suggest these immunoprecipitation results were artifact (data not shown). Thus the findings shown in Fig. 5 support the implications of the biotinylation data shown in Fig. 4 that the α- and β2-subunits do not assemble in the MDCK cells, although α1 does associate with endogenous β1-subunits. It has previously been shown that α1β2 heterodimers can form in the absence of β1 (2, 6).

Cellular effects of butyrate on MDCK cells. To further characterize the impact of butyrate on MDCK cells, we studied its effect in the background MDCK cell line. A dramatic decrease of the monolayer transepithelial resistance (RTE) is associated with butyrate treatment (Fig. 6A). Normally, a stable high RTE develops as MDCK cells grow together into polarized monolayers and tight junctions are formed. The addition of butyrate causes these polarized cell layers to lose their high RTE. This loss of RTE is reversible on removal of butyrate (data not shown), consistent with previous work which demonstrated that MDCK monolayers remain polarized in the presence of low levels of butyrate (22, 24).

As we previously observed in the tet-induced MDCK β2-myc cell line (Fig. 4A), a reduction of the total cellular Na-K-ATPase β1 occurs after butyrate treatment of the background MDCK cell line (Fig. 6B, bottom). A smaller but consistent reduction of the α-subunit in the total lysates was also observed (Fig. 6B, top). It should be noted that equal protein concentrations were loaded in Fig. 6B, so the proportionately larger reduction in the endogenous β1-subunit cannot be explained by...
inhibited cell growth or cell death in the butyrate condition. Whether this observation reflects different degradation rates for the two subunits is the subject of ongoing work.

During butyrate treatment, both the endogenous \( \alpha \)- and \( \beta_1 \)-subunits of the Na-K-ATPase in MDCK cells remain localized to the basolateral surface (Fig. 6C). A reduced amount of surface-labeled endogenous Na-K-ATPase is seen in the presence of butyrate (Fig. 6C), which is expected given the loss of Na-K-ATPase subunits, especially \( \beta_1 \)-subunits during butyrate treatment. The conservation of the basolateral localization of the endogenous subunits during butyrate treatment is in sharp contrast to the loss of polarization of the overexpressed \( \beta_2 \)-myc isoform in Fig. 4B.

**Butyrate causes mislocalization of Na-K-ATPase overexpressed \( \beta_2 \)-subunits.** To determine whether the loss of basolateral polarization of the \( \beta_2 \)-myc subunit by butyrate (Fig. 4B) is isoform specific, butyrate treatment and surface biotinylation were performed with MDCK \( \beta_1 \)-flag cells. These cells overexpress \( \beta_1 \)-flag from the same chromosomal site previously used for \( \beta_2 \)-myc expression. Figure 7A, bottom, demonstrates that butyrate treatment enhances the tet-induced overexpression of the \( \beta_1 \)-flag in MDCK \( \beta_1 \)-flag cells, analogous to the increase of \( \beta_2 \)-myc observed in Fig. 4A. Under the tet-induced butyrate-stimulated condition, the core glycosylated form of the \( \beta_1 \)-flag subunit (\( \beta_1 \)-flagC) is the major form of \( \beta_1 \) detected (Fig. 7A, middle and bottom), which is not the case in the absence of butyrate-stimulated overexpression (see Fig. 1B). The glycosylation status of this form has been confirmed by deglycosylation experiments with Endo H and PNGase F (data not shown). A decreased mobility of the \( \beta_1 \)-flag carrying higher-order glycosylation (\( \beta_1 \)-flagC) is also observed (Fig. 7A, middle and bottom).

Surface labeling of tet-induced MDCK \( \beta_1 \)-flag cells reveals that the endogenous Na-K-ATPase subunits and the expressed \( \beta_1 \)-flag are selectively located in the basolateral surface in the absence of butyrate, as expected (Fig. 7B). However, just as with the \( \beta_2 \)-myc, overexpressed \( \beta_1 \)-flag subunits are detected at the apical surface as well as the basolateral surface in the presence of butyrate (Fig. 7B, middle). Unlike the mislocalized \( \beta_2 \)-myc, the endogenous \( \alpha \)-subunit is clearly mis-targeted to the apical surface along with the mislocalized \( \beta_1 \)-flag (Fig. 7B, top). To confirm that this apical detection is not due to an overall loss of cell polarity, Western blot detection was also performed for E-cadherin, which remains at the basolateral surface (Fig. 7B, bottom) in the butyrate-treated tet-induced MDCK \( \beta_1 \)-flag cells.

**DISCUSSION**

In the present work, we overexpressed epitope-tagged forms of the \( \beta_1 \)- and \( \beta_2 \)-isoforms of the Na-K-ATPase \( \beta \)-subunit in a tet-inducible MDCK expression system to compare the processing and polarized targeting of the Na-K-ATPase subunits. We found that both the \( \beta_1 \)- and \( \beta_2 \)-isoforms are localized exclusively to the basolateral membrane at the cell surface under normal growth conditions. When either the \( \beta_1 \)- or \( \beta_2 \)-isoform is expressed in the presence of butyrate, the overexpressed \( \beta \)-subunit is detected at both the apical and basolateral surfaces.

**Effect of \( \beta \)-subunit overexpression on endogenous Na-K-ATPase levels.** It has been previously reported that overexpression of the \( \beta_1 \)-subunit leads to a corresponding increase in the expression of endogenous \( \alpha \) in some cell systems in which the \( \alpha \) level is a limiting factor in Na-K-ATPase production (10, 25, 27, 28). However, we have seen that the tet-induced expression of \( \beta_2 \)-myc or \( \beta_1 \)-flag in our MDCK cell system does not lead to a significant change in expression of either endogenous \( \alpha \) or \( \beta_1 \) Na-K-ATPase subunits. Our findings suggest that expression...
of the β-subunit is not a limiting factor for the formation and expression of Na-K-ATPase heterodimers in MDCK cells. This, in turn, suggests that the α-subunit is the limiting factor for pump expression and that unassociated β-subunits might be expressed in excess to the 1:1 ratio of αβ necessary for the formation of the Na-K-ATPase heterodimer (23). Since an additional role for the β-subunit as an adhesion molecule has been suggested (18, 31), it is important to determine whether there are unassociated β-subunits present in cells and whether the β-subunits perform a function beyond their role in the sodium pump.

Surface localization of the β2-isoform expressed in MDCK cells. Under normal cell growth conditions, the β2-isoform is strictly localized to the basolateral compartment when expressed in MDCK monolayers, just like the β1 isoform. We showed that the presence of butyrate in the growth media is the likely cause of β2-isoform apical localization reported in previous work (39). This effect is not isoform specific, as butyrate disrupts the polarization of expressed β-subunits when either the β1- or β2-isoforms of the β-subunit are overexpressed. Our finding and those of others (34) demonstrate that there is no difference in the localization of the β2-isoform when expressed in MDCK cells, contrary to previous claims. Although our work does not directly address the role that the β2-isoform may play in PKD, it is clear that the expression of the β2-isoform is not sufficient to account for mistargeting of the Na-K-ATPase in an otherwise normal system.

Cellular effects of butyrate in MDCK cells. Butyrate is a small fatty acid normally located in the digestive tract as a metabolic product of intestinal flora in vivo where it is the primary energy source for colonocytes. In vitro, butyrate has been observed to induce a number of cellular alterations, which vary between cell lines. The primary effect attributed to butyrate is inhibition of histone deacetylase activity, which leads to alterations in protein expression and can lead to cell cycle arrest, and eventually induce apoptosis (for a review, see Refs. 17, 21).

We observed a decrease in the total cellular level of the endogenous β-subunit in the presence of butyrate. Interestingly, it has been demonstrated that the presence of the Na-K-ATPase β-subunit is necessary for normal cell-cell contacts in MDCK cells (27) and suggested that interacting β-subunits between cells may serve an adhesive role (31). It has been previously noted that the introduction of β1 into MSV-transformed MDCK cells allows those cells to regain elevated R<sub>TE</sub> values (26). The loss of R<sub>TE</sub> we observe when MDCK cells are treated with butyrate along with the drop in the β-subunit level fit well with this finding and together suggest that the presence of the β1-subunit may contribute to the high cellular R<sub>TE</sub> in MDCK cells.

A reduction of basolateral short-circuit current (I<sub>sc</sub>) attributed to decreased Na-K-ATPase activity has also been found in butyrate-treated MDCK cells (24). We showed that prolonged treatment with butyrate decreases the total level of endogenous pump subunits (Figs. 4A, 6B, and 7A) and can reduce the Na-K-ATPase at the cell surface (Figs. 4A and 6C). This suggests that butyrate may stimulate the internalization and degradation of the Na-K-ATPase as well as potentially influencing transcriptional regulation of expression.

Although butyrate decreases the levels of endogenous β-subunits, it increases expression off the CMV promoter (24, 37) and therefore leads to stimulated expression of the β2-myc or β1-FLAG (Figs. 4A and 7A) in the tet-induced MDCK cell system. A number of observations suggest that it is unlikely that the inappropriate apical localization of these subunits (Figs. 4B and 7B) is simply a result of the butyrate-enhanced expression overloading the cellular sorting and targeting mechanism. First, in the absence of butyrate, we do not observe inappropriate delivery of overexpressed β-subunits even after prolonged tet induction which increases expression (data not shown). Second, in surface labeling experiments, we do not detect the core glycosylated β1-FLAG at the plasma membrane (Fig. 7B), although the cellular level of β1-FLAG is greatly increased in the presence of butyrate (Fig. 7A). Finally, we do not observe apical localization or decreased surface accessibility of E-cadherin in butyrate-treated cells, which indicates that the sorting and delivery mechanisms are functioning correctly for this basolateral membrane protein. Our finding of maintained E-cadherin polarization in the presence of butyrate is consistent with previous observations in human colon cancer cells (1). Based on the results presented here, it appears that butyrate specifically alters the delivery of newly expressed Na-K-ATPase β-subunits and α1β1 heterodimers when expressed in MDCK cells but does not cause inappropriate localization of other basolaterally directed proteins. From our current results, it is not clear whether the changes in glycosylation that we observe in the presence of butyrate (Figs. 4 and 7) are related to the mistargeting. However, a precedent for glycosylation of β-subunits influencing polarization has been previously established (33).

Isoform-specific association of Na-K-ATPase subunits. Through the use of butyrate, we observed the mistargeting of endogenous MDCK Na-K-ATPase α1-subunit to the apical surface with exogenously overexpressed β1-subunit (Fig. 7B). However, the endogenous α1 is not similarly mistargeted with the β2-subunit expressed under the same conditions (Fig. 4B). Furthermore, co-IP experiments under conditions in which α1β1 heterodimers are observed fail to show β2 association with α (Fig. 5). These findings suggest that the α1-subunit in MDCK cells has a higher affinity for the β1-isoform than for the β2-isoform and are consistent with reports of lower affinity between subunits in an α1β2 than an α1β1 or α2β2 heterodimeric configuration of the Na-K-ATPase when expressed in amphibian oocytes or in insect cell lines (2, 29, 30).

In summary, we established that the β2-isoform of the Na-K-ATPase is targeted to the basolateral surface of MDCK cells. We also show that the small fatty acid butyrate causes the inappropriate delivery of overexpressed β1- and β2-subunits to the apical surface of MDCK cells. This finding identifies a novel effect of butyrate in the polarized delivery of these important membrane proteins.

ACKNOWLEDGMENTS

We thank the late Dr. R. B. Gunn (Emory University School of Medicine at Atlanta) for generosity in providing the MDCK/FpIn cell line, Dr. J. Kyte (University of California, San Diego) for the gift of the anti-KETYY antibody, and Dr. R. Mercer (Washington University, St. Louis) for the gift of cDNA. We also thank Dr. S. Lutesenko (Oregon Health Sciences University) and Dr. J. Bystriansky (University of Illinois at Chicago) for critical comments on the manuscript.
REFERENCES