Hormonal Regulation of the Polarized Function and Distribution of Na/H Exchange and Na/HCO_3 Cotransport in Cultured Mammary Epithelial Cells

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Abstract. The time course for development of polarized function and morphological distribution of pH regulatory mechanisms has been examined in a mouse mammary epithelial cell line (31EG4). Monolayers grown on permeable supports had tight junctions when grown 3-4 days in the presence of the lactogenic hormones dexamethasone (D, a synthetic glucocorticoid) and insulin (I), or in D, I, and prolactin (P), but there were no tight junctions in the absence of D. Microspectrofluorimetry of the pH-sensitive dye BCECF was used to measure pH (pH_i) in cells mounted in a two-sided perfusion chamber to distinguish pH regulatory activity at the apical and basolateral membranes. Na/H exchange was assayed as the Na-dependent, amiloride-sensitive component of pH_i recovery from an acid load induced by a pulse of NH_3/NH_4-containing solution. When monolayers were grown 3–4 d in the presence of P, D, and I, Na/H exchange was restricted to the basolateral membrane. In contrast, in the absence of P, Na/H exchange was present on both the apical and basolateral membranes. After 5-6 days, in the presence or absence of P, Na/H exchange was present only on the basolateral membrane. An antibody to the NHE-1 isoform of the Na/H exchanger was used to determine its morphological distribution. In all hormone conditions the antibody recognized a protein of approximately 110 kD (Western blot), and confocal immunofluorescence microscopy of this antibody and of an anti-ZO-1 (the marker of the tight junctions) antibody showed that the morphological distribution of the Na/H exchanger was similar to the functional distribution under all hormonal treatments. In addition, a putative Na/HCO_3 cotransport system was monitored as a Na-dependent, amiloride-insensitive pH_i recovery mechanism that was inhibited by 200 μM H_2DIDS. After treatment with D+I (but not with I alone) cotransport appeared exclusively on the basolateral membrane, and the polarized expression of this transporter was not altered by P. We conclude that when mammary cells are grown in D+I-containing media, the Na/H exchanger is expressed initially (i.e., after 3–4 d) on both the apical and basolateral membranes and later (5–6 d) on only the basolateral membrane. P (in the presence of D+I) selectively speeds this polarization, which is determined by polarized distribution of the exchanger to the apical and/or basal membrane and not by the activation and/or inactivation of transporters. Since the Na/HCO_3 cotransporter (which requires D+I for expression and is unaffected by P) is expressed only in the basolateral membrane, we suggest that polarization of different pH regulatory mechanisms may be independently regulated in mammary epithelial cells.

THE polarized distribution of cytoskeletal components and membrane lipids and proteins (13, 29, 38, 39) is essential for an epithelium to perform vectorial transport. Several different mechanisms for development of polarity of membrane proteins have been discovered: direct targeting of newly synthesized proteins from the Golgi complex to the appropriate membrane domain, rerouting of proteins from one membrane domain to the other, and delivery of a protein to both the apical and basolateral domains, with selective inactivation of the protein in one membrane and stabilization on the opposite membrane by interaction with the cytoskeleton (14, 16, 18, 29).

In most polarized epithelia the common pH regulatory proteins Na/H exchange, Na/HCO_3 cotransport, and Cl/HCO_3 exchange are polarized to the basolateral membrane (29, 31, 43, 46). However, one or all of these regulators of intracellular pH (pH_i) is also present in the apical membrane of particular epithelia (e.g., apical Na/H exchange in the intestine [8, 25, 30, 44], renal proximal tubule [12, 20, 24], and the retinal pigment epithelium [23]). In some cases,

one or the other transporter is present simultaneously in both apical and basolateral membranes of the same cell (e.g., the rabbit ileum [19], the salamander renal proximal tubule [4], and the rabbit descending limb [21]). It has also been shown that an H^+ATPase and Cl/HCO_3 exchanger may even reverse polarity in response to changing acid–base conditions (4, 36, 37), although there is some disagreement on this issue (17). In LLC-PK1 cells two pharmacologically distinct Na/H exchangers which polarize to opposite sides of the cell have been identified (5, 6, 15, 45), and the apical Na/H exchangers are probably genetically (32, 34, 43, 44) and physiologically (5, 8, 9, 15, 30) distinct from those in the basolateral membrane.

The mammary epithelium represents a dynamic, alternative model to examine the various mechanisms that develop polarity because in vivo the proliferation, differentiation and vectorial ion transport of this endothelium are regulated by lactogenic hormones (11). During lactation the pH of milk is 6.8, and it is relatively depleted of Na (1, 11). Also, prolactin increases Na absorption in primary cultures of mammary epithelial cells grown on floating collagen gels (1). Thus, it was interesting to test whether lactogenic hormones regulate the polarity and activity of Na-dependent pH_i regulatory mechanisms in the mammary gland.

In the present study we have characterized the time course for development of polarity of two different endogenous pH_i regulatory mechanisms in mouse mammary epithelial monolayers (31EG4 cells), taking advantage of the fact that development of high transepithelial electrical resistance (i.e., tight junctions) in these cells is regulated by glucocorticoids (48). monolayers were grown on permeable supports in the presence of insulin and dexamethasone with or without prolactin over a time course of up to 6 d. Microspectrofluorimetry of the pH-sensitive dye 2′-7′-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein (BCECF)[1] was used to examine the hormonal regulation of the polarization of both Na/H exchange and a Na- and HCO_3-dependent and H_2 DIDS-sensitive pH regulator (which we have termed Na/HCO_3 cotransport) to the apical and basolateral membranes of these cells over this 6-day period. Na/H exchange activity was initially detected on both apical and basolateral membranes and then became polarized to the basolateral side of the cell. Development of this functional polarity was speeded by the lactogenic hormone prolactin. To determine whether the development of this functional polarity was due to changes in activity of the Na/H exchanger or to the physical redistribution of the protein, antibodies to the human Na/H exchanger (anti-NHE-1) and to the tight junction–associated protein ZO-1 were used (with confocal microscopy) to localize the exchanger to the apical and basolateral sides of the cells. The confocal images demonstrated a similar polarization of the Na/H exchanger protein (i.e., relative to the tight junction protein ZO-1) as the functional polarization demonstrated. In contrast, Na/HCO_3 cotransport appeared exclusively on the basolateral side of the cells, and the time course and polarity of its appearance were similar in either the presence or absence of prolactin. All of these changes occurred well after the formation of tight junctions.

1. *Abbreviations used in this paper*: BCECF, 2′,7′-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein; D, dexamethasone; I, insulin; NMG, *N*-methyl-D-glucamine; P, prolactin; pH_i, intracellular pH.

Materials and Methods

Cell Culture, Electrical Resistance, and Paracellular Permeability

The hormonal regulation of development of a tight monolayer of 31EG4 cells has been previously described (48). Briefly, 31EG4 cells were routinely cultured on 100 mm plastic dishes (Corning Inc., Corning, NY) in a humidified, 37°C, 95/5%:air/CO_2 environment, and passaged at 1:6 dilution every 7 d in DME/F12 (50:50) supplemented with 5% FBS, insulin 5 μg/ml, and gentamicin sulfate 50 μg/ml. For experiments to investigate the polarized distribution of pH_i regulatory mechanisms, the cells were seeded at a density of 5,000 cells/mm^2 and grown on permeable supports (0.49-cm^2 area; Anocell 10™ filters; Applied Scientific, San Francisco, CA) in a 24-well plate with 350-μl of medium (DME/F12 [50:50] supplemented with 2% FBS, gentamicin sulfate 50 μg/ml) on the apical side and 650 μl basolaterally. Combinations of insulin (I) (5 μg/ml), dexamethasone (D, a synthetic glucocorticoid) (1 μm), and/or prolactin (P) (5 μg/ml) were added to the media as described in the text. The media were changed daily.

To determine the junctional integrity of the monolayers, transepithelial electrical resistance was measured daily (before feeding) with chopstick electrodes and an EVOM (Epithelial Voltohmmeter; World Precision Instruments, New Haven, CT). For uniformity, measurements were made after the media and cells had equilibrated at room temperature for 15 min. Values were connected for background resistance of the filter and media. As described previously by Zettl et al. (48), cells grown 3–6 d in insulin-containing media (I) had resistances (60 ± 15 Ω·cm^2, n = 12) that were not significantly above those of the filter alone, and transepithelial permeability of the monolayers to mannitol was high: 12% of the [^{14}C] mannitol added to one side permeated to the other side over 4 hours. In contrast, all DI and PDI monolayers used in this study had resistances between 1,000–3,000 Ω·cm^2, and permeabilities of these DI or PDI monolayers to [^{14}C] mannitol were 10-fold lower than the I-treated monolayers (48).

Solutions

A Hepes-buffered Ringer's solution (titrated to pH 7.4 at 37°C and equilibrated with air) was used for all experiments. This solution contained (in mM): 140.0 NaCl, 3.0 KCl, 2.0 KH_2PO_4, 1.0 $MgSO_4$, 1.0 $CaCl_2$, 10.0 glucose, and 10.0 Hepes. To acid load cells for pH regulation studies, 30 mM NH_4Cl was added to the Na-containing Ringer's solution. In Na-free solutions, Na was replaced mole-for-mole by *N*-methyl-D-glucamine (NMG). A high [K] solution (identical to the NaCl-Hepes solution except all the Na was replaced by K) was used for calibration of the BCECF fluorescence in terms of pH_i. In some experiments amiloride (Sigma Immunochemicals, St. Louis, MO) was used at a final concentration of 1 mM, while in others H_2DIDS ([H_2] 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; Molecular Probes, Eugene, OR) was added to the solution as a powder to give a final concentration of 200 μM. For solutions containing both amiloride and H_2DIDS a separate stock solution was made for each compound at double the final concentration, and they were mixed 50:50, vol/vol. Nigericin (Sigma Immunochemicals) was used at a final concentration of 20 μM (from a 10 mM stock solution in dimethylformamide/ethanol 3:1, vol/vol) for calibrating BCECF fluorescence.

Solutions used for gel electrophoresis, Western blotting, and immunomicroscopy were prepared as follows: PBS and PBS with Ca^{2+} and Mg^{2+} were purchased from Biowhittaker Cell (Walkersville, MD). Lysis buffer contained 10 mM Tris, pH 8.0, 1% SDS, Aprotinin (1 μg/ml), pepstatin (1 μg/ml), and PMSF (0.2 μM). TBST-5% contained 50 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20, and 5% non-fat dry milk. For immunomicroscopy the PBS contained Ca^{2+} and Mg^{2+}. In addition, PBS/BSA contained 0.1% BSA and PBS/BSA/Tween contained 0.1% BSA and 0.05% Tween 20.

Loading Cells with BCECF

Monolayers were loaded in growth media containing 10 μM BCECF-AM (from Molecular Probes, in a 10 mM stock solution in DMSF) for 25–30 min at 37°C in 5% CO_2/95% air atmosphere. The filters were washed free of dye with the standard Na Ringer's solution and mounted in the chamber with continuous perfusion for 10–20 min to compensate for inhibitory effects caused by the dye loading process (27). Before measurements of pH_i were begun, monolayers were inspected in brightfield for confluency. There were no apparent changes as a result of the dye loading procedure, and the resistance of the monolayers did not change as a consequence of BCECF

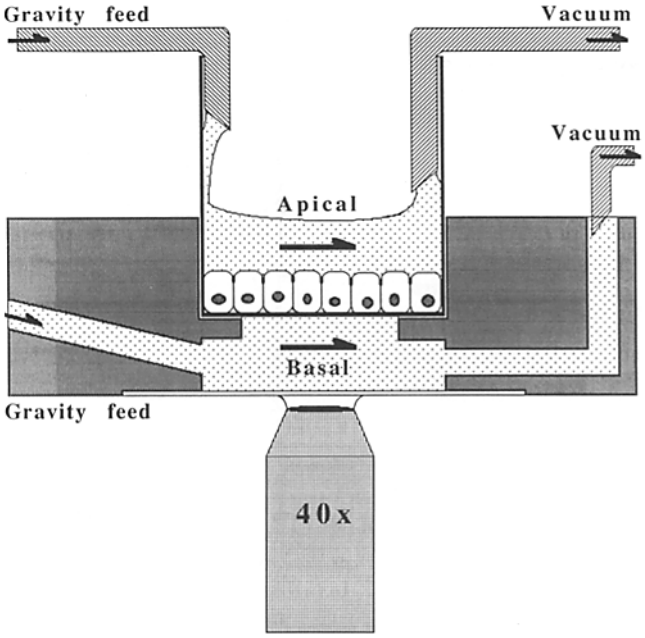

Figure 1. Schematic drawing of the chamber used for the present study. Immediately before experiments the feet were cut off the bottom of Anocell 10™ filters, and they were sealed into the chamber with vacuum grease. The chamber was placed in a 37 ± 1°C water-jacketed holder on the stage of an inverted microscope. A 40× objective focused on the cells in the monolayer across a glass coverslip, the solution in the basal chamber, and the filter. The apical chamber was the filter insert itself. See text for details.

loading. In epifluorescence BCECF was evenly distributed throughout the cytoplasm.

Mounting Monolayers in a Double-Sided Perfusion Chamber

The chamber used in this study (see Fig. 1) was constructed out of Kel-F (A.I.N. Plastics Inc., Berkeley, CA) and designed to fit an intact Anocell 10™ filter. The Anocell 10™ filter was chosen because it is constructed with aluminum oxides (similar to, but harder than, glass) instead of polycarbonate. These filters are optically clear and also do not flex during dual-sided perfusion. Before each experiment the resistance of the monolayer on the filter was measured to insure confluency, and the feet were trimmed off the bottom of the filter with a razor blade. The filter was then mounted in the chamber, sealed on the perimeter with vacuum grease, and placed in a water-jacketed brass holder that maintained a temperature of 37 ± 1°C on the stage of a Zeiss IM35 microscope (Carl Zeiss, Oberkochen, Germany). Various gravity-driven perfusion solutions were kept in 60 ml syringes connected to two eight-way Hamilton valves (one for apical solutions and the other for basal solutions) mounted in a heated (40°C) box. The runoff of the solution was collected by aspiration. The rate of perfusion of the apical (150 μl) and basal (30 μl) solutions was adjusted so that the turnover of each chamber occurred every 2–3 s. The perfusion syringes were refilled at the same rate they were drained by a second set of syringes suspended above them to maintain constant, and equal, hydrostatic pressures.

Microspectrofluorimetry of BCECF and pH_i Calibration

The methods for using BCECF to measure pH_i have been described previously (26, 47). Once the cells had been loaded with the dye and the filter was mounted in the chamber on the stage of the microscope, dye was excited by a 75 W xenon bulb, and the appropriate wavelength was selected by alternating two filters (440 ± 5 and 490 ± 5 nm) mounted in a paddle wheel driven by a stepping motor at 2 Hz. The excitation beam passed through a neutral density filter and an iris aperture to confine the illuminated field to the desired size. A Zeiss water-immersion objective (40×, 0.75 numerical aperture) provided the necessary working distance to focus on the monolayer across the coverslip, basal-side perfusion chamber and the filter (~1.2 mm). The emitted fluorescence intensity passed through a 520–550-nm bandpass emission filter and an image plane pinhole, which limited the collected light to a group of 10–15 cells, before hitting a photomultiplier tube. An IBM/AT computer and UMANS-software (by Chester M. Regen, Biorad, Cambridge, MA) controlled the stepping motor and collected, stored, and processed the data. Background fluorescence of unloaded monolayers on the filter was <5% of either the 490- or 440-nm signal from loaded cells, so no correction was made for background fluorescence.

After some experiments an in situ calibration of pH_i from the BCECF 490/440 ratio was performed using the high [K]-nigericin technique (28, 41). Average calibration curves and resting pH_i's were determined on three to five fields in several different monolayers for each hormone condition, and these calibration curves were used for other experiments in which calibration curves were not determined. This was justified because BCECF yielded a linear relationship between pH_i and the fluorescence intensity ratio (490/440) between pH_i 6.4–7.8 (data not shown). The control pH_i for cells in each hormone condition was determined on day 4: 1 = 7.43 ± 0.02 (n = 10), DI = 7.40 ± 0.04 (n = 9), and PDI = 739 ± 0.02 (n = 11). There was no significant difference among the resting pH_i values for any of the experimental conditions.

Confocal Immunofluorescence Microscopy Using Anti-NHE-1 and Anti-ZO-1 Antibodies

We used an affinity-purified antibody raised against a cytoplasmic segment of the human Na/H exchanger (so-called NHE-1 [34, 35, 43], a gift from Dr. Sergio Grinstein, Hospital for Sick Children, University of Toronto, Toronto, Canada) and confocal immunomicroscopy to localize the Na/H exchanger in 31EG4 cells to the apical and/or basolateral sides of the monolayer. Optical slices and the resulting images were obtained from the apical side down through the cells in 0.5-μM sections to the basolateral side of the monolayer. Since one of the main goals was to determine the apical versus basolateral distribution of the exchanger, cells were stained with antibodies to both ZO-1 (to determine the location of the tight junctions, which define the boundary between the apical, and basolateral membranes of the cells) and to NHE-1 to localize the Na/H exchanger.

Polyclonal antibodies against the NHE-1 isoform of the Na/H exchanger were raised by injecting rabbits with a fusion protein constructed with a β-galactosidase of *Escherichia coli* and the last 157 amino acids of the human transporter (35). The antibodies were affinity purified by adsorption to nitrocellulose strips containing the fusion protein, followed by elution in 0.2M glycine, pH 2.2, and rapid neutralization with Tris. The affinity-purified antibodies were stored at −20°C in 0.2% gelatin with 50% glycerol.

Anti-ZO-1 antibodies were used to show the tight junction (40). These antibodies were isolated from hybridoma supernatant (R26.4C; The Developmental Studies Hybridoma Bank, Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and Department of Biology, University of Iowa, Iowa City, IA, under contract NOl-HD-6-2915 from the NICHD). Hybridomas were cultured in spinner flasks in RPMI plus nutridoma for 9 d. The supernatant was enriched by a 50% NH_3SO_4 cut. It was then dialyzed against 0.2 M Na/HCO_3, pH 8.5, and stored in 0.5 M NaCl with NaN_3.

Immunolocalization, using the anti-NHE-1 and anti-ZO-1 antibodies and confocal laser scanning microscopy, was performed on monolayers that were treated identically to those used for studying pH_i regulation in the dual-perfusion chamber. Monolayers were grown on filters in DI or PDI conditions for 3–4 or 5–6 d, washed three times in PBS with Ca^{2+} and Mg^{2+}, fixed in acetone/methanol (50:50, vol/vol) at −20°C for 5 min, air dried, and then incubated with PBS/BSA/Tween. The fixed monolayers were then washed three times in PBS with Ca^{2+} and Mg^{2+} and incubated for 1 h in the PBS/BSA solution containing both the anti-NHE-1 antibody (diluted 1:200) and the anti-ZO-1 antibody (diluted 1:50).

Monolayers were then washed two more times in PBS/BSA/Tween and once in PBS/BSA. This was followed by a 1-h incubation with FITC-labeled goat anti-rat IgG (Sigma Immunochemicals; 1:200 in PBS/BSA) to visualize ZO-1. Finally, the cells were incubated 1 h with biotin-labeled goat anti-rabbit IgG (1:200 in PBS/BSA) followed by three washes in PBS/BSA and a 30-min incubation with Texas red-labeled avidin (diluted 1:1,000 in a buffer containing 150 mM NaCl and 10 mM Hepes) to visualize the Na/H exchanger. The biotin-avidin Vectastain ABC kit was purchased from Vector Laboratories (Burlingame, CA). The monolayers were then washed

three times in PBS/BSA and once in PBS/BSA/Tween, removed from the plastic culture insert and mounted on slides with Hanker-Yates, an antibleaching agent (Polysciences, Inc., Warrington, PA).

Confocal images were obtained with an MRC 600 system coupled to a Zeiss Axioplan epifluorescence microscope using a Zeiss 40x Plan-Neofluar multi-immersion objective (0.9 NA). Paired images were obtained with a dual filter set for fluorescein and Texas red. To illustrate the spatial relationship between the two different labels in the same specimen (in this case fluorescein for ZO-1, and Texas red for NHE-1) paired series of optical sections were collected, one for each label. These images were subsequently superimposed and assigned pseudocolors representing the original color of the label used for immunofluorescence.

For each specimen a series of digitized optical sections was collected starting from just above the apical membrane stepping toward the basal side of the monolayer and the filter in 0.5-μm steps. After contrast enhancement, several sections were reassembled to generate images representing thicker sections of the specimen above, in the plane, and below the region of the tight junction (i.e., ZO-1 staining). Projections representing a 1.5-μm-thick section above ZO-1 were constructed from the three images obtained immediately before the appearance of ZO-1 (e.g., see Fig. 8, *a*, *e*, *h*, and *k*). In the plane of ZO-1, eight sections (4.0 μm) were reassembled into one image (Fig. 8, *f*, *i*, and *l*), except for Fig. 8, *b* and *c*, where the image in the plane of ZO-1 was split into two four-section images to show the distribution of the Na/H exchanger in this critical region. Below ZO-1 three sections were reassembled to represent a 1.5-μm-thick section of the lateral membrane (see Fig. 8, *d*, *g*, *j*, and *m*). The reassembled projections were then superimposed and colorized. All sections from 1.5-μm above the first appearance of ZO-1 through the 1.5-μm below ZO-1 were used to construct the reassembled images, and all images were processed identically. The final color confocal micrographs were printed with a Kodak XL-7700 dye-sublimation printer (Eastman Kodak Co., Rochester, NY).

Gel Electrophoresis and Western Blotting

31EG4 cells were grown on permeable filter supports for 4 d, washed twice with PBS (with Ca^{2+} and Mg^{2+}), twice more with PBS, and then harvested with hot (95°C) lysis buffer. The harvested cells were then boiled for an additional four minutes. Extracts were sheared with 10 passages through a 26-gauge needle. Protein content was determined by DC (detergent compatible) protein assay (Bio-Rad Laboratories, Cambridge, MA). For each hormonal treatment 25 μg of cell extract was subjected to 8% SDS-PAGE (22) and then transferred to nitro ME (Micron Separations, Inc., Westboro) (42). Nitrocellulose was blocked overnight at 4°C with TBST-5%. Primary rabbit antibody directed against NHE-1 was diluted to 1:500 in TBST-1% and incubated overnight at 4°C; secondary antibody directed against rabbit IgG and conjugated to HRP (Cappel, Malvern, PA) was diluted 1:6,000 in TBST-1% and incubated for 1 h. Further washes were performed with TBST-1%. Detection of the signal was by ECL on Hyperfilm-ECM (Amersham Corp., Arlington Heights, IL), 1-2-min exposure.

Statistics

Where applicable, values have been presented as means ± S.E.M.

Results

Dual Perfusion Microfluorimetry on I-treated, Leaky Monolayers

To investigate the polarity of Na/H exchange, the sidedness of Na- and amiloride-dependent pH_i recovery was monitored following an acid load. A typical experiment on I-treated cells is shown in Fig. 2. As shown in the inset, changes of apical and basolateral solutions were denoted by designations above and below, respectively, the pH_i trace. This format has been used for all of the figures shown. 31EG4 mammary cell monolayers were acidified with a basal prepulse of 30 mM NH_4Cl^* and subsequent treatment with Na-free solutions on both sides of the monolayer. (Cells acidified to a greater extent when NH_4 was pulsed on only one side of the monolayer than when NH_4 was applied to both sides. The more rapid acidification was presumably due to an accumulation of H's, resulting from the entry of NH_4 across the basolateral membrane and concurrent exit of NH_3, but not H, across the apical membrane. This protocol reduced the total time of NH_4 exposure required for cellular acidification and was used in all of the experiments shown.) When Na was added to the apical side, pH_i recovered, and this effect was completely blocked by application of amiloride to either the apical or the basolateral side of the cells. Thus, pH_i recovery induced by application of Na solution to one side of the monolayer was inhibited by amiloride applied to the opposite side. These results showed that these I-treated cells did indeed have a Na/H exchanger, but the high paracellular permeability of the monolayer (see Materials and Methods and reference 48) made it impossible to determine whether the Na/H exchanger was polarized to the apical or basolateral membrane.

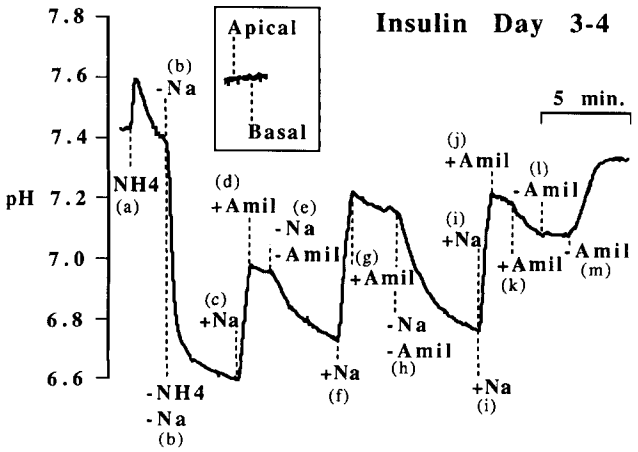

Figure 2. Na/H exchange is present in monolayers cultured for 4 d in insulin-containing media, but functional polarity cannot be detected. As shown in the inset, changes of apical and basolateral solutions were denoted by designations above and below, respectively, the pH_i trace. This format has been used for all of the figures shown. The "+" and "−" signs refer to addition or removal, respectively, of 30 mM NH_4Cl, 1 mM amiloride or Na Ringer's (in this case "−Na" refers to treatment with Na-free Ringer's solution). Cells were acidified by treatment with a basal prepulse of 30 mM NH_4Cl (*a*) and subsequent perfusion on both sides with Na-free solutions (*b*). Returning Na Ringer's to the apical perfusate (*c*) induced a rapid pH_i recovery which was entirely inhibited by addition of 1 mM amiloride to the apical perfusate (*d*). There was a drop in pH_i when amiloride was removed and Na-free solution simultaneously replaced the normal Na Ringer's (*e*). Application of Na Ringer's to the basal side (*f*) induced a second rapid recovery which was largely inhibited by application of amiloride to the basal side (*g*). Subsequent removal of amiloride and Na resulted in another rapid drop in pH_i (*h*). Simultaneous addition of Na to both the apical and basal sides resulted in a rapid pH_i recovery (*i*) that was completely inhibited by application of amiloride to only the apical side (*j*). There was a small drop in pH_i when amiloride was added to the basal perfusate (*k*), and pH_i did not recover when apical amiloride was removed (*l*). Removal of basal amiloride resulted in complete recovery to resting pHi (*m*). This trace is representative of five similar experiments on 3- or 4-d-old monolayers.

Polarity of Na-dependent pH_i Regulators in Tight, 3–4-d-old DI-treated Monolayers

After only 3–4 d in culture with dexamethasone and insulin (DI), 31EG4 cells had differentiated into monolayers with

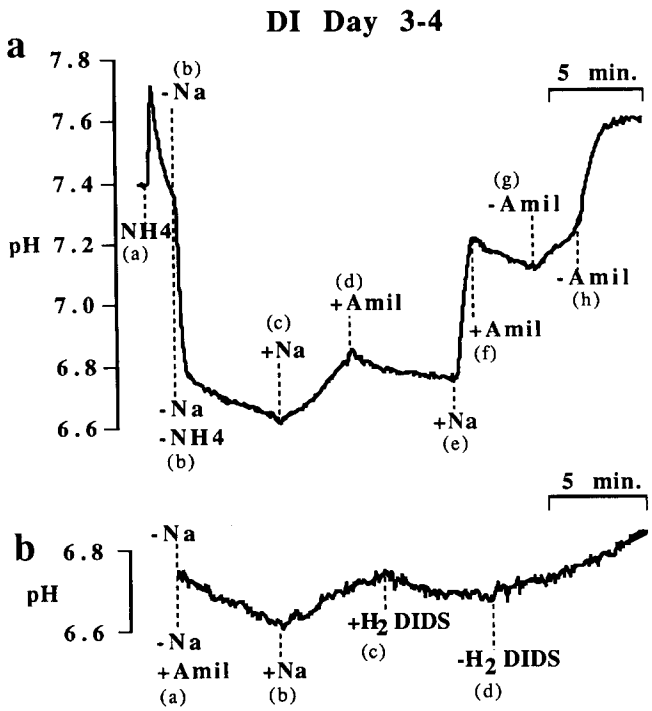

Figure 3. Cells grown for 4 d in DI have Na/H exchange in both apical and basal membranes. A similar protocol to Fig. 2 was used. Cells were acidified by perfusing the basal side with 30 mM NH_4Cl (*a*) followed by removal of NH_4Cl and perfusion of both sides of the monolayer with Na-free Ringer's (*b*). In Na-free solutions the cells continued to acidify slowly for some time. Readdition of Na Ringer's to the apical perfusate (*c*) induced a pH_i recovery that was completely inhibited by 1 mM amiloride applied to the same (*apical*) side (*d*). In the continued presence of apical amiloride, addition of Na solution to the basal perfusate (*e*) induced a second rapid pH_i recovery, which was entirely inhibited (in fact, slightly reversed) by 1 mM amiloride added to the basal perfusate (*f*). Subsequent removal of amiloride from only the apical perfusate (*g*) resulted in a pH_i recovery even though amiloride was present in the basal perfusate. Finally, when amiloride was removed from the basal perfusate (*h*), there was an increase in the pH_i recovery rate resulting in a return to near resting pH_i. Trace is representative of 21 similar experiments on 3- or 4-d-old monolayers. (*b*) 75% of the experiments conducted on 4 d DI monolayers demonstrated a basal Na-dependent, amiloride-insensitive component of pH_i recovery that was blocked by H_2DIDS, the well known inhibitor of Na/HCO_3 cotransport. The trace begins following an NH_4Cl pulse with Na-free solutions bathing the apical side and Na-free plus 1 mM amiloride solutions on the basal side (*a*). Basal addition of Na Ringer's in the presence of amiloride (*b*) induced a slow pH_i recovery. Further, addition of 200 μM H_2DIDS to the basal solution (*c*) reversed pH_i recovery, and this effect was reversible upon wash-out of H_2DIDS (*d*). Trace is representative of five similar experiments.

resistances of 1,000–3,000$\Omega \cdot cm^2$ and very low paracellular permeability, indicative of the formation of functional tight junctions (48). Fig. 3 *a* shows an experiment with one of these DI monolayers. After the acid loading treatment with NH_4 and perfusion with Na-free solutions on both the apical and basal sides, addition of Na Ringer's to the apical side caused pH_i to recover towards the control level, and this effect was inhibited by amiloride addition to the apical side. When Na solutions were perfused onto the basal side (in the presence of apical amiloride), pH_i recovered, and this effect was blocked by basal amiloride. This experiment shows that the monolayer was tight to both Na and amiloride and that Na/H exchange was present on both the apical and basal membranes. Using this same type of protocol on other 3–4 d DI-treated monolayers, we found Na/H exchange on the apical side in 83% of the cells and on the basolateral membrane in 100% of the cells (Table I).

Table I.

		Day 3–4	Day 5–6
Na/H exchange			
DI	Ap	83% (24/29)	9% (1/11)
	Bl	100% (32/32)	100% (10/10)
PDI	Ap	11% (2/17)	0% (0/9)
	Bl	100% (38/38)	100% (10/10)
Na/HCO_3 cotransport			
DI	Ap	0% (0/15)	0% (0/8)
	Bl	75% (12/16)	100% (7/7)
PDI	Ap	0% (0/12)	0% (0/5)
	Bl	72% (18/25)	100% (6/6)

Values in parentheses are the number of times Na/H exchange or Na/HCO_3 cotransport was detected in the apical or basolateral membranes, followed by the number of times tested. The percent values were calculated by dividing the first number (times detected) by the second (times tested), and the percentages represent an estimate of the probability of finding these mechanisms at the apical (*Ap*) or basal (*Bl*) membrane at either day 3–4 or day 5–6.

Other experiments demonstrated a Na-dependent, amiloride-insensitive transporter present on the basolateral (but not the apical) membrane of 75% of the 3–4 d DI-treated monolayers (see Fig. 3 *b*). In monolayers that had been acidified by NH_4Cl treatment and perfused with Na-free solutions, addition of Na solution to the basal side in the presence of amiloride often induced pH_i recovery (12 out of 16 experiments). The rate of pH_i recovery increased further when amiloride was removed from the basolateral solution, indicating that Na/H exchange was also present on the basolateral membrane. Further, as shown in Fig. 3 *b*, when the monolayers were treated with Na solution + amiloride + 200 μM H_2DIDS, the well known blocker of Na/HCO_3 cotransport (2), pH_i recovery was totally blocked. (According to the known models of pH_i regulation, the Na-dependent, amiloride-insensitive pH_i recovery from an acid load could be due to either Na/HCO_3 cotransport (3) or Na-dependent Cl/HCO_3 exchange (33). If the Na-dependent, amiloride-insensitive recovery were due to Na-dependent Cl/HCO_3 exchange, and then reducing (Cl) in the bathing solution should cause pH_i to increase as Cl exits the cell and HCO_3 enters. This maneuver had absolutely no effect on pH_i of 31EG4 cells (not shown). We have therefore concluded that 31EG4 cells have neither Na-independent nor Na-dependent Cl/HCO_3 exchange and that the Na-dependent, amiloride-insensitive, H_2DIDS-inhibitable pH_i recovery shown in Figs. 3 *a* and 4 *b* is due to the presence of Na/HCO_3 cotransport on the basolateral membrane.) For simplicity of discussion we will refer throughout the text to this mechanism as a Na/HCO_3 cotransporter, though we realize that the Na and HCO_3 dependency and H_2DIDS sensitivity are alone insufficient evidence to make a firm conclusion regarding the specific mechanism involved.

This putative Na/HCO_3 cotransporter was never present on the apical membrane of 31EG4 cells, but it was present

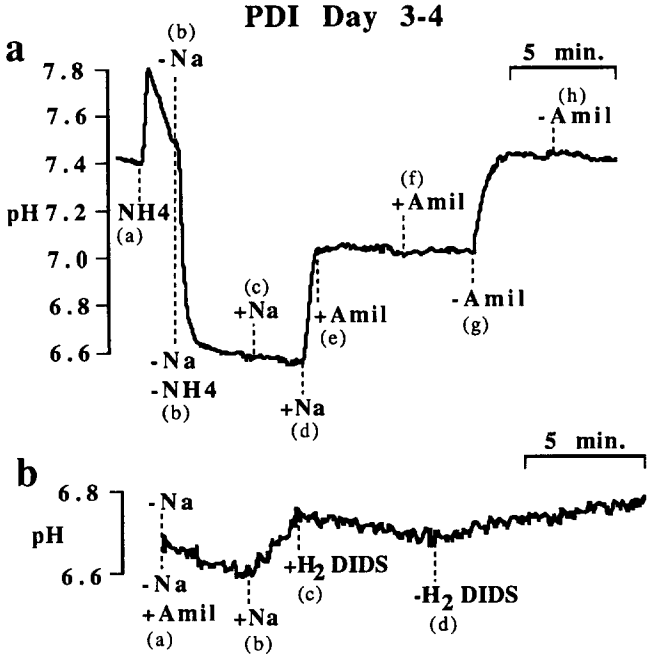

Figure 4. Monolayers grown for 4 d in PDI have Na/H exchange only in the basolateral membrane. These cells were treated identically to the cells in Fig. 3. except that P was present with D and I in the growth media. Cells were acidified with NH_4Cl in the basal perfusate (*a*) and subsequent removal of NH_4Cl and perfusion with Na-free solutions on both the apical and basal sides (*b*). When Na Ringer's was perfused on the apical side (point c), pH_i did not increase. Perfusion of Na solution on the basal side (*d*) caused a rapid increase in pH_i, which was entirely inhibited by basal application of amiloride (*e*). Further application of amiloride to the apical side in the presence of basal amiloride (*f*) had no effect on pH_i. Finally, when amiloride was removed from the basal perfusate (*g*), there was a rapid pHi recovery, while removal of amiloride from the apical perfusate (*h*) had no effect on pH_i. Trace is an example of 13 similar experiments on 3- or 4-d-old monolayers. (b) 72% of the experiments conducted on day 4 PDI monolayers demonstrated a Na-dependent, amiloride-insensitive component of pH_i recovery that was blocked by H_2DIDS. The trace begins following acidification with NH_4Cl (see Figs. 2–4) with Na-free solutions bathing the apical side and Na-free plus 1 mM amiloride solutions on the basal side (*a*). Basal addition of Na Ringer's in the presence of amiloride (*b*) induced a slow pH_i recovery. Further addition of 200 μM H_2DIDS to the basal solution (*c*) reversed pH_i recovery, and this effect was reversible upon wash-out of H_2DIDS (*d*). Trace is representative of four similar experiments.

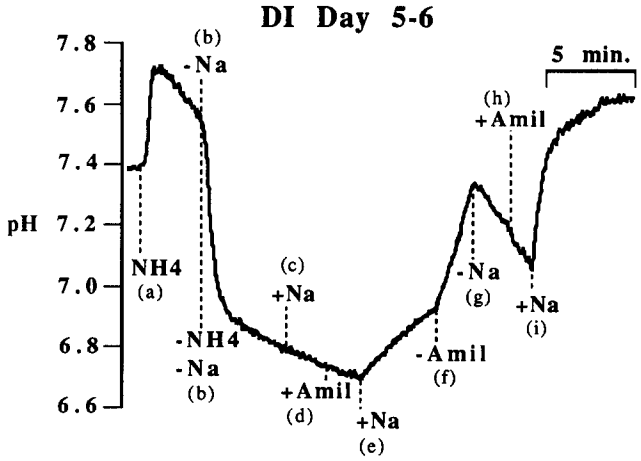

Figure 5. Monolayers cultured for five d in DI have Na/H exchange as well as another Na-dependent, amiloride-resistant mechanism in the basolateral membrane only. Cells were treated with NH_4Cl (*a*) and then perfused with Na-free solution on both apical and basal sides (*b*). Subsequent perfusion with Na solution on the apical side (*c*) and with amiloride on the basal side (*d*) had no effect on pH_i. In the presence of basal amiloride and apical Na, perfusion with Na Ringer's on the basal side (*e*) caused a slow pH_i recovery. Removal of the basal amiloride (*f*) caused a rapid recovery that was reversed by changing the basal solution to Na-free (*g*). Subsequent application of apical amiloride (*h*) did not affect pH_i, nor did it inhibit the complete pH_i recovery upon the readdition of Na Ringer's to the basal perfusate (*i*). Note that in the presence of Na solutions containing amiloride (*e-f*), pH_i still recovered toward baseline at a considerable rate (see text for details). Trace is representative of eight similar experiments on 5- or 6-d-old monolayers.

on the basolateral membrane in 75% of the 3–4 d DI-treated monolayers (Table I). The Na/HCO_3 cotransporter was never observed in I-treated monolayers (Fig. 1).

Two other aspects of these experiments should be noted. First, in those cells in which Na/H exchange was present on the apical side, the relative activity of the exchanger (based on rates of pHi recovery at pH_i 6.8) was, on average, about 10-times higher on the basolateral side of the cells. We do not know if this difference in rate is due to functional inactivation, different density of expression of the exchanger on the two membranes, or that the basolateral membrane has a larger surface area than the apical membrane. In those monolayers that exhibited basal Na/HCO_3 cotransport, the rate of pH_i recovery due to the basolateral Na/H exchanger was about 10 times that of the basolateral Na/HCO_3 cotransporter. Because our experiments were conducted in air-equilibrated solutions that were nominally HCO_3-free (where $[HCO_3] = 250$ μM), the rates of pH_i recovery due to the Na/HCO_3 cotransporter are slower than they would be in HCO_3-containing solutions. In experiments conducted on 3–4 d DI monolayers using solutions containing 25 mM HCO_3 and 5% CO_2 gas (pH 7.4), the rates of pH_i recovery due to the Na/HCO_3 cotransporter were 10–15-times faster than those observed in the experiments using Hepes-buffered, HCO_3-free solutions. However, even in experiments conducted in HCO_3-containing solutions, there was no evidence for Na/HCO_3 cotransport on the apical side of the cells. Thus, the conclusions about targeting and expression of functional Na/HCO_3 cotransporters studied here in Hepes-buffered solutions are also valid for HCO_3-containing solutions.

Second, the amiloride sensitivity of apical and basal Na/H exchange appeared to be approximately the same. In seven different experiments, cells were acidified in the standard way, treated with Na Ringer's containing 1 mM amiloride, and then the (amiloride) was sequentially lowered in either the apical or basolateral solution. When 3–4 d DI cells were acidified, 50 μM amiloride completely inhibited the Na-induced pH_i recovery on either side of the monolayer, and recovery occurred when this concentration of amiloride was washed out of the solution (data not shown). Similarly, 50 μM amiloride completely inhibited the basal exchanger in PDI cells (data not shown). In renal and intestinal epithelia, where distinct apical and basal Na/H exchange mechanisms have been identified (5, 6, 15, 19, 43, 44), the apical ex-

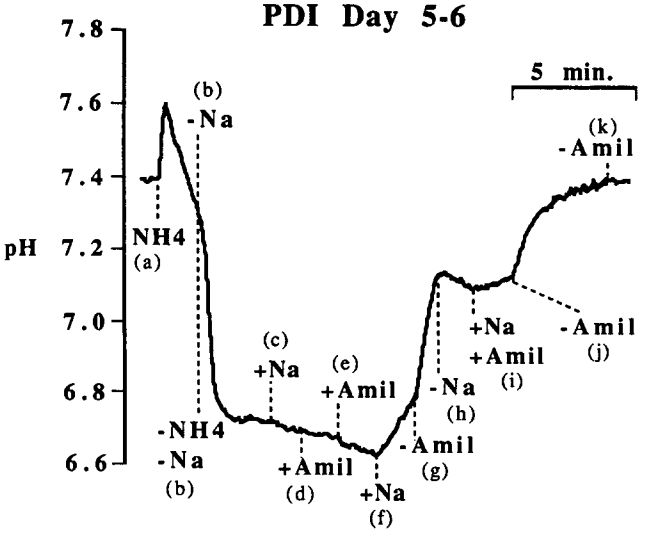

Figure 6. Monolayers cultured for 5 d in PDI still have Na/H exchange and a Na-dependent, amiloride-resistant mechanism in the basolateral membrane only. After NH_4Cl treatment (*a*) and then perfusion with Na-free solutions on both sides (*b*), cells acidified. Apical Na Ringer's (*c*) had no effect on pH_i, even upon the subsequent addition of basal amiloride (*d*) and then apical amiloride (*e*). In the presence of amiloride on both sides, basal Na solution (*f*) caused a slow pH_i recovery which increased upon the removal of basal amiloride (*g*). The recovery was entirely reversed by Na-free solution in the basal perfusate (*h*). When Na Ringer's solution containing amiloride was added to the basal perfusate (*i*), there was a slow pH_i recovery which increased dramatically upon removal of amiloride (*j*). This recovery was not inhibited by apical amiloride, and removal of apical amiloride (*k*) did not affect pH_i. Trace is representative of 5 similar experiments on 5- or 6-d-old monolayers.

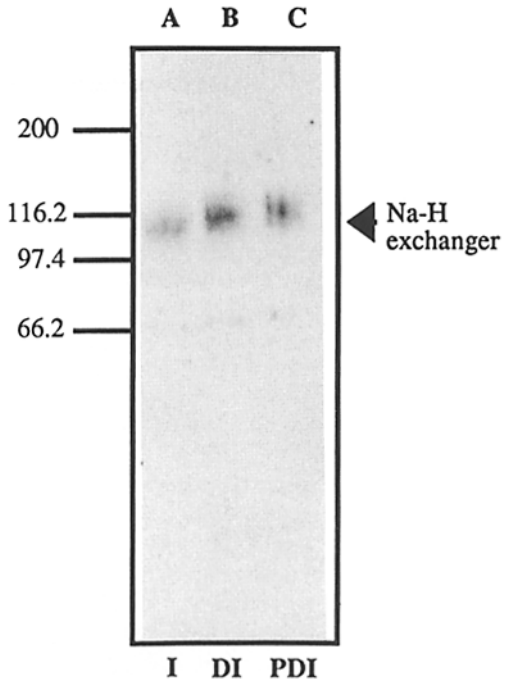

Figure 7. Anti-NHE-1 antibody recognizes (Western blot) a single protein of ~110 kD in 31EG4 cells. 31EG4 cells were cultured on permeable supports for 4 d in media containing either I (lane *a*), DI (lane *B*), or PDI (lane *C*). Extracts were subjected to 8% SDS-PAGE, and then transferred to nitrocellulose. Molecular weight markers are: myosin (*200 kD*), *E. coli* β-galactosidase (*116.2 kD*), rabbit muscle phosphorylase b (*97.4 kD*), and BSA (*66.2 kD*).

changer is typically 10–200 times less sensitive to amiloride than the basal one, i.e., IC_{50}'s of 100 μM vs. 5 μM amiloride, respectively (7). Since the basal and apical exchangers of 31EG4 cells were both completely blocked by 50 μM amiloride in 140 mM Na Ringer's, it appears that the exchangers in the apical and basolateral membranes of 31EG4 cells are the amiloride-sensitive type, like those found in unpolarized cells and in the basolateral membrane of other epithelial cells (7, 43).

Effects of Prolactin on Polarity of Na/H Exchange and Na/HCO_3 Cotransport in 3–4-d-old Monolayers

PDI-treated cells were treated identically to the cells in Fig. 3 except that prolactin (P, 5 μg/ml) was present with D and I in the growth media. The PDI-treated monolayers had similar morphology and transepithelial electrical resistances to the DI monolayers (48), but, in contrast to the DI monolayers, Na/H exchange was usually detected in only the basolateral membrane. A typical protocol is shown in Fig. 4 *a*. After acid-loading, addition of Na to the apical perfusate of PDI cells usually had no effect on pH_i, while addition of Na solution to the basal side caused a rapid increase in pH_i that was inhibited by basal application of amiloride. Amiloride addition to, or removal from, the apical perfusate had no effect on pH_i. However, removal of amiloride from the basal perfusate resulted in a rapid pH_i recovery (in the presence of apical amiloride, a control for leakiness of the layer). Na/H exchange was present on the apical membrane in only 2 of 17 cases. In contrast, Na/H exchange was present on the basolateral membrane in every one of 38 experiments. In the two experiments in which there was a detectable Na/H exchanger on the apical membrane, the rates of amiloride-sensitive pH_i recovery (measured between pH_i 6.6 and 6.8) were roughly 10 times slower than the rates exhibited on the basolateral side. We also examined the polarity of Na/H exchange after short term application of prolactin. After a 24-h pre-treatment of two different DI monolayers (day 3–4), Na/H exchange was still present on both the apical and basolateral sides (not shown).

Similar to the DI-treated cells, Na/HCO_3 cotransport was present on the basolateral, but not the apical, membrane in 72% of the PDI monolayers. An example using the same protocol as Fig. 3 *b* is seen in Fig. 4 *b* (also Table I). Cells were acidified and Na-free solution perfused on both the apical and basal sides. Amiloride in the basal perfusate had no effect on pH_i, and subsequent addition of Na to the basal perfusate induced an amiloride-insensitive pH_i recovery that was reversibly inhibited by H_2DIDS.

As summarized in Table I for 3–4 d PDI monolayers, the apical membrane only rarely exhibited any pH_i regulatory ability, and, in those few cases, rates of pH_i recovery were small compared to those induced by the Na/H exchanger and the Na/HCO_3 cotransporter at the basolateral membrane. These results suggest that prolactin enhanced the development of polarity of Na/H exchange, but did not affect the appearance or the polarity of Na/HCO_3 cotransport.

Na/H Exchange and Na/HCO_3 Cotransport in 5–6-d-old DI- and PDI-treated Monolayers

The polarization of the two pH_i regulatory mechanisms was also examined in monolayers after 5–6 d of culture in either DI or PDI. As shown in Fig. 5 for 5–6 d DI-treated monolayers, addition of Na solution to the apical side had no effect on pH_i recovery after an acid load. To control for the possibility that an apical Na/H exchanger could be masked by passage of Na across the basolateral membrane on another Na/H exchanger (and consequent H accumulation equal to the H lost across the apical side as Na entered), amiloride was added to the basal perfusate. In 11 different experiments (seven with basal amiloride present) apical Na/H exchange was present in only one. Basal Na/H exchange was present in 10 of 10 experiments. Thus, even in the absence of prolactin, Na/H exchange in DI-treated momolayers became functionally polarized over time: it was present on the apical membrane 83% of the time on day 3–4 and only 9% of the time by days 5–6 (Table I).

The 5–6 d DI-treated momolayers also exhibited a Na-dependent, amiloride-insensitive component of pH_i recovery. In the presence of basal amiloride (and apical Na) the readdition of Na to the basal perfusate elicited a pH_i recovery that was, like the pH_i recovery exhibited on days 3–4, inhibited by H_2DIDS (not shown). This apparent Na/HCO_3 cotransport activity was present in 100% of the 5–6 d cultures and localized to the basolateral side (Table I). Thus, in DI-treated cells the Na/HCO_3 cotransporter becomes more prevalent as the monolayers aged: it was present on the basolateral membrane 100% of the time by day 5–6 as opposed to 75% on day 3–4.

The results for 5–6 d PDI-treated monolayers were nearly identical. As shown in Fig. 6, apical Na had no effect on pH_i even when amiloride was added to the basal perfusate. Addition of Na to the basal side induced an amiloride-insensitive recovery which increased in rate when amiloride was removed. The 5–6 d PDI-treated monolayers exhibited no apical Na/H exchange (0 out of 9 attempts), and Na/HCO_3 cotransport (basal side only) was present 100% of the time.

Localization of the Na/H Exchanger with Immunofluorescence

After the formation of tight junctions, functional polarization of Na/H exchange could be established by several mechanisms. One possibility is that the Na/H exchanger continued to be delivered to the apical membrane but eventually became functionally inactivated over time. Alternately, the Na/H exchanger could have become selectively targeted and restricted to the basolateral membrane. To distinguish between these two mechanisms, monolayers were double stained for the Na/H exchanger (NHE-1) and the tight junction-associated protein ZO-1, then examined by confocal microscopy. The tight junction defines the boundary between the apical and basolateral membranes of the cells. Therefore, as the focal plane moves apical to basolateral, staining above the tight junction is defined as apical and below as basolateral.

Analysis by Western blot (Fig. 7) demonstrated that the anti-NHE-1 antibody recognized a single band of ≈110 kD in all three hormone conditions, which is consistent with the predicted molecular weight for the glycosylated Na/H exchanger (35) and also with the amiloride-sensitivity data indicating that there was only one type of exchanger operating in these cells.

The images shown in Fig. 8 illustrate sections taken from apical through basolateral regions of the cells (left to right). Four conditions comparing DI- and PDI-treated monolayers at day 3 and then at day 5 are presented in Fig. 8; 3-d-old DI cells (*a–d*), 3-d-old PDI cells (*e–g*), 5-d DI cells (*h–j*), and 5-d PDI cells (*k–m*). Fig. 8, *n* and *o* are controls showing nonspecific binding of the secondary antibodies. Fig. 8 *n* shows cells treated with the fluorescein-conjugated anti-rat secondary only, and *o* shows cells treated with the fluorescein-conjugated IgG and the goat anti-rabbit biotin secondary IgG followed by Texas red avidin.

ZO-1 staining was evident as a fine ring (*green*) that outlined single cells near the apical side of the monolayer. The pattern of ZO-1 staining indicated that the morphology of either DI or PDI monolayers was less regular at day 3 than at day 5. At day 3 some areas of the monolayer were beginning to rise up and become more columnar than others. This is evident in the 3 d DI series (Fig. 8, *a–d*) in which we have split the eight section (4.0 μm) ZO-1 image into two four-section (2.0 μm) images. The cells in the center of Fig. 8, *a–d* were slightly taller than the surrounding cells, so ZO-1 appears and disappears first in the center of panels *b* and *c* and is present only on the edge of image *d*. Although the subsequent ZO-1 images (Fig. 8, *f, i,* and *l*) each contain eight sections (a total of 4.0 μm), the actual thickness of the ZO-1 band (as seen in Fig. 8, *a–d*) in any condition was ~2.0 μm. In day 5 DI or PDI monolayers the ZO-1 staining pattern appeared more regular (Fig. 8, *i* and *j*), and the monolayers were slightly taller than day 3 monolayers (i.e., 10–12 vs. 8–10 μm).

Staining of the Na/H exchanger (*red*) exhibited distinct patterns that depended on the time and hormonal treatment. In 3–4-d DI monolayers, which usually exhibit apical Na/H exchange pH_i regulatory activity (Table I), there were closed, circular patches of NHE-1 staining above ZO-1, indicative of the presence of the Na/H exchanger in the apical membrane (Fig. 8 *a*). In Fig. 8, *a–d*, the cells in the center of the images were slightly taller than those in the periphery, and the confocal sections show Na/H staining above ZO-1 in both Fig. 8 *a* and *b*. Thus, there was apparent apical staining in most of these DI-treated cells. There was also circumferential staining (with the same diameter as the ZO-1 staining) at the level of (Fig. 8, *b* and *c*) and below (Fig. 8 *d*) the level of the tight junction, indicative of the presence of the Na/H exchanger in the basolateral membrane. This pattern was not as distinct as ZO-1, which could be due to folding of the lateral membrane which we have observed in electron microscopy (not shown). We also observed diffuse intracellular staining of the exchanger in the plane of ZO-1 (Fig. 8, *b* and *c*), which may represent staining of the Na/H exchanger in intracellular vesicles below the apical membrane.

In 3-d PDI cells (Fig. 8, *e–g*), which only infrequently exhibit apical Na/H exchange activity (Table I), apical NHE-1 staining appeared only occasionally, and to a much lesser extent than in 3-d DI monolayers. The images shown for the 3-d PDI monolayer exhibited the most apical staining (i.e., above ZO-1) of any 3-d PDI preparations used for these experiments: there were only three cells in this image that ex-

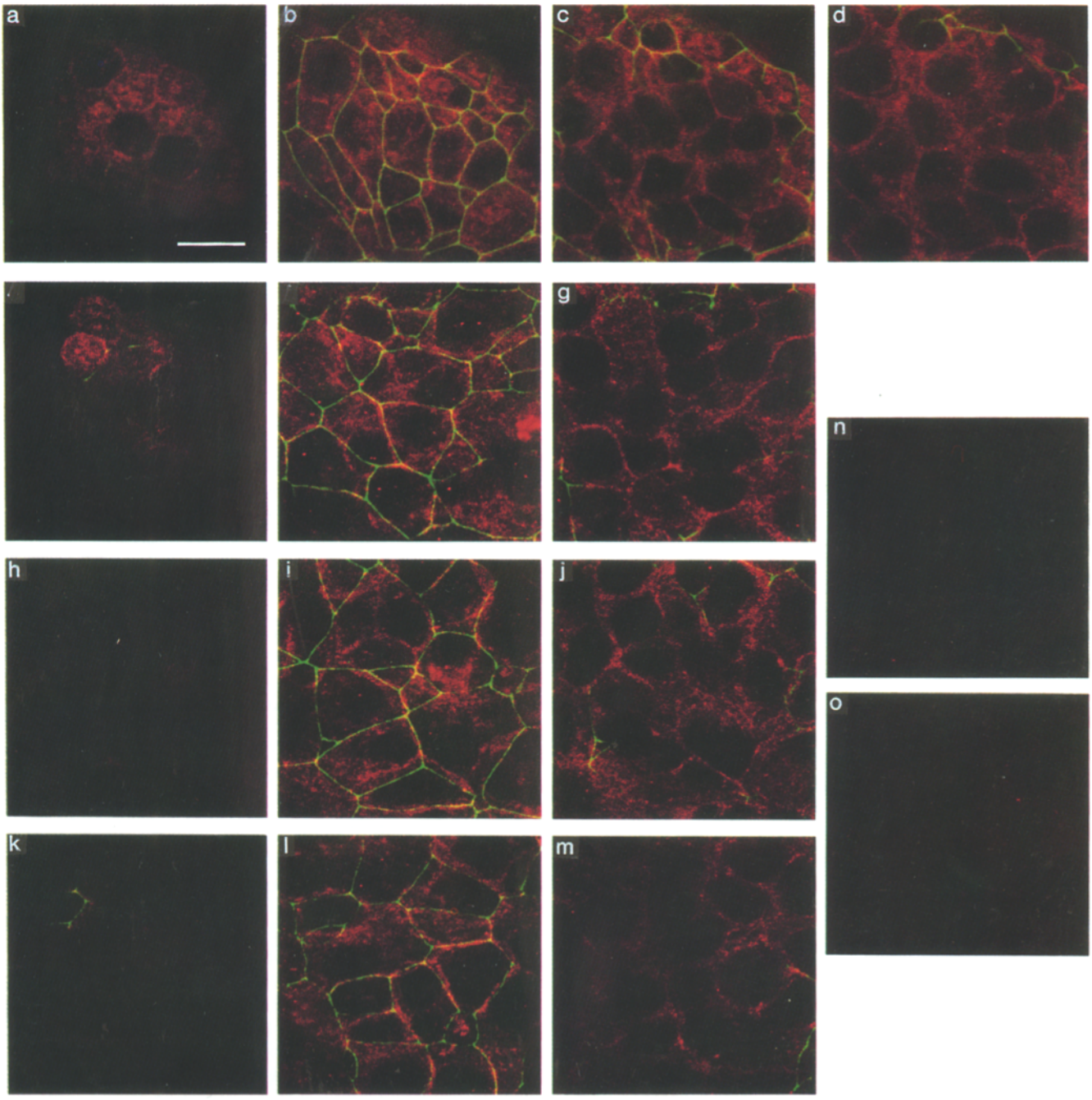

Figure 8. Immunolocalization of Na/H exchange with confocal microscopy demonstrates that the time course of protein distribution is similar to time course of development of functional polarity. After 3- and 5-d incubation in either DI or PDI, monolayers were fixed and co-stained with anti-NHE-1 and anti-ZO-1 as described in Materials and Methods. Specimens were viewed with confocal microscopy. Each series of images (from left to right) shows the region above ZO-1, in the plane of ZO-1 and below ZO-1. *a–d* are from a typical 3-d DI monolayer. *a* is above ZO-1; *b* and *c* are in the plane of ZO-1; and *d* is below ZO-1. In this series the plane of ZO-1 was shown using two panels because the monolayer was slightly elevated (1.0–2.0 μm) in the center of field of view. *e–g* are from a 3-d PDI monolayer, and represent the most staining we could identify in this condition. *h–j* are from a 5-d DI monolayer. *k–m* are from a 5-d PDI monolayer. *n* and *o* are controls that were not treated with primary antibody. *n* is the brightest section obtained on a monolayer treated with only the fluorescein-conjugated secondary goat anti-rat IgG, and *o* is the brightest section from a monolayer treated with both the fluorescein-conjugated secondary and the goat anti-rabbit biotin secondary IgG followed by Na/H avidin. Bar, 10 μm.

hibited any apical staining of NHE-1, and this was the most apical staining that was observed in any of our experiments. This was in contrast to the DI-treated cells in which nearly all the cells exhibited apical NHE-1 staining (Fig. 8, *a* and *b*). Staining of NHE-1 at the basolateral sides of these cells was similar to that exhibited by the 3-d DI monolayers.

In 5-d DI (Fig. 8, *a–j*) or 5-d PDI (Fig. 8, *k–m*) monolayers, both of which exhibit basal, but not apical,

Na/H exchange pHi regulatory activity (Table I), there was characteristic ringlike, lateral staining below ZO-1, but there was no evidence of apical NHE-1 staining. The apparent intracellular staining was also reduced in these 5-d cells compared to both 3-d DI and 3-d PDI cells.

Discussion

Functional and Morphological Polarity of the Na/H Exchanger, but Not Na/HCO_3 Cotransport, Are Regulated by Prolactin

This study combined a physiological assay and immunolocalization to examine the hormonal regulation of development of polarity of endogenous pHi regulatory proteins in mouse mammary epithelial cells. After 3–4 d of treatment of 31EG4 monolayers with dexamethasone and insulin, Na/H exchange was present 83% of the time on the apical membrane and 100% of the time on the basal membrane, but by day 5–6 Na/H exchange was functionally polarized to the basal membrane. The time course of functional polarization was clearly enhanced by prolactin: Na/H exchange was present predominantly on the basal membrane of PDI monolayers on days 3–4, and there was essentially no Na/H exchange on the apical membrane by day 5–6 of prolactin treatment. Immunolocalization studies indicated that the time course of polarization of Na/H exchanger protein was similar to the time course for the development of functional polarity, and that this process also appeared to be speeded by prolactin. These findings suggest that in 31EG4 cells functional polarity of Na/H exchange was established largely by regulating the morphological distribution of the protein. It has previously been demonstrated in endometrial and mammary carcinoma cell lines that prolactin increases the polarized surface expression of an apocrine membrane antigen to the apical membrane (10). In 31EG4 cells prolactin appears to enhance basolateral polarization of the Na/H exchanger, which may ultimately play an important role in determining the ion transport activity across the mammary epithelium. Since dexamethasone induced tight junction formation in these monolayers by day 2–3 in either DI or PDI (48; also see Materials and Methods), it appears that final establishment of functional polarity of Na/H exchange occurs well after the formation of tight junctions in this system.

The 31EG4 mammary cells also exhibited an amiloride-insensitive, Na-dependent, H_2DIDS-inhibitable pH_i recovery that we suggest is due to Na/HCO_3 cotransport. Several distinct differences suggest that the Na/HCO_3 cotransporter was polarized by a different mechanism than the Na/H exchanger. Unlike Na/H exchange, Na/HCO_3 cotransport was not present in insulin-treated monolayers, suggesting that the appearance of the cotransporter required dexamethasone. Also, the time course of polarization of the Na/HCO_3 cotransporter was quite different from that exhibited for the Na/H exchanger. Na/HCO_3 cotransport appeared (from functional studies) exclusively on the basolateral membrane in ~75% of either DI or PDI monolayers at 3–4 d and in 100% of 5–6 d-old DI or PDI monolayers. In contrast, the Na/H exchanger was first expressed and functional on both the apical and basolateral membranes and subsequently became polarized to the basolateral membrane. The time course of polarization of the exchanger, but not the cotransporter, was speeded by prolactin.

In vivo the lactogenic hormones regulate vectorial transport of Na and protons in the mammary gland (1, 11). A possible mechanism would be hormonal regulation of the polarity and functional activity of Na-dependent pH_i regulatory mechanisms during development of the mammary epithelium. However, because both the Na/H exchanger and the Na/HCO_3 cotransporter become polarized to the basolateral, and not the apical, membrane, it seems unlikely that these mechanisms contribute directly to either Na absorption or H secretion in the intact mammary gland. However, the fact that prolactin selectively speeds the basal polarization of the Na/H exchanger might indicate the importance of the polarity of this mechanism for proper function of the mammary gland.

Our use of dual perfusion microfluorimetry to study development of functional polarity of Na/H exchange required that the monolayers have tight junctions. When the monolayers were leaky, as with I-treated monolayers or with DI and PDI monolayers before tight junctions had formed on day 3, it was impossible to determine whether the Na/H exchanger was present on the apical, basal, or both sides of the cells. We are confident for several reasons that the apical expression of Na/H exchange seen in DI-treated monolayers was not a result of Na leaking paracellularly to the basal exchanger. Both the DI and PDI monolayers were quite impermeable to paracellular passage of [^{14}C] mannitol (48). Also, apical Na/H exchange occurred in the presence of 1 mM amiloride in the basal perfusate. Finally, the effect was specific to Na/H exchange. If Na were traveling paracellularly, Na/HCO_3 cotransport would not have appeared to be located exclusively on the basolateral side.

Implications for Epithelial Sorting Mechanisms

Several models have been proposed for sorting of plasma membrane proteins in epithelial cells. There is evidence both for direct targeting of proteins to the specific membrane domain of the cell and also for delivery to both membranes with subsequent rerouting to polarize proteins to the appropriate membrane (18, 29, 38). What sorting mechanism(s) appears to be operating in 31EG4 mammary cells? Since the Na/H exchanger was initially present on both membranes and subsequently found only on the basolateral side, while Na/HCO_3 cotransport was only found on the basolateral side, it is tempting to hypothesize that two different sorting mechanisms may be operating in this cell type: the first mechanism would initially direct Na/H exchange to both membranes and then re-route it to the basal side, and the second would target the Na/HCO_3 cotransporter directly to the basolateral side. According to this model, prolactin influences only the mechanism that is responsible for polarizing Na/H exchange, while the Na/HCO_3 cotransporter appears in response to glucocorticoids and is directly targeted to the basolateral membrane. All of this sorting occurs after the induction of tight junctions. Further testing of this model awaits an antibody to the Na/HCO_3 cotransporter, and a detailed analysis of the fate of apically directed Na/H exchangers.

Another possibility that we considered was that there are two different exchangers expressed in these cells, and that prolactin speeds the down regulation of expression of the apical exchanger. However, because the apical and basal Na/H exchangers in day 4 DI monolayers as well as the basal Na/H

exchange in day 4 PDI monolayers had roughly equivalent sensitivities to amiloride, it seems unlikely that two genetically distinct exchangers are initially expressed in 31EG4 monolayers (7, 15). Further, the immunolocalization data indicated that the functional polarity of Na/H exchange is determined largely by regulating the distribution of a single type of Na/H exchanger and that prolactin somehow speeds this polarization process.

Several models could explain these findings. Based on the immunofluorescence, the most likely possibility is that prolactin accelerates polarization by halting the delivery of Na/H exchanger to the apical membrane. It is possible, though, that this technique lacks the sensitivity to identify very low quantities of protein in the apical membrane and that functional polarity is established by regulating the activity, rapid turn-over, and basolateral stabilization (by interaction with the membrane cytoskeleton) of a single type of exchanger that is continually delivered to both apical and basolateral membranes. This model is similar to what has been recently discovered for the Na/K-ATPase in monolayers of MDCK cells (16). In any case, the differences in the time course and hormonal regulation of development and polarity of the Na/H exchanger and Na/HCO_3 cotransport in 31EG4 cells indicate that polarized membrane function within a single cell type might arise from multiple, individually regulated sorting mechanisms.

The dual perfusion chamber used in this study was designed and built in collaboration with Dr. Joseph Bonanno. We are grateful for his generosity and for useful discussions throughout the process from design to implementation. We also thank Paul Negulescu for useful suggestions on experimental protocol, Duncan Stuart for assistance with the confocal microscopy, and Sergio Grinstein for sharing the NHE-1 antibody. This paper represents work submitted in partial fulfillment of the requirements for the Ph.D. degree to M. D. Sjaastad from the University of California at Berkeley.

This work was supported by National Institutes of Health grants to T. Machen (DK-19520 and DK-38864) and G. L. Firestone (DK-42799 and CA-05388). Mike Sjaastad and Karen Zettl were predoctoral trainees supported by an NIH Systems and Integrative Biology Training Grant (GM-07379).

Received for publication 16 February 1993 and in revised form 21 April 1993.

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