Hormonal Regulation of the Polarized Function and Distribution of Na/H Exchange and Na/HCO₃ 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 (pHi) 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 pHi recovery from an acid load induced by a pulse of NH₃/NH₄⁺-containing solution. When monolayers were grown 3-4 days 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/HCO₃ cotransport system was monitored as a Na-dependent, amiloride-insensitive pH recovery mechanism that was inhibited by 200 μM H₂DIDS. 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/HCO₃ 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/HCO₃ cotransport, and Cl/HCO₃ exchange are polarized to the basolateral membrane (29, 31, 43, 46). However, one or all of these regulators of intracellular pH (pHi) 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 Na+/ATPase and Cl/HCO₃ 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 regulatory mechanisms in the mammary gland.

In the present study we have characterized the time course for development of polarity of two different endogenous Na/H exchangers 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 (0.49-μm pore, 0.4-μm thick; Applied Scientific, San Francisco, CA) in a 24-well plate with 350-μl of medium (DME/F12 [50:50] supplemented with 1% FBS, gentamicin sulfate 50 μg/ml) on the apical side and 650 μl basolaterally. Solutions used for gel electrophoresis, Western blotting, and immunocytochemistry 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 immunocytochemistry the PBS contained Ca²⁺ and Mg²⁺. In addition, PBS/8.0, 150 mM NaCl, 0.05% Tween-20, and 5% non-fat dry milk. For immunocytochemistry the PBS contained Ca²⁺ and Mg²⁺. In addition, PBS/BSA contained 0.1% BSA and PBS/BSA/Tween contained 0.1% BSA and 0.05% Tween 20.

### 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). Both 31EG4 cells were routinely cultured on 100 mm plastic dishes (Corning Inc., Corning, NY) in a humidified, 37°C, 95%/5% air/CO₂ environment, and passed 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 examine the polarized distribution of Na/H regulatory mechanisms, the cells were seeded at a density of 5,000 cells/cm² and grown on permeable supports (0.49-μm pore area; pore size 0.4-μm thick; 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. 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 Ωcm², 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 [¹⁴C] mannitol added to one side permeated to the other side over 4 h. In contrast, all D1 and PDI monolayers used in this study had resistances between 1,000–3,000 Ωcm², and permeabilities of these D1 or PDI monolayers to [¹⁴C] mannitol were 10-fold lower than the I-treated monolayers (46).

#### 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 KH₂PO₄, 1.0 MgSO₄, 1.0 CaCl₂, 100 glucose, and 10.0 Hepes. To acid load cells for pH regulation studies, 30 mM NH₄Cl 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. In some experiments amiloride (Sigma Immunochemicals, St. Louis, MO) was used at a final concentration of 1 mM, while in others H₂DIDS (H₂2, 4,4'-disothiocyanostilbene-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 H₂DIDS a separate stock solution was made for each component, 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 immunocytochemistry were prepared as follows: PBS and PBS with Ca²⁺ and Mg²⁺ 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 immunocytochemistry the PBS contained Ca²⁺ and Mg²⁺. 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 DMSO) for 25–30 min at 37°C in 5% CO₂/95% 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 procedure (27). Before measurements of pH, the filters were incubated in brightfield for confuency. 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.

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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, intracellular pH.
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 do not flex during dual-sided perfusion. Before each experiment the resistance of the monolayer on the filter was measured to insure confuency, 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 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.

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.

Microspectrofluorimetry of BCECF and pHj Calibration

The methods for using BCECF to measure pHj have been described previously (25, 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 UMAN-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 pHj from the BCECF 490/440 ratio was performed using the high [K]-nigericin technique (28, 41). Average calibration curves and resting pHj'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 pHj and the fluorescence intensity ratio (490/440) between pHj 6.4–7.8 (data not shown). The control pHj for cells in each hormone condition was determined on day 4: 1 = 7.43 ± 0.02 (n = 10), D1 = 7.40 ± 0.04 (n = 9), and D1-D2 = 7.39 ± 0.02 (n = 11). There was no significant difference among the resting pHj 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 3IEG4 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 NHE1 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 (R264C; 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 NOI-HD-6-2915 from the NICHD). Hybridomas were cultured in spinner flasks in RPMI plus fumonisin B1 for 9 d. The supernatant was enriched by a 50% NH4SO4 cut. It was then dialyzed against 0.2 M Na/HC03, pH 8.5, and stored in 0.5 M NaCl with NaN3.

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 pHj regulation in the dual-perfusion chamber. Monolayers were grown on filters in D1 or D2 conditions for 3–4 or 5–6 d, washed three times in PBS with Ca2+ and Mg2+, 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 Ca2+ and Mg2+ 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 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 Hanks-Yates, an anti-bleaching 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, color of the label used for immunofluorescence.

Zeiss Axioplan epifluorescence microscope using a Zeiss 40x Plan-Neofluar multi-immersion objective (0.9 NA). Paired images were obtained 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 (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).

**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 pHi trace. This format has been used for all of the figures shown. The “+” and “−” signs refer to addition or removal, respectively, of 30 mM NHCl, 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 NHCl (a) and subsequent perfusion on both sides with Na-free solution (b). Returning Na Ringer’s to the apical perfusate (c) induced a rapid pH recovery which was entirely inhibited by addition of 1 mM amiloride to the apical perfusate (d). There was a drop in pH 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 (h). Simultaneous addition of Na to both the apical and basal sides resulted in a rapid pH recovery (i) that was completely inhibited by application of amiloride to only the apical side (j). There was a small drop in pH when amiloride was added to the basal perfusate (k), and pH did not recover when apical amiloride was removed (l). Removal of basal amiloride resulted in complete recovery to resting pH (m). This trace is representative of five similar experiments on 3- or 4-d-old monolayers.

**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 pHi 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 pHi trace. This format has been used for all of the figures shown.

3IEG4 mammary cell monolayers were acidified by treatment with a basal prepulse of 30 mM NHCl (a) and subsequent treatment with Na-free solutions on both sides of the monolayer. (Cells acidified to a greater extent when NH4 was pulsed on only one side of the monolayer than when NH4 was applied to both sides. The more rapid acidification was presumably due to an accumulation of H's, resulting from the entry of NH4 across the basolateral membrane and concurrent exit of NH3, but not H, across the apical membrane. This protocol reduced the total time of NH4 exposure required for cellular acidification and was used in all of the experiments shown.) When Na was added to the apical side, pHi recovered, and this effect was completely blocked by application of amiloride to either the apical or the basolateral side of the cells. Thus, pHi 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.

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

After only 3-4 d in culture with dexamethasone and insulin (DI), 3IEG4 cells had differentiated into monolayers with
resistances of 1,000–3,000Ω·cm² and very low paracellular permeability, indicative of the formation of functional tight junctions (48). Fig. 3a shows an experiment with one of these DI monolayers. After the acid loading treatment with NH₄Cl and perfusion with Na-free solutions on both the apical and basal sides, addition of Na Ringer’s to the apical side caused pHi 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), pHi 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).

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. 3b). In monolayers that had been acidified by NH₄Cl treatment and perfused with Na-free solutions, addition of Na solution to the basolateral side in the presence of amiloride often induced pHi recovery (12 out of 16 experiments). The rate of pHi 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. 3b, when the monolayers were treated with Na solution + amiloride + 200 μM H₂DIDS, the well known blocker of Na/HCO₃ cotransport, pH recovery was totally blocked. (According to the known models of pH regulation, the Na-dependent, amiloride-insensitive pH recovery from an acid load could be due to either Na/HCO₃ cotransport (3) or Na-dependent CI/HCO₃ exchange (33). If the Na-dependent, amiloride-insensitive recovery was due to Na-dependent CI/HCO₃ exchange, and then reducing (CI) in the bathing solution should cause pH to increase as CI exits the cell and HCO₃ enters. This maneuver had absolutely no effect on pH of 31EG4 cells (not shown). We have therefore concluded that 31EG4 cells have neither Na-independent nor Na-dependent CI/HCO₃ exchange and that the Na-dependent, amiloride-insensitive, H₂DIDS-inhibitable pH recovery shown in Figs. 3a and 4b is due to the presence of Na/HCO₃ cotransport on the basolateral membrane. For simplicity of discussion we will refer throughout the text to this mechanism as a Na/HCO₃ cotransporter, though we realize that the Na and HCO₃ dependency and H₂DIDS sensitivity are alone insufficient evidence to make a firm conclusion regarding the specific mechanism involved.

This putative Na/HCO₃ cotransporter was never present on the apical membrane of 31EG4 cells, but it was present...
Further application of amiloride did not increase. Perfusion of Na solution on the basal side (d) caused a slow increase in pHi, which was entirely inhibited by amiloride on the apical side (e). Application of apical 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 pHi, nor did it inhibit the complete pHi 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), pHi 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.

transporter. Because our experiments were conducted in air-equilibrated solutions that were nominally HCO₃⁻-free (where \([\text{HCO}_3^-] = 250 \, \mu\text{M}\) ), the rates of pHi recovery due to the Na/HCO₃ cotransporter are slower than would be in HCO₃⁻-containing solutions. In experiments conducted on 3-4 d DI monolayers using solutions containing 25 mM HCO₃⁻ and 5% CO₂ gas (pH 7.4), the rates of pHi recovery due to the Na/HCO₃ cotransporter were 10-15-times faster than those observed in the experiments using HEPES-buffered, HCO₃⁻-free solutions. However, even in experiments conducted in HCO₃⁻-containing solutions, there was no evidence for Na/HCO₃ cotransport on the apical side of the cells. Thus, the conclusions about targeting and expression of functional Na/HCO₃ cotransporters studied here in HEPES-buffered solutions are also valid for HCO₃⁻-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 \(\mu\text{M}\) amiloride completely inhibited the Na-induced pHi 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 \(\mu\text{M}\) amiloride completely inhibited the basolateral Na/H exchange 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 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 NH₄Cl (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 pHi. In the presence of basal amiloride and apical Na, perfusion with Na Ringer's on the basal side (e) caused a slow pHi 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 pHi, nor did it inhibit the complete pHi 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), pHi 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.
Effects of Prolactin on Polarity of Na/H Exchange and Na/HCO₃ 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 pHᵢ, while addition of Na solution to the basolateral side caused a rapid increase in pHᵢ, that was inhibited by basal application of amiloride. Amiloride addition to, or removal from, the apical perfusate had no effect on pHᵢ. However, removal of amiloride from the basolateral perfusate resulted in a rapid pHᵢ 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 pHᵢ recovery (measured between pHᵢ 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/HCO₃ 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 pHᵢ, and subsequent addition of Na to the basal perfusate induced an amiloride-insensitive pHᵢ recovery that was reversibly inhibited by H₂DIDS.

As summarized in Table I for 3–4-d PDI monolayers, the apical membrane rarely exhibited any pHᵢ regulatory ability, and, in those few cases, rates of pHᵢ recovery were small compared to those induced by the Na/H exchanger and the Na/HCO₃ 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/HCO₃ cotransport.
Na/H Exchange and Na/HCO₃ Cotransport in 5–6-d-old DI- and PDI-treated Monolayers

The polarization of the two pH 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, 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 prolatin, Na/H exchange in DI-treated monolayers 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 monolayers also exhibited a Na-dependent, amiloride-insensitive component of pH recovery. In the presence of basal amiloride (and apical Na) the readdition of Na to the basal perfusate elicited a pH recovery that was, like the pH recovery exhibited on days 3–4, inhibited by H₂DIDS (not shown). This apparent Na/HCO₃ 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/HCO₃ 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 even when amiloride was added to the basal perfusate. Addition of Na to the basolateral 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/HCO₃ 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 eighth 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 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-
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.

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 pH regulation 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/HCO₃ Cotransport, Are Regulated by Prolactin

This study combined a physiological assay and immunolocalization to examine the hormonal regulation of development of polarity of endogenous pH 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 exchange 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 the formation of tight junctions in this system.

The 31EG4 mammary cells also exhibited an amiloride-sensitive, Na-dependent, H₂DIDS-inhibitable pH, recovery that we suggest is due to Na/HCO₃ cotransport. Several distinct differences suggest that the Na/HCO₃ cotransporter was polarized by a different mechanism than the Na/H exchanger. Unlike Na/H exchange, Na/HCO₃ 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/HCO₃ cotransporter was quite different from that exhibited for the Na/H exchanger. Na/HCO₃ 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, regulatory mechanisms during development of the mammary epithelium. However, because both the Na/H exchanger and the Na/HCO₃ 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 [³⁵C]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/HCO₃ 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/HCO₃ 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/HCO₃ 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/HCO₃ 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/HCO₃ 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 3IEG4 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/HCO3 cotransport in 3IEG4 cells indicate that polarized membrane function within a single cell type might arise from multiple, individually regulated sorting mechanisms.

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