Na-K-ATPase regulates tight junction permeability through occludin phosphorylation in pancreatic epithelial cells

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nonreceptor tyrosine kinase c-Yes (5), and protein phosphatases that include the serine/threonine protein phosphatase 2A (PP2A) (17). The PP2A holoenzyme consists of a catalytic subunit (C), a structural subunit (A), and a regulatory subunit (B). Several B subunit families that modulate PP2A catalytic activity, substrate specificity, and subcellular localization have been identified (30). PP2A containing the Bα regulatory subunit is a major PP2A isoform involved in cell growth and cytoskeletal regulation in numerous cell types and is localized to and regulates TJ functions (17).

We have established the human pancreatic adenocarcinoma cell line HPAF-II as a polarized cell culture model to study TJs in pancreas (20). HPAF-II cells are ductal pancreatic cancer cells that express Muc 1 and Muc 4 mucus genes and secrete high levels of Muc1 mucin. Using this cell line, we now provide evidence for the first time that in mammalian cells Na-K-ATPase is localized to the apical junctions (in addition to its well-described basolateral localization) and associates with PP2A. Inhibition of the Na-K-ATPase ion transport function reduced PP2A activity, hyperphosphorylated occludin, and induced rearrangement of TJ strands. The resulting increase in TJ permeability to ionic and nonionic molecules suggests that Na-K-ATPase activity is required in controlling the TJ gate function in pancreatic epithelial cells.

MATERIALS AND METHODS

Cell culture, TER, and antibodies. Human HPAF-II cells were kindly provided by Dr. Reber (University of California, Los Angeles, CA) and were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25 U/ml penicillin, 25 μg/ml streptomycin, and 100 μM nonessential amino acids as described (20). For experiments, the cells were seeded on Costar Transwells with 0.4-μm pore size (Corning, Corning, NY) and allowed to grow until a transepithelial electrical resistance (TER) of more than 1,000 Ω·cm² developed, usually 3–6 days. Coimmunoprecipitation and glutathione S-transferase (GST)-pull-down assays were done using confluent monolayers grown on 100-mm culture dishes.

For K⁺-free conditions, HPAF-II cells were washed twice with K⁺-free buffer (140 mM NaCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 10 mM glucose, pH 7.4, 10% FBS dialyzed against the K⁺-free buffer) and then incubated with this buffer generally for 3 h unless noted otherwise (21, 22), a time point at which the cells are fully viable and the inhibition of Na-K-ATPase is completely reversible. For K⁺ depletion, K⁺-free buffer was replaced by culture medium and the cells were incubated at 37°C, 5% CO₂ for the times indicated. Control cells received a media change at the same time. For ouabain treatments, cells were treated with varying concentrations of ouabain (Sigma Chemical, St. Louis, MO) dissolved in DMSO added to the culture medium usually for 7 h. HPAF-II cells treated with DMSO alone were used as control cells for these experiments.

TER was measured as described previously (20–22) with an EVOM Epithelial Voltmeter (World Precision Instruments, Sarasota, FL). To obtain the TER values (in Ω·cm²), the background resistance value of a blank filter without cells was subtracted from the measured values and then the values were normalized to the area of the filter.

Antibodies against ZO-1, occludin, claudin-4, and E-cadherin were obtained from Invitrogen (Carlsbad, CA) and β-catenin, anti-PP2A catalytic α antibodies were from BD Biosciences (San Jose, CA). Antibodies against NaK-α (M8-P1-A3 for immunoprecipitations and M7-PB-E9 for immunoblotting) and NaK-β (M17-P5-F11) were kindly provided by Dr. William Ball Jr., University of Cincinnati Medical Center, Cincinnati, OH. Horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA). FITC-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA), and gold-conjugated secondary antibodies from Ted Pella (Redding, CA).

Immunoblot analysis, immunoprecipitations, and β- phosphatase treatments. Cell lysates were prepared as described earlier (23). Briefly, confluent monolayers of HPAF-II cells grown on Transwells were lysed in a buffer containing 95 mM NaCl, 25 mM Tris, pH 7.4, 0.5 mM EDTA, 2% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μg/ml each of antipain, leupeptin, and pepstatin. The lysates were clarified by centrifugation at 13,000 rpm for 10 min. The supernatants were collected, and total protein was estimated by using the Bio-Rad DC reagent (Bio-Rad Laboratories, Hercules, CA) as per manufacturer’s instructions. Equal amounts of protein (100 μg) were separated by SDS-PAGE. Primary antibodies were diluted 1:1,000 in PBS containing 10% nonfat dry milk. Horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies were used at a dilution of 1:2,000 in PBS/10% nonfat dry milk. Protein bands were detected ECL (Perkin Elmer Life Sciences, Boston, MA).

β-Phosphatase (β-PPase) treatment of occludin and claudin-4 was done as described previously (21). Occludin or claudin-4 was immunoprecipitated from control or Na-K-ATPase-inhibited cells lysed in a buffer containing 10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 0.2 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 5 μg/ml each of antipain, leupeptin, and pepstatin. The immunoprecipitates were washed and then incubated with β-PPase (New England Biolabs, Beverly, MA) at 30°C for 30 min according to the manufacturer’s instructions. Proteins were resolved by SDS-PAGE and immuno blotted as described above.

GST-pull-down assays and coimmunoprecipitations. In vitro binding assays were done as described (1, 3). Briefly, the cytoplasmic domain of Na-K-β containing amino acids 1–35 (NaK-β NGST) and the NH₂-terminus of NaK-α containing amino acids 1–93 (NaK-α N-GST) were cloned in pGEX-5x vector (Invitrogen, Carlsbad, CA) and transformed into Escherichia coli BL21 cells. Expression of recombinant protein was induced by the addition of 0.25 mM isopro pylglyactoside for 2 h. Bacterial cell pellets were lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM MgCl₂, 250 μg/ml lysozyme, 1 mM PMSF, and 10 μg/ml each of antipain, leupeptin, and pepstatin. The GST-fusion protein was bound to glutathione-coupled agarose beads (Pharmacia Biotech, Piscatawy, NJ) for 1 h and 4°C of the amount of coupled fusion protein was estimated by using Coomassie-stained SDS-PAGE gels. HPAF-II cell lysates were prepared in a buffer containing 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM sodium glycereophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 5 μg/ml each of antipain, leupeptin, and pepstatin. The lysates were clarified by centrifugation at 10,000 rpm for 10 min at 4°C. The protein concentrations of the cleared supernatants were determined, and equal amounts of protein were incubated with purified GST-fusion proteins at 4°C for 16 h. Bound proteins were detected by immunoblotting as described above.

For coimmunoprecipitations, equal protein from cell lysate prepared as described above were incubated on a rotator overnight with antibody bound to protein A agarose beads at 4°C. The proteins bound to the beads were separated by SDS-PAGE and coimmunoprecipitating proteins were analyzed by immunoblotting.

TEM, freeze fracture, and immunoelectron microscopy. For transmission electron microscopy (TEM), HPAF-II monolayers were grown to confluence on Transwells. Control or Na-K-ATPase-inhibited cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 h at room temperature. The samples were then processed by conventional procedures for electron microscopy. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Freeze fracture analysis was done as described previously (20). Briefly, confluent monolayers of HPAF-II cells grown on Transwells.
were fixed in 2% glutaraldehyde in PBS for 30 min at 4°C. After rinsing with Dulbecco’s PBS, the cells were scraped from the filters and infiltrated with 25% glycerol in 0.1 M cacodylate buffer. The cells were pelleted, frozen in liquid nitrogen, and freeze fractured at −115°C in a Balzers 400T freeze-fragment unit (Balzers, Liechtenstein). The replicas were cleaned with sodium hypochlorite and examined with a Philips 301 electron microscope (Philips, Eindhoven, Holland).

For immunoelectroscopy, HPAF-II cells grown on 35-mm tissue culture dishes were fixed in ice-cold methanol. After being washed with PBS-1 mM MgCl₂·0.1 mM CaCl₂·0.2% bovine serum albumin (PBS-CM-BSA), the cells were incubated with primary antibodies against NaK-β and occludin for 16 h at 4°C. The cells were washed three times with PBS-CM-BSA containing 0.075% saponin (Sigma Chemical) and then incubated for 1 h at room temperature with gold-conjugated anti-mouse (10 nm; NaK-β) and anti-rabbit (5 nm; occludin) secondary antibodies diluted 1:6 in PBS-CM-saponin. After washing with PBS-CM-saponin, the cells were fixed in 2% glutaraldehyde in cacodylate buffer and scraped off the plate, and cell pellets were processed by conventional electron microscope procedures. To compare the staining intensity of NaK-β at the apical junctional region and at the lateral plasma membrane, the numbers of 10-nm gold particles were counted in 32 and 29 fields of 0.25 μm × 0.25 μm localized to the respective regions. The data represent the means ± SE of number of gold particles per field.

Paracellular diffusion studies. Confluent monolayers of HPAF-II cells with TER values of at least 1,000 Ω·cm² were chosen for these studies. Cells in culture medium, treated with ouabain or under K⁺-depleted conditions, were assessed for paracellular diffusion of 3H-inulin or 3H-mannitol as described previously (20, 21). 3H-inulin (2 μCi) or 3H-mannitol (2 μCi) (Amersham, Arlington Heights, IL) in 500 μl of culture medium with or without ouabain or K⁺-free medium were added to the apical side of the filter; the basal compartment medium was collected. The samples were counted in a liquid scintillation counter and permeability was calculated with $P = (X_b / X_i) / (A / l)$ where $X_b$ are counts per minute in the basal chamber; ($X_i$), the initial concentration in the apical chamber, $A$ is the area of the filter in centimeters squared, and $l$ is the time in minutes, as described previously (21, 22).

PP2A activity. Control and Na-K-ATPase inhibited HPAF-II cells on Transwells were lysed in a buffer containing 20 mM imidazole-HCl, pH 7.0, 2 mM EDTA, 2 mM EGTA, 1 mM benzamidine, 1 mM PMSF, 10 μg/ml trypsin inhibitor, and 10 μg/ml each of antipain, leupeptin, and pepstatin. Equal amounts of protein were used for immunoprecipitation of the catalytic subunit of PP2A. PP2A activity was determined by dephosphorylation of the specific phosphopeptide R-K-pY-T-I-R by using the Ser/Thr Phosphatase Assay Kit 1 (Upstate Biotechnology, Lake Placid, NY) according to manufacturer’s instructions, and free phosphate released was determined by using the Malachite Green Assay included in the kit.

Immunofluorescence and confocal microscopy. Immunofluorescence and confocal microscopy were done as described previously (20, 22, 23). HPAF-II cells grown on Transwells were fixed in methanol at −20°C and then incubated with primary antibodies diluted 1:1,000 in PBS-CM-BSA for 1 h at room temperature. FITC-conjugated secondary antibodies were used at a dilution of 1:100. Epifluorescence pictures were captured with a SPOT charge-coupled device camera and SPOT imaging software, version 4.0.4 (Diagnostic Instruments, Sterling Heights, MI) attached to an Olympus AX70 microscope. Confocal microscopy was done with an Olympus laser scanning confocal microscope, and images were generated by the Fluoview Image Analysis software (version 2.1.39) as described (22).

RESULTS

To test whether Na-K-ATPase enzyme activity is necessary for TJ function in HPAF-II cells, we inhibited the enzymatic activity of Na-K-ATPase by two independent methods, using the specific pharmacological inhibitor ouabain, and by K⁺ depletion (21, 22). Whereas ouabain binds to and inhibits the enzyme irreversibly, Na-K-ATPase can be reactivated after K⁺ depletion by addition of K⁺ to test for reversibility of observed effects. TER, a measure of the ion permeability of TJs, was drastically decreased in K⁺-depleted cells (Fig. 1A). Whereas the TER of untreated control cells was 1,694 ± 43 Ω·cm², the TER of K⁺-depleted cells dropped to 896 ± 60 Ω·cm² within 1 h and reduced to 289 ± 9 Ω·cm² after 2 h. This TER decrease was reversible upon K⁺ repletion. In ouabain-treated cells, TER decreased in a dose-dependent manner (Fig. 1B), indicating that inhibition of Na-K-ATPase leads to increased ion permeability of TJs in HPAF-II cells. TJ permeability to nonionic molecules in Na-K-ATPase-inhibited cells was determined by tracer studies using 3H-inulin (hydrodynamic radius ~10–14 Å) and 3H-mannitol (hydrodynamic radius ~4 Å) (Fig. 1C). Ouabain caused a dose-dependent increase of TJ permeability for both inulin and mannitol, with 50 μM ouabain having an effect similar to K⁺ depletion. The TJ permeability to inulin and mannitol was reversible upon K⁺ repletion. Taken together, these results demonstrated that inhibition of Na-K-ATPase increases the permeability of both ionic and nonionic molecules through the paracellular space in HPAF-II cells.

We then tested whether the observed permeability changes are associated with altered TJ organization. Immunofluorescence of the TJ proteins ZO-1, occludin, and claudin-4 in ouabain-treated and K⁺-depleted cells did not reveal significant differences compared with control cells at the light microscopic level (Fig. 2A). Furthermore, confocal microscope vertical sections showed lateral localization of β-catenin in control and Na-K-ATPase-inhibited cells, indicating that the polarity is not affected (Fig. 2B). However, TEM revealed that, upon Na-K-ATPase inhibition by both K⁺ depletion and ouabain, the TJ contact points were reduced compared with the extensive TJs observed in control cells (Fig. 3A). K⁺-repleted cells showed a morphology similar to untreated control cells. In freeze-fracture electron micrographs, the TJ network in control HPAF-II cells was somewhat variable, with most segments forming a network of TJ strands (Fig. 3B, control), whereas ~10% of the remaining junctional length was comprised of segments composed of condensed TJ strands (data not shown). In K⁺-depleted cells the frequency of segments with condensed strands increased to 20%, which upon K⁺ repletion returned to ~8% of the total TJ length.

To test whether the rearrangement of TJ strands upon inhibition of Na-K-ATPase is associated with any changes in TJ protein levels, we performed immunoblot analysis of ZO-1, occludin, and claudin-4 (Fig. 4A). We did not find any substantial differences in the levels of either of these TJ proteins or in the levels of the adherens junction proteins E-cadherin and β-catenin, both of which have been shown to regulate TJs (Fig. 4A). Furthermore, there was no difference in β-catenin immunofluorescence staining pattern and intensity (Fig. 2B), β-catenin tyrosine phosphorylation (data not shown), or the amounts of β-catenin coimmunoprecipitating with E-cadherin.
between control and Na-K-ATPase inhibited cells (data not shown), suggesting that increased TJ permeability in Na-K-ATPase inhibited cells might not be due to compromised adherens junction function.

Since we did not observe any change in TJ protein expression, we checked for posttranslational modifications in TJ proteins. In MDCK cells, occludin migrates as multiple band clusters of slow-migrating high-molecular-mass occludin forms that are phosphorylated on Ser residues and of fast-migrating, low-molecular-mass, dephosphorylated occludin species (37). In control HPAF-II cells, occludin migrated as a doublet (Fig. 4A). Following inhibition of Na-K-ATPase, the intensity of the high-molecular-mass occludin form increased with a concomitant decrease in the low-molecular-mass occludin form (Fig. 4A, lanes 2 and 6). To test whether occludin is being hyperphosphorylated upon inhibition of Na-K-ATPase, we treated occludin immunoprecipitates with λ-protein phosphatase (λ-PPase) before immunoblot analysis. In the absence of λ-PPase, control cells showed two distinct bands (Fig. 4B). Upon λ-PPase treatment, the molecular mass of the upper band (arrow) shifted to a mass similar to the lower band, confirming that the upper band is the hyperphosphorylated form of occludin. In K⁺-depleted cells, the lower mass band almost completely disappeared, indicating that most of the occludin in Na-K-ATPase inhibited cells was hyperphosphorylated, as assessed by its sensitivity to λ-PPase treatment. In K⁺-repleted cells, the intensity and phosphorylation of occludin was similar to that of untreated control cells. Ouabain showed a dose-dependent effect in the levels of hyperphosphorylated occludin. We observed a similar occludin hyperphosphorylation in several other human epithelial cell lines with inhibited Na-K-ATPase function (data not shown). Although altered phosphorylation of other TJ proteins has been shown to be associated with compromised TJ function (2), we did not observe hyperphosphorylation of claudin-4 (Fig. 4C) or ZO-1 (data not shown) under electrophoretic conditions in which a molecular mass shift upon phosphorylation of both of these proteins has been observed previously (17). These data indicated that increased TJ permeability in Na-K-ATPase-inhibited HPAF-II cells might be associated with hyperphosphorylation of occludin.

Occludin has been shown to be phosphorylated at serine/threonine as well as tyrosine residues and is a target for a number of protein kinases and protein phosphatases (9). Immunoprecipitation of occludin and immunoblotting by use of anti-phosphotyrosine antibody revealed no band, suggesting that increased phosphorylation of occludin in Na-K-ATPase inhibited cells is not due to tyrosine phosphorylation (data not shown). A previous report by Nunbhakdi-Craig et al. (17) had identified occludin as a target of aPKCζ-mediated serine/threonine phosphorylation in MDCK cells. However, Na-K-ATPase inhibition in HPAF-II cells did not change aPKCζ activity, and we were not able to confirm that occludin hyperphosphorylation upon Na-K-ATPase inhibition in these cells was due to increased aPKCζ activity (data not shown). To identify other kinases possibly involved in the phosphorylation of occludin, we treated HPAF-II cells with inhibitors of kinases such as PKC (bisindolylmaleimide), Erk1/2 (PD98059) as well as the phosphatidylinositol 3-kinase pathway (LY294002), before the inhibition of Na-K-ATPase. None of the above mentioned inhibitors could prevent the hyperphosphorylation of occludin in Na-K-ATPase inhibited HPAF-II cells (data not shown).
Next, we decided to test the role of PP2A, since recent studies revealed that PP2A is localized to TJs and is involved in the regulation of the TJ function in mammalian cells (17). In HPAF-II cells, the specific PP2A inhibitor calyculin A caused a dose-dependent increase in occludin phosphorylation (Fig. 5A), and at 25 nM concentration most of the occludin was phosphorylated. The effect of calyculin on occludin phosphorylation was reversible following washout of the inhibitor (Fig. 5B). As observed in Na-K-ATPase inhibited cells, occludin hyperphosphorylation in calyculin A-treated cells was accompanied by increased TJ permeability (data not shown). These results suggested that inhibition of PP2A might be involved in the hyperphosphorylation of occludin, and we tested PP2A activity in Na-K-ATPase function-compromised cells using a spectrophotometric enzyme assay. In control cells, total PP2A activity in PP2A catalytic subunit immunoprecipitates was determined as 6.85 ± 0.33 pmol of phosphate release, which was reduced to 4.11 ± 0.40 pmol of phosphate in immunoprecipitates of K⁺-depleted cells, a value similar to the activity in calyculin A-treated cells (Fig. 5C). The inhibition of PP2A activity by K⁺ depletion was reversible upon addition of K⁺. These results indicated that inhibition of Na-K-ATPase results in reduced PP2A activity, which in turn increases the occludin phosphorylation, leading to increased TJ permeability. Since PP2A is localized to TJs, we hypothesized that occludin might directly bind to PP2A to regulate the phosphorylation of this protein. However, our coimmunoprecipitation experiments did not detect PP2A associated with occludin (data not shown). Strikingly, we found that PP2A readily coimmunoprecipitated with NaK-β and to a lesser extent with NaK-α, whereas control IgG did not show any binding (Fig. 5D). It is possible that reduced NaK-α binding to PP2A is due to reduced immunoprecipitation efficiency of the NaK-α subunit antibody. To rule out this possibility, we tested the association of PP2A with NaK-α using a PP2A catalytic subunit antibody for coimmunoprecipitation analysis. NaK-α was clearly detected in the PP2A immunoprecipitates. GST-pull-down assays further confirmed that PP2A associates with the NH₂-terminus of NaK-β in cell lysates of HPAF-II cells (Fig. 5E). The association of NaK-β with PP2A was found in both control and Na-K-ATPase inhibited cells (data not shown). Although the NH₂-terminus of NaK-α did not pull down PP2A, a recent study revealed that the NaK-α loop 4–5 binds to PP2A in a yeast two-hybrid screen (18). These results indicate that PP2A is in a complex with Na-K-ATPase and that normal Na-K-ATPase function is necessary to maintain PP2A activity.

Since PP2A is localized to TJs and since it forms a complex with Na-K-ATPase, we tested whether Na-K-ATPase is also

Fig. 2. Localization of tight junction proteins and epithelial polarity upon inhibition of Na-K-ATPase. A: immunofluorescence of TJ marker proteins zonula occludens (ZO)-1, occludin, and claudin-4 reveal similar staining patterns in control, K⁺-depleted (-K⁺) (3 h), K⁺-repleted (-K⁺/+K⁺) (3 h depletion, 12 h repletion), and ouabain-treated (50 μM) (7 h) HPAF-II cells. B: polarized distribution of the basolateral marker protein β-catenin in confocal XY and XZ (vertical) sections in control and Na-K-ATPase inhibited cells. Bars, 10 μm.
localized to the TJs using immunogold labeling and electron microscopy. NaK-β (10-nm gold particles) and occludin (5-nm gold particles) distinctly codistributed at the apical junctions that include both tight and adherens junction regions (Fig. 6 C). In addition and as expected, NaK-β was also localized to the entire lateral plasma membrane but clearly excluded from the desmosomes or the apical plasma membrane, indicating a specific immunolabeling (Fig. 6, A and C). Quantitative analysis of the electron micrographs revealed a threefold difference in NaK-β labeling intensity with $1.7 \pm 0.3$ gold particles per unit area detected at the apical junctions vs. $5.6 \pm 0.6$ per unit area localized to the basolateral plasma membrane below the adherens junction (Fig. 6, B and C). The intensity labeling for occludin was $6.4 \pm 0.5$ gold particles per unit area in the apical junction region and $1.1 \pm 0.3$ gold on the lateral membrane. The nonspecific areas contained $0.1 \pm 0.1$ of each 10-nm and 5-nm gold particles per area unit, suggesting specific immunolabeling of NaK-β and occludin on the plasma membrane domains. We were not able to confirm the localization of NaK-α to the TJ region owing to the failure of our antibody to detect NaK-α by immuno-EM. How-

Fig. 3. Altered tight junction ultrastructure upon Na-K-ATPase inhibition in HPAF-II cells A: transmission electron microscopy of control, K⁺-depleted (−K⁺) (3 h), K⁺-repleted (−K⁺/+K⁺) (3 h depletion, 12 h repletion), and ouabain-treated (50 μM) (7 h) HPAF-II cells. Insets are higher magnification of the TJ regions of each panel. Bar, low magnification, 0.5 μm; Bar, inset, 0.2 μm. B: freeze-fracture replica of control, K⁺-depleted (−K⁺) and K⁺-repleted (−K⁺/+K⁺) HPAF-II cells. Compressed TJ strands in K⁺-depleted cells are indicated (arrowheads). Bars, 80 nm.

Fig. 4. Na-K-ATPase inhibition results in hyperphosphorylation of the tight junction protein occludin. A: immunoblot analysis of 100 μg whole cell lysates from Na-K-ATPase-inhibited HPAF-II cells for TJ proteins ZO-1, occludin, and claudin-4, and adherens junction proteins E-cadherin and β-catenin. *Shift in molecular weight of occludin observed in Na-K-ATPase-inhibited cells. B: occludin is hyperphosphorylated in Na-K-ATPase-inhibited cells. Occludin was immunoprecipitated from control, K⁺-depleted (−K⁺) (3 h), K⁺-repleted (−K⁺/+K⁺) (3 h depletion, 12 h repletion), and ouabain-treated (50 μM) (7 h) HPAF-II cells. The immunoprecipitates were either untreated or treated with λ-protein phosphatase (λ-PPase), separated by SDS-PAGE and immunoblotted for occludin. Arrow indicates phosphorylated occludin. C: λ-PPase treatment of claudin-4 immunoprecipitates does not result in a shift in electrophoretic mobility.
ever, these results strongly indicate that at least NaK-β is localized to the apical junctions in polarized pancreatic epithelial cells (in addition to the basolateral plasma membrane).

**DISCUSSION**

In this study, we provide the first evidence that in mammalian cells Na-K-ATPase is localized to the apical junctional complex and associates with PP2A, a protein known to regulate TJ function and localized to TJs. We demonstrate that inhibition of Na-K-ATPase results in the hyperphosphorylation of occludin in a PP2A-dependent manner. Hyperphosphorylation of occludin was accompanied by altered TJ structure at the electron microscopy level and increased permeability to both ionic and nonionic solutes. Thus, these results demonstrate that normal Na-K-ATPase enzyme activity is necessary for the proper regulation of the phosphorylation status of occludin by PP2A compatible for its function to regulate the permeability of TJs. On the basis of these results we suggest that Na-K-ATPase is a key regulator of the TJ gate function in pancreatic epithelial cells.

Na-K-ATPase appears to regulate TJ permeability through different mechanisms in different cell types. Upon inhibition of Na-K-ATPase activity in MDCK cells, TJ permeability increases but is accompanied by dephosphorylation of occludin (S. A. Rajasekaran, unpublished observations). Interestingly, occludin dephosphorylation in MDCK cells is associated with increased TJ permeability (17). In primary cultures of polarized retinal pigment epithelial cells (RPE), inhibition of Na-K-ATPase decreased TER and increased permeability to non-ionic molecules (21) similar to HPAF II cells. Although there was a striking similarity in the TJ morphology by light and electron microscopy in RPE and HPAF II cells, changes in the...
phosphorylation of occludin were not detected in RPE cells (21). Whether this is due to the apical localization of Na-K-ATPase in RPE cells remains to be determined. In mouse blastocysts, inhibition of Na-K-ATPase by K+/H11001 depletion as well as ouabain treatment increased permeability and compromised localization of ZO-1 and occludin at the plasma membrane (35). Thus it appears whereas Na-K-ATPase has a conserved role in the regulation of TJ function in mammalian cells, the mechanism by which the effect is manifested is distinctly different in different cell types.

During Ca²⁺-induced TJ biogenesis, translocation of occludin and ZO-1 from the cytosol to the plasma membrane is accompanied by increased phosphorylation of occludin (8, 25, 31, 37). Using MDCK cells and a Ca²⁺ switch assay, Nunbhakdi-Craig et al. (17) demonstrated that enhanced PP2A activity prevents TJ assembly whereas inhibition of PP2A increased phosphorylation of occludin, ZO-1, and claudin-1 and promoted localization of these proteins to the TJ and TJ assembly. These studies suggested a critical role for PP2A in the regulation of TJs during their biogenesis. In contrast, our studies were performed in HPAF-II cells with established TJs. Our results reveal that inhibition of PP2A activity increases occludin phosphorylation as observed in the study by Nunbhakdi-Craig et al. but in sharp contrast disrupted TJ structure and function. These differences could be due to diverse signaling in TJ biogenesis and the maintenance of established TJs of fully polarized cells or due to cell type-specific or species-specific differences. Indeed, we observed that in other human epithelial cells such as Caco-2 and RT-4 occludin was hyperphosphorylated upon inhibition of Na-K-ATPase whereas occludin was dephosphorylated in Na-K-ATPase-inhibited MDCK cells of canine origin (data not shown). Since Na-K-ATPase is a key enzyme necessary for the survival of a cell, it is possible that different cell types have evolved different
strategies to adapt to the inhibition of this enzyme. However, these results clearly manifest a critical role for PP2A in the regulation of TJ function. Although recent studies indicate that claudins play a critical role in the structure and functions of TJs (24, 34), the finding that increased occludin phosphorylation significantly alters their permeability suggests that occludin might be involved in the fine regulation of the TJ permeability by integrating signals obtained from Na-K-ATPase via PP2A, either through protein-protein interactions or by responding to an increase in intracellular Na\(^+\) upon inhibition of the pump activity.

One of the striking findings reported in this study is the localization of NaK-\(\beta\) to the apical junctional complex. The quantification of the immunogold labeling indicates that the density of Na-K-ATPase localized to the TJ is less than its density at the basolateral plasma membrane. However, the significance of NaK-\(\beta\) localization at the apical region is not known. The fact that Na-K-ATPase binds to PP2A, a protein localized to TJs and that regulates occludin phosphorylation, suggests that Na-K-ATPase, occludin, and PP2A might be in a complex at the apical junctional region. Consistent with this idea, a recent study indicated that Na-K-ATPase cosediments with fractions enriched in occludin but not ZO-1 and claudin during epithelial polarization (36). These results further support our idea that Na-K-ATPase, PP2A, and occludin might form an independent complex at the TJ region. The Na-K-ATPase-PP2A-occludin complex might form a membrane microdomain involved in cell signaling activity that regulates fine-tuning of TJ permeability. At present, it is not known whether NaK-\(\alpha\) is localized to the TJ as well or whether NaK-\(\beta\) is independently present at TJs. Future experiments are necessary to further validate this point.

This study has relevance to pancreatic diseases such as pancreatic cancer and pancreatic cancer. We showed that in HPAF-II cells Na-K-ATPase is a potent inhibitor of PP2A and its level of inhibition is similar to calyculin A, a specific inhibitor of PP2A, suggesting that in pancreatic epithelial cells Na-K-ATPase plays an important role in the regulation of PP2A activity. Perturbation of Na-K-ATPase function might lead to reduced PP2A activity and might be associated with loss of TJ functions in pancreatic diseases such as pancreatic cancer. Disruption of the TJ paracellular permeability has been implicated in the caerulein-induced acute pancreatitis (7, 26). However, whether this increase in paracellular permeability is associated with changes in PP2A and Na-K-ATPase activity needs to be determined.

In a recent study we presented evidence that NaK-\(\beta\), independently of Na-K-ATPase activity, triggers the formation of a scaffolding complex containing phosphatidylinositol 3-kinase, annexin II, and Rac1, which eventually signals downstream to suppress cell motility (3). In addition to these proteins, it is also known that Na-K-ATPase binds to signaling proteins like IP3R (38), PLC-\(\gamma\)1 (38), and Src (11, 33) and to the spectrin-ankyrin cytoskeleton (14, 16). These results are consistent with the idea that Na-K-ATPase might function as a scaffolding signaling platform. We have shown earlier that the level of NaK-\(\beta\) is reduced in a highly transformed, poorly differentiated pancreatic carcinoma cell line lacking TJs (6). Reduced expression of NaK-\(\beta\) in carcinoma might, therefore, result in the deregulation of this scaffolding complex, leading to loss of TJs and gain of invasive and metastatic behavior of pancreatic cancer cells.

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