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The Apical Membrane Glycocalyx of MDCK Cells

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Abstract. The microenvironment near the apical membrane of MDCK cells was studied by quantitation of the fluorescence of wheat germ agglutin attached to fluorescein (WGA). WGA was shown to bind to sialic acid residues attached to galactose at the α-2,3 position in the glycocalyx on the apical membrane. Young MDCK cells (5–8 days after splitting) showed a patchy distribution of WGA at stable sites that returned to the same locations after removal of sialic acid residues by neuraminidase treatment. Other lectins also showed stable binding to patches on the apical membrane of young cells. The ratio of WGA fluorescence emission at two excitation wavelengths was used to measure near-membrane pH. The near-membrane pH was markedly acidic to the pH 7.4 bathing solution in both young and older cells (13–21 days after splitting). Patches on the apical membrane of young cells exhibited a range of near-membrane pH values with a mean ± SEM of 6.86 ± 0.04 (n = 121) while the near-membrane pH of older cells was 6.61 ± 0.04 (n = 120) with a uniform WGA distribution. We conclude that the distribution of lectin binding sites in young cells reflects the underlying nonrandom location of membrane proteins in the apical membrane and that nonuniformities in the pH of patches may indicate regional differences in membrane acid-base transport as well as in the location of charged sugars in the glycocalyx.

Key words: pH — Sialic acid — Neuraminidase — Lectin — Microenvironment

Introduction

The extracellular surface of the apical membrane of most epithelial cells has a 70–100 nm thick glycocalyx resulting from the heavy glycosylation of membrane proteins (Rosenberg, 1995). The structure of the sugar chains on the cell membrane influences a wide variety of interactions including: cell-cell recognition (Varki, 1997); the binding and internalization of pathogenic bacteria (Varki, 1997), viruses (Niles & Cohen, 1991; Zimmer et al., 1995; Keppler et al., 1998) and toxins (Varki, 1997); as well as the adhesion of calcium oxalate crystals to renal epithelial cells (Lieske et al., 1996; Lieske & Deganello, 1999). The glycocalyx could alter the local microenvironment at the apical surface because of the presence of a large number of negatively charged sugar moieties (Varki, 1997). It has been reported for several decades that the apical surface of some intestinal epithelia is markedly acidic to the lumenal solution (Lucas, 1983; McNeil et al., 1987; Shimada, 1987), and that this acid region alters the transport of ionizable solutes (Holtug et al., 1992). It is unclear whether fixed negative charges in the glycocalyx of the apical cell membrane contribute to the low pH microclimate or whether the presence of fixed negative charges on the sugars influences the kinetics or voltage sensitivity of membrane ion channels (Thornhill et al., 1996; Bennett et al., 1997). In a previous study from our laboratory of the lateral intercellular spaces of MDCK cell epithelium, it was reported that these spaces were acidic to the bathing solutions by about 0.4 pH units and that the pH difference was unaffected by a variety of inhibitors of acid extrusion (Chatton & Spring, 1994). Subsequently, it was demonstrated that the lower pH in these spaces was a consequence of buffering by the fixed negative charges of the glycocalyx (Dzekunov & Spring, 1998).

In the present investigation, we utilized a fluorescein-labeled lectin, wheat germ agglutinin (WGA), as a

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near-membrane pH meter to measure the pH close to the apical membrane of MDCK cells of different ages. Young MDCK cells (5–8 days after seeding) exhibited stable, discrete sites that bound WGA and other fluorescently labeled lectins while older cells (13–21 days after seeding) showed more uniform binding. The average pH measured by the fluorescent WGA was acidic to the bathing solution in both young and old cells. Near-membrane pH of the WGA-binding sites on young cells was quite heterogeneous, and the pH of individual sites was influenced differently by the substitution of lithium for sodium. Enzymatic removal of the sialic acid moieties from the glycosylation groups of young cells revealed that new, fully glycosylated proteins were subsequently inserted at the same locations of the cell membrane. WGA binding sites on young cells did not change location or diffuse measurably over a period of hours.

Materials and Methods

Cell Culture

Low-resistance MDCK cells (passages 60–74 from the American Type Culture Collection, Rockville, MD) were maintained using Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (Gibco, Grand Island, NY) and 2 mM glutamine, but without riboflavin, antibiotics, or phenol red. For experimental purposes the cells were grown to confluence on glass coverslips and were used after 5–23 days.

Experimental Solutions and Perfusion System

HEPES-buffered perfusion medium contained (in mM): 14 HEPES, 142 Na^+, 5.3 K^+, 1.8 Ca^{2+}, 0.8 Mg^{2+}, 136.9 Cl^-, 5.6 glucose. The pH of the HEPES solutions was adjusted to 7.4 or to 6.4 at 37°C. The HEPES solutions were gassed with room air. The bicarbonate buffered solution contained (in mM): 24 HCO_3^-, 142 Na^+, 5.3 K^+, 1.8 Ca^{2+}, 0.8 Mg^{2+}, 126.9 Cl^-, 5.6 glucose and was gassed with 5% CO_2/95% air at 37°C to maintain pH 7.4. In experiments with low sodium, 128 mM Na^+ in HEPES-buffered medium was replaced by 128 mM Li^+. The osmolarity of all solutions was 292–300 mOsm/kg.

For the pH measurements the apical surface of monolayers was perfused with experimental solution at 37°C in a chamber mounted on the stage of microscope as previously described (Harris et al., 1994). The perfusion solution was switched rapidly by computer-controlled pinch valves.

Chemicals

Wheat germ agglutinin fluorescein isothiocyanate (WGA) or tetramethylrhodamine conjugates were obtained from Molecular Probes (Eugene, OR). All other fluorescein-labeled lectins were purchased from Vector Laboratories (Burlingame, CA). N-acetylneuraminic acid (type VI from *E. coli*) and Neuraminidase (type V from *Clostridium perfringens*) were from Sigma (St. Louis, MO).

Labeling Apical Cell Surface with Fluorescent Lectins

Fluorescent lectins were applied to the apical surface of MDCK cells for 30 min at 37°C. Any unattached dye was then washed out by perfusion. Since lectins bind to specific sugar residues of the glycocalyx, the fluorescent markers remain for several hours on the apical surface of the monolayers.

Fluorescence Microscopy for Lectin Colocalization

Typically two lectins marked with fluorescein and rhodamine dyes respectively, were applied to the apical surface of MDCK cells. The lectin binding sites were observed by confocal laser scan microscopy (model 410, Carl Zeiss, Thornwood, NY). The confocal images of each lectin were collected in separate channels and were displayed in false color as green for fluorescein and red for rhodamine.

Fluorescence Microscopy for pH Measurements

WGA conjugated with fluorescein was employed as a pH indicator probe. The experiments were performed on the stage of an inverted microscope (Diaphot, Nikon, Melville, NY) modified for simultaneous transmitted light differential interference contrast and low-light-level fluorescence. Epifluorescence illumination for excitation ratio imaging was achieved at 458 and 488 nm using a 100×/1.3 N.A. objective lens (Nikon). The microscope output port was connected to a 1K × 1K progressive-scan, intensified CCD camera (Model ICCD-1001, Video Scope, Sterling, VA). The images were obtained at a maximum rate of 15/sec, digitized at 8 bits, and stored on computer for later offline analysis. The sequence of events (e.g., solution valves, intensifier gain, laser power, illumination shutters, image storage) during the experiment was controlled by computer using a custom-made program.

pH Calibration

The pH calibration curve was generated by measurement of the ratio of fluorescence emission intensities with two excitation wavelengths (458 and 488 nm) of WGA dissolved in buffered solutions with known pHs. A 5 μl drop of solution was placed on a microscope slide and a glass coverslip was sealed to the slide. For each of the 5 different pH calibration standards from 5.5 to 8.0, a ratio pair of images was taken and the fluorescence intensity ratio was determined.

Image Analysis

The fluorescent images were analyzed for pH by choosing several regions from each pair of images and calculating the intensity ratio for each region for comparison with the pH calibration curve (Metamorph, Universal Imaging, West Chester, PA) . The regions selected in young cells corresponded to individual patches on the apical cell membrane whereas in older cells, the selected regions were rectangles covering a substantial portion of the apical surface of a single cell.

Statistics

Data are presented as mean ± SEM. Significance was determined using the paired *t*-test or Student *t* test, and *P* value less than 0.05 was considered statistically significant.

Results

Wheat Germ Agglutinin Binding

WGA binds specifically to N-acetylneuraminic (sialic) acids and N-acetyl-glucosamine residues in the glycoca-

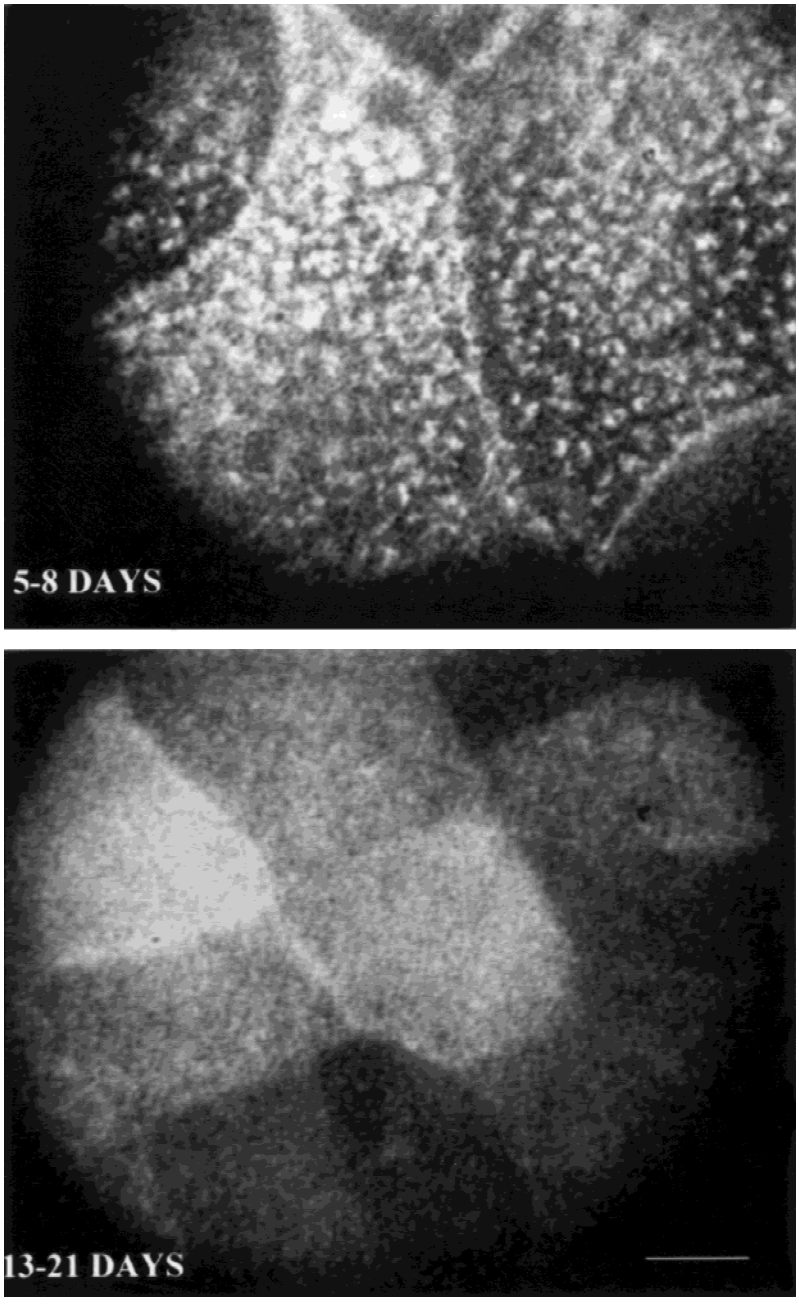

Fig. 1. Wheat germ agglutinin (fluorescein label) binding to the apical membrane of young MDCK cells, 5–8 days after splitting (top), and older cells, 13–21 days after splitting (bottom). The apical membrane smooth surface area of the young cells is about 4.3 times that of the older cells. Scale bar = 10 μm for both panels.

lyx (Rosenberg, 1995; Varki, 1997). The distribution of WGA (12 μg/ml) on the apical side of MDCK epithelial monolayers differed as a function of cell type and time after seeding. Young cells (5–10 days after seeding) spread out markedly to achieve confluence and bound WGA in irregularly distributed patches on the apical surface as well as in a discontinuous ring at the level of the tight junctions (Fig. 1, top). Older cells (15–23 days after seeding) were taller, showing a greatly reduced apical area and more uniform binding of WGA with little or no discernable staining of the tight junctions (Fig. 1, bottom). Individual patches could not be readily delineated in the older cells although the staining had a granular appearance. Some cells in both cultures failed to bind WGA at all.

Because WGA is a multivalent protein, its binding to monovalent sugar residues could, in principle, lead to glycoprotein and glycolipid aggregation. To check whether WGA binding itself causes the formation of patches on the apical surface of young MDCK monolayers, we perfused cells with a low lectin concentration (0.6 μg/ml) on the stage of our fluorescence microscope and observed the time course of WGA binding to the glycocalyx. After around 5 min of perfusion clearly detectable fluorescent patches appeared on the cell surface and additional perfusion with fluorescent lectin did not

significantly increase size of patches. On older cells, incubation with WGA showed a more uniform fluorescence intensity with a granular appearance but no clumps of dye on the surface of monolayers. We concluded that the presence of lectin does not cause the patch formation on the cell surface, and only reflects the preexisting grouping of glycosylated proteins and lipids.

Binding of Other Lectins

To find out which type of the sugar residue predominantly binds WGA on the apical glycocalyx of MDCK cells, we applied other lectins that detect only N-acetylneuraminic acid or N-acetyl-glucosamine. Thus incubation of young MDCK cells from 20 to 40 min with succinylated WGA (15 μg/ml), an agglutinin that does not bind to sialic acid residues, but retains its specificity toward N-acetyl-glucosamine, did not show any detectable fluorescence in young cells. In older cells, a few (3–5) very faintly stained cells were detectable in each culture. Application of *Sambucus Nigra* lectin (SNA), a lectin that links preferentially to sialic acid attached to terminal galactose in the α-2,6 position and far less to the α-2,3 position (Varki, 1997), at a concentration of 20 μg/ml for up to 50 min also did not demonstrate any binding sites on young epithelia. We concluded that WGA binds mostly to α-2,3 linked sialilated glycoconjugates on the apical surface of MDCK cells.

Since the dark areas of the apical cell surface represent regions either devoid of sialic acid residues or inaccessible to WGA, we examined the binding of other fluorescently labeled lectins with different sugar specificity: *Concanavalin A* (12 μg/ml), a lectin that recognizes mannose and glucose; *Soybean* agglutinin (SBA, 15 μg/ml), specific for galactose and N-acetyl-galactosamine; *Arachis hypogaea* lectin (PNA, 12 μg/ml), specific for terminal galactose only (Varki, 1997). The young cells showed a distinct patchy distribution of binding of all three of these lectins that differed from that for WGA, indicating the presence of all of the relevant sugar residues in the MDCK cell glycocalyx.

Simultaneous staining (Fig. 2, left panel) of young MDCK epithelia with WGA-rhodamine (red) and PNA-fluorescein (green) showed the presence of both on 90% of cells. The localization of WGA and PNA was determined by confocal microscopy to ensure that the lectins were on the apical surface of monolayers and were not internalized. From the image analysis of optical sections of fluorescently labeled cells we conclude that both lectins were not quickly internalized, did not pass the tight junctions and, indeed, are confined to the apical glycocalyx of MDCK cells (Fig. 2, x-z sections). However, the distribution of each lectin on the apical side of monolayers differed substantially. Binding sites of both lectins were plentiful on the apical surface of young cells and in many places they were located very close to each other and appeared to be colocalized (yellow spots, Fig. 2). WGA binding sites formed perijunctional rings, whereas no fluorescence from PNA was detected in the region of the tight junctions. Labeling of older monolayers (Fig. 2, right panel) with these lectins showed, as has been reported in detail previously (Kersting et al., 1993) that some cells in the monolayer preferentially bound WGA while others only labeled with PNA.

Neuraminidase

Additional evidence that sialic acid residues are the major ligands for WGA on the apical surface of MDCK cells came from experiments with neuraminidase, an enzyme that cleaves the O-glycosidic linkages between the terminal neuraminic acids and the subterminal sugars. Perfusion of WGA labeled MDCK cells with a buffer solution containing 2 mg/ml neuraminidase for 5–10 min eliminated the fluorescence completely. Treatment of MDCK cells with a lower dose of the enzyme, 0.5 mg/ml, for 10–15 min reduced the fluorescence intensity by about 50% (Fig. 3) with little or no significant subsequent changes in the fluorescence pattern over the next 20–30 min. After washout of the enzyme solution, the binding of WGA to the enzymatically treated monolayer was evaluated by periodically perfusing a WGA-containing solution for 5 min and measuring the fluorescence intensity. The fluorescence intensity gradually increased as a function of time at the same locations on the apical surface as those seen before enzyme treatment until initial intensity levels were achieved after approximately 3 hr (3 monolayers). This result is consistent with the conclusion that new, fully glycosylated proteins were inserted into the apical membrane in identical locations to those that had previously bound WGA before the enzyme treatment.

Near-membrane pH

Labeling cells with WGA-fluorescein permitted measurement of the pH of sialoglycoproteins and glycolipids on the apical surface. A fluorescein-lectin conjugate has been previously employed as a near-membrane pH indicator on the basolateral membrane of multiple cells in the guinea pig intestine (Kirschberger et al., 1999). The negatively charged and possibly viscous environment of the apical glycocalyx created by sialic acids (Rosenberg, 1995; Varki, 1997), can, in principle, change the spectral characteristics of the dye and introduce a systematic error in the pH estimate. To determine whether WGA-fluorescein was suitable for the measurement of glycocalyx pH, we measured the ratio of fluorescence emis-

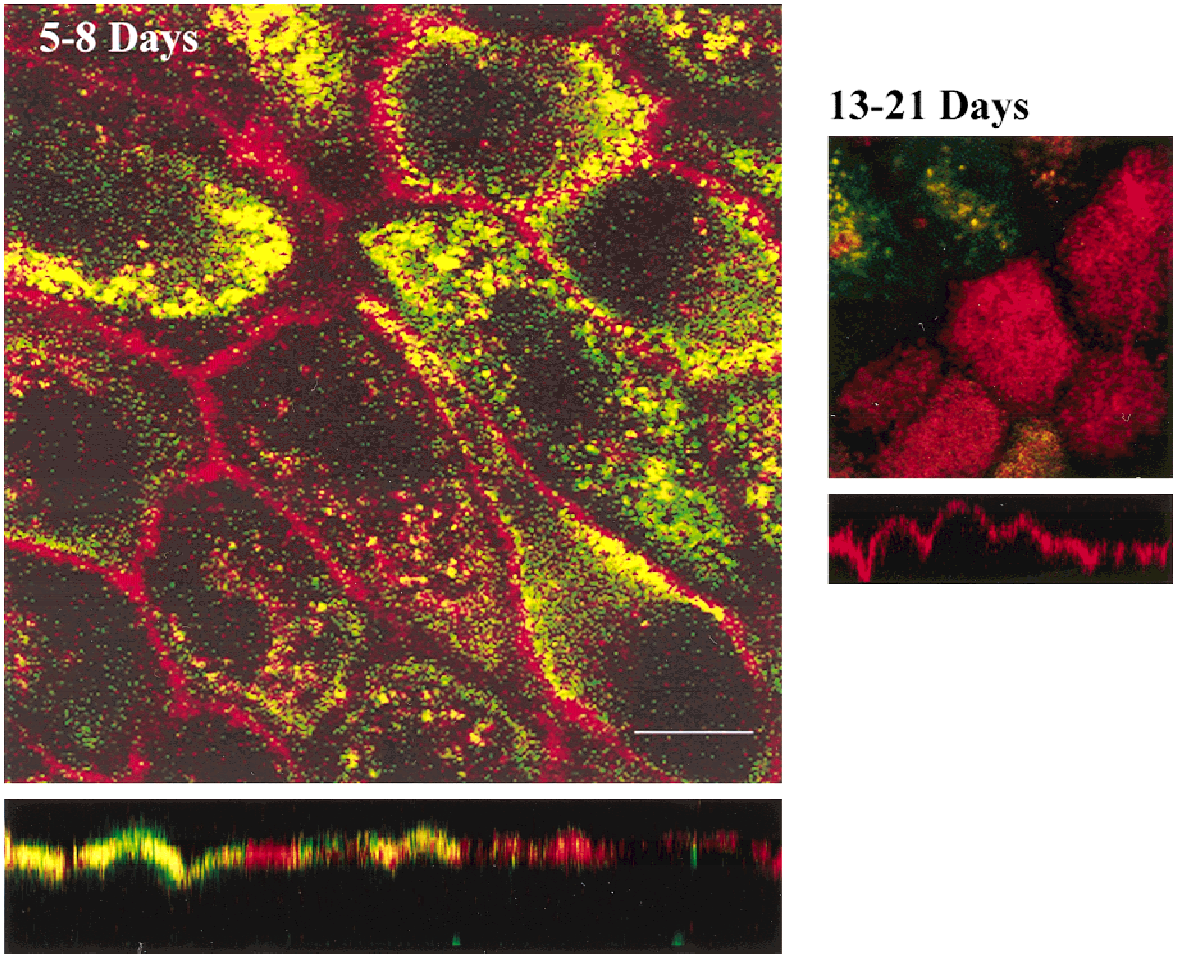

Fig. 2. Confocal microscope optical sections showing binding of wheat germ agglutinin (rhodamine label) in red and that of peanut lectin (fluorescein label) in green to the apical surface of young cells (5–8 days after splitting) and older cells (13–21 days after splitting). An x-z scan is shown below the respective images. Scale bar = 10 μm for all images.

sion during excitation at 488 or 458 nm in HEPES-buffered solution at 5 different pHs from 8.0 to 5.5 in the presence and absence of neuraminic acid (Fig. 4). The similarities of the two curves indicated that neuraminic acid in concentration up to 8.5 mM did not change the pH sensitivity of fluorescein.

In experiments on young cells with patchy distribution of WGA-binding sites, pH values reported by WGA-fluorescein were remarkably different from patch to patch. As shown in Fig. 5, a typical cell exhibited patches in which the pH varied from 6.40, acidic to the bathing solution, to quite alkaline values, 7.82. Average glycocalyx pH of young (5–8 days) MDCK cells was 6.86 ± 0.04 (n = 121 cells), significantly acidic to the pH 7.4 HEPES perfusion solution (Fig. 6, solid bar, control). The crosshatched bar in Fig. 6 shows that lowering perfusate pH to 6.4 gave a slightly alkaline glycocalyx pH 6.55 ± 0.04 (n = 78 cells). On older cells (13–21 days), with more uniform lectin distribution on apical surface, glycocalyx pH was measured from large regions of the apical surface of individual cells. The pH of glycocalyx of older cells averaged 6.61 ± 0.04 (n = 120 cells) when the bathing solution pH was 7.4, significantly more acidic than in young cells (Fig. 6, solid bar, control). When the perfusate pH was reduced to 6.4, the glycocalyx pH of older cells fell to 6.28 ± 0.03 (n = 71 cells), significantly more acidic than the perfusate.

Because the pH values reported by the WGA-fluorescein in young cells were so low and differed so much from patch to patch, we were concerned about their validity. We reasoned that partial removal of sialic acid residues by a low dose of neuraminidase should increase the pH of the patches toward that of the perfusate. Indeed, treatment of the young cells with neuraminidase (0.5 mg/ml for 15 min) significantly reduced the presence of WGA on apical surface, as shown previously (Fig. 2), and increased near-membrane pH to 7.27 ± 0.06 in the pH 7.4 solution (Fig. 6, enzyme). Although only

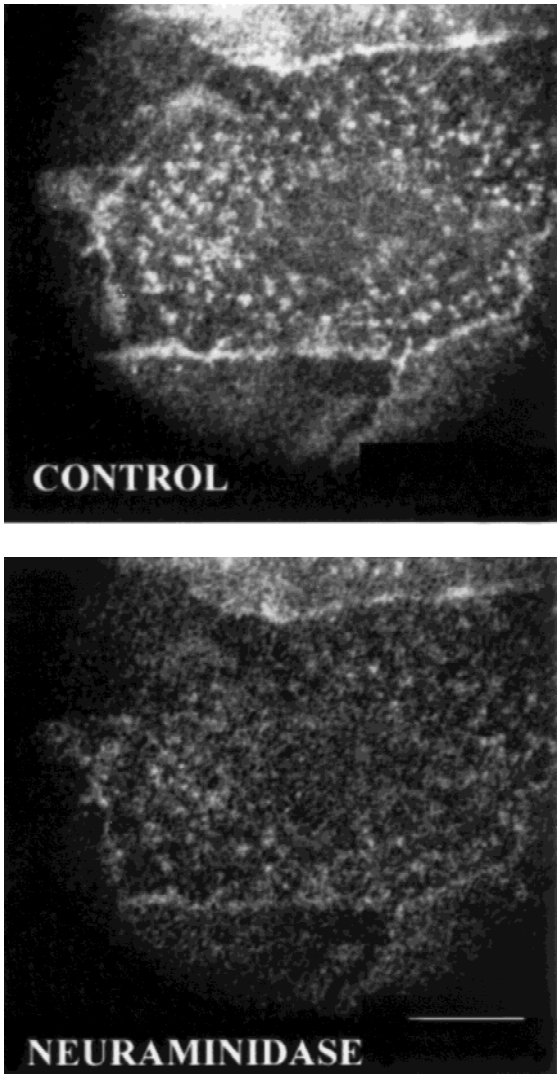

Fig. 3. Young MDCK cell stained with WGA-fluorescein before and immediately after treatment with a low dose of neuraminidase. *See* text for details. Scale bar = 10 μm.

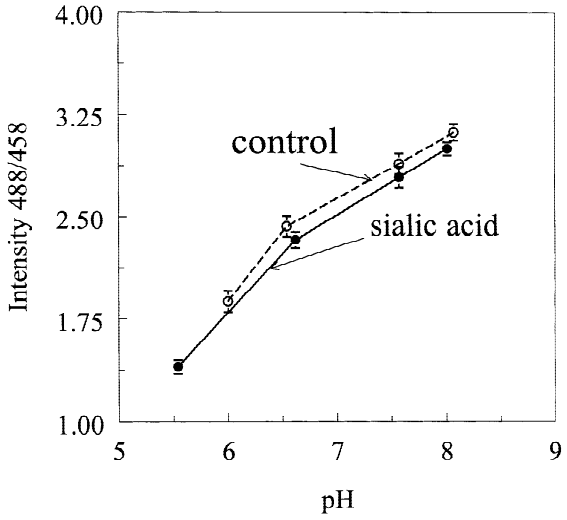

Fig. 4. Calibration of WGA-fluorescein in HEPES buffer without (open circles) or with sialic acid (filled circles). Ratio of fluorescence emission intensities for excitation at 488 or 458 nm is shown as a function of solution pH. In sialic acid containing solutions, the pH was adjusted by changing the concentration of sialic acid. In control HEPES buffer, pH was altered by the addition of HCl.

about half of the sialic acid sites were removed by the enzyme, the result supported our conclusion that any error in our pH measurement could not have been larger than 0.13 pH units as this was the difference between the pH 7.4 buffer and the near-membrane value. When the perfusate pH was 6.4, the near-membrane pH was 6.87 ± 0.03, alkaline to the perfusate. This result showed that a systematic underestimate of near-membrane pH was not the basis for the acid values recorded under control conditions.

Older cells did not exhibit a change in near-membrane pH (Fig. 6) after enzymatic digestion of sialic acid residues although WGA fluorescence was substantially diminished. This result led us to conclude either that sufficient sialic acid residues remained after enzyme treatment to stabilize near-membrane pH or that other charged sugars provided the requisite buffering. The stability of the pH in older cells was consistent with previous observations showing increased glycocalyx buffer capacity as cells become more developed (Dzekunov & Spring, 1998).

Sodium Substitution

To ascertain whether a relationship exists between acid extrusion systems and near-membrane pH, we replaced most of the perfusate Na (128 mM) by Li to inhibit Na/H exchangers, Na-dependent cotransporters and Na channels. Young epithelia (6–10 days old) with a broad range of control pHs showed varied responses to the replacement of Na by Li (Fig. 7, top). Although the average pH changed significantly from 6.60 ± 0.09 (9 monolayers) to 6.83 ± 0.07 (9 monolayers), one group of patches acidified compared to control values (Fig. 7, top). Switching the perfusion medium back to Na demonstrated a somewhat heterogeneous result, as well, with the mean value changing significantly from 6.71 ± 0.1 (5 monolayers) to 6.44 ± 0.1 (5 monolayers). On older cells (20–23 days), the removal of Na and replacement by Li resulted in uniform alkalinization of near-membrane pH from 6.33 ± 0.04 (8 monolayers) to 6.56 ± 0.04 (8 monolayers), an effect that was fully reversible (Fig. 7, bottom).

Near-Membrane pH is Similar in Bicarbonate Buffer

Because bicarbonate transporters are an important regulator of pH in many epithelial cells, the use of the HEPES

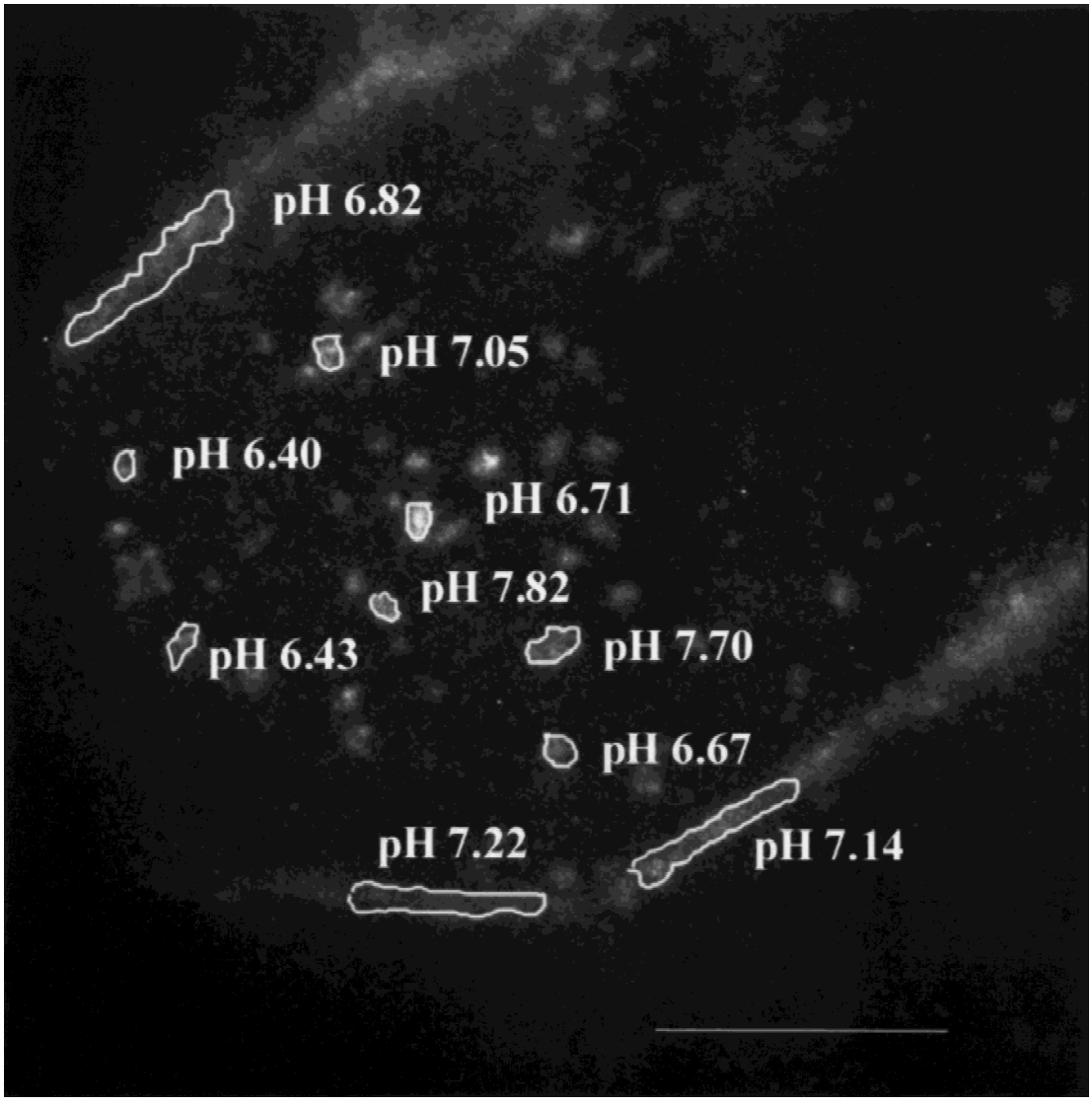

Fig. 5. A representative example of near-membrane pH measured by WGA-fluorescein fluorescence of young cells from the ratio of emission intensities at the two excitation wavelengths (488 nm and 458 nm). Numerical pH values calculated for individual patches are shown adjacent to each patch. Scale bar = 10 μm.

perfusion buffer instead of bicarbonate could produce artifactual changes of near-membrane pH. To test this possibility we perfused 6 day-old MDCK monolayers with bicarbonate buffer (pH 7.4) and measured the near-membrane pH. As shown in Fig. 8, the distribution of pH values in bicarbonate buffer did not differ significantly from that in HEPES. The mean values of pH were 6.76 ± 0.05 (n = 305 cells in 6 monolayers) in HEPES buffer and 6.63 ± 0.05 (n = 314 cells in 2 monolayers) in bicarbonate buffer.

Discussion

The goal of this study was the use of near-membrane pH measurements of the apical glycocalyx of MDCK epithelial cells to gain additional understanding of the nature of the microclimate on the cell surface. We found that the apical surface pH was substantially below that of the bulk medium as a result both of buffering by the glycocalyx and of sodium-dependent acid-base transport. In addition, the surface pH of young cells was remarkably heterogeneous, presumably reflecting a nonuniform underlying distribution of proteins and acid extrusion sites. Our results also gave us insights into the nature and distribution of the charged sugar groups that constitute the apical glycocalyx.

It has previously been shown by a number of investigators using pH-sensitive microelectrodes that the mucosal surface pH of intestinal epithelia was acidic to the pH of the luminal solution, resulting in a so-called ‘acid

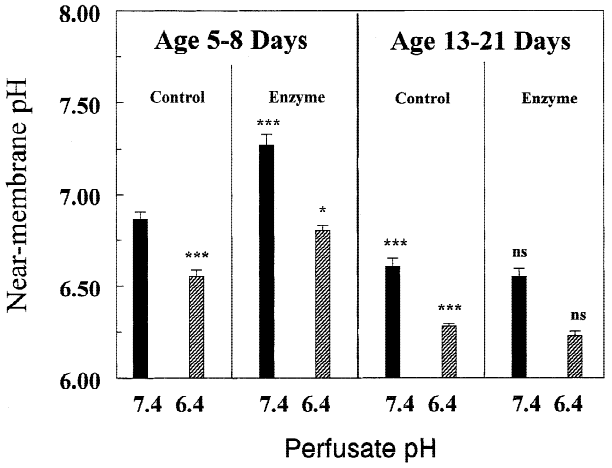

Fig. 6. Near-membrane pH measured with HEPES perfusates at several representative sites on the apical surface of young cells stained with WGA-fluorescein in young cells (left side) and older cells (right) as a function of perfusate pH and treatment with neuraminidase (enzyme). Statistical significance indicated by: * = <0.05, *** = <0.001, *ns* = not significant.

microclimate' (Lucas, 1983; Rechkemmer et al., 1986; Shimada, 1987; McNeil et al., 1987; Holtug et al., 1992). In contrast, Genz, von Engelhardt & Busche (1999) recently reported an alkaline surface pH in guinea-pig colon using 5-(N-hexadecanoyl) aminofluorescein (denoted H-110 by Molecular Probes, Eugene, OR) as a near-membrane pH indicator. H-110 inserts its acyl tail directly into the plasma membrane with the result that the pH is determined very close to the membrane. They attributed the low pH values in the previous microelectrode experiments to artifacts associated with the difficulties of positioning the electrodes near the cell membrane surface. In previous experiments with H-110 on MDCK cells (Timbs & Spring, 1996), we reported that the dye readily labeled the basolateral membrane of MDCK cells and that the fluorescence emission spectrum shifted substantially to the red when the dye was in the membrane. We also observed that antifluorescein antibody only partially reduced H-110 fluorescence, consistent with the conclusion that some of the fluorescein head groups were facing into the cytoplasm as well as toward the external solution (M.M. Timbs and K.R. Spring, *unpublished observations*). Since the intensity of fluorescein fluorescence decreases as pH falls, a substantial alkaline error is introduced into the estimates of near-membrane pH by even a small fraction of the dye facing the more alkaline cell interior. For this reason, we deemed H-110 unreliable as a probe of near-membrane pH in MDCK cells and question the validity of the measurements by Genz et al. (1999).

Lectins, sugar-binding proteins widely used for carbohydrate recognition, conjugated with a pH-sensitive fluorescent dye are a useful tool for near-membrane pH measurements (Kirschberger et al., 1999). From the variety of available lectins we chose WGA, because: (i) its binding to the apical surface of MDCK epithelia was already known (Kersting et al., 1993); (ii) it recognizes terminal N-acetylneuraminic acids, which are known to make a major contribution to the net negative charge of glycocalyx (Varki, 1997). Another potential WGA ligand is N-acetyl-glucosamine. However, experiments with succinylated WGA, specific for N-acetyl-glucosamine, showed that the WGA binding site on the apical surface of MDCK cells was sialic (N-acetyl-neuraminic) acid. The lack of fluorescence from monolayers stained with the lectin SNA indicates, that all terminal neuraminic acid residues must have the α2-3 connection to the next galactose, possibly as an apical membrane sorting signal as suggested for human colon carcinoma (HT-29) cells (Huet et al., 1998).

The heterogeneity of pH values seen in the patches on the surface of young MDCK cells was consistent with the conclusion that the acid extrusion sites on the membrane are not uniformly distributed. Not all WGA-positive sites were acidic and not all of the acidic sites were sensitive to the reduction in perfusate Na concentration. Such variability was not detectable in older cells, presumably because of the higher density of WGA-positive sites on the apical membrane. The surface pH of older cells was not sensitive to the removal of sialic acid residues by neuraminidase. The two probable explanations for this lack of sensitivity to the enzyme are that other charged groups in the glycocalyx provided buffering, or that the enzyme could not get access to all of the sialic acid residues. We previously reported that older MDCK cells have a higher buffering capacity due to greater development of the glycocalyx (Dzekunov & Spring, 1998).

Application of several other fluorescent lectins to the apical side of MDCK epithelia showed the presence of mannose, glucose, galactose and N-acetyl-galactosamine in the glycocalyx. It was remarkable how the lectin binding pattern varied with cell age. In young cells, all lectins that bound exhibited a patchy distribution on cell surface (Fig. 1, top), consistent with the clustering of glycoproteins and glycolipids. Simultaneous labeling of young cells with WGA-rhodamine and PNA-fluorescein (Fig. 2) showed topological differences in carbohydrate distribution: neuraminic acid residues (WGA-positive) were highly expressed in tight junction regions and on the apical surface, whereas terminal galactoses (PNA-positive) were only observed on the apical surface.

These observations support the argument that binding of these multivalent lectins did not cause the formation of patches but merely reflects the natural sugar distribution in glycocalyx of MDCK cells. Regional and spotlike distribution of carbohydrates was described in the glycocalyx of intact, nonfixed outer hair-cells

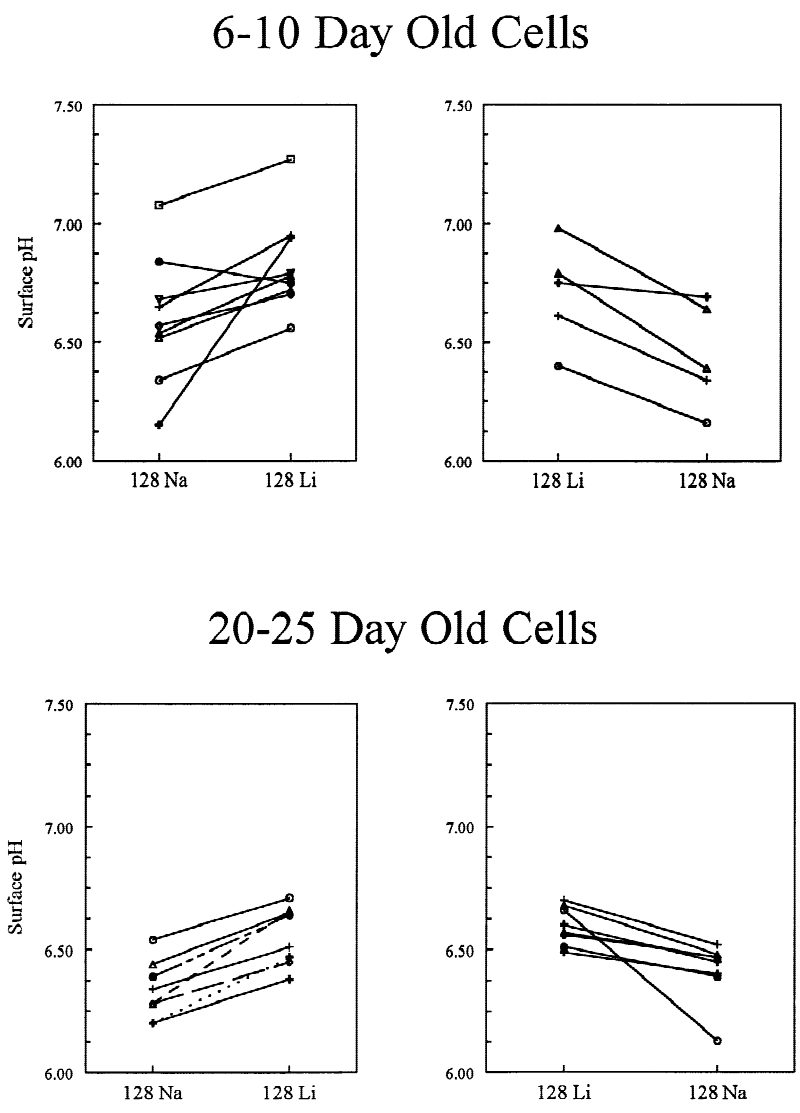

Fig. 7. Near-membrane pH measured with WGA-fluorescein in young cells (top panels) and older cells (bottom panels) when 128 mM of the perfusate sodium was replaced by lithium (left panels) or when the perfusate was returned to sodium (right panels). Error bars omitted for clarity. *See* text for details.

(OHCs) from the guinea-pig cochlea (Plinkert et al., 1992). By means of lectin binding N-acetyl-D-glucosamine was shown in the entire OHC membrane, whereas mannose and glucose were mainly observed at the cuticular plate and at the basal cell pole. These authors suggested that the spot-like arrangement of N-acetyl-D-galactosamine near the cuticular plate may be associated with proteins of the *zonula adherens.*

On older MDCK cells, WGA and PNA staining looked more uniform without discernable differences between tight junctions and the apical surface, as previously described (Kersting et al., 1993). The apical membrane of older cells is highly infolded compared to that of young cells and any carbohydrate clusters may be more difficult to detect due to the density of glycosylation and limits of the spatial resolution of the light microscope. The apparent smooth surface area from light microscopic images of a young MDCK cell is as high as 400 μm^2 while that in an older cell is usually about 50 μm^2. Each cell in an older monolayer predominantly binds only one type of lectin (e.g., WGA or PNA), a result that has been used to discriminate between the principal or intercalated cell populations (Kersting et al., 1993). Many young MDCK cells showed distinct labeling with both lectins as well as indications that other lectins occupy sites different from those recognized by WGA or PNA.

The discrete, punctate distribution of WGA-fluorescein on the apical surface of young MDCK cells presumably reflects the clustering of proteins that have glycosylation chains terminating with a sialic acid residue. Thus, the lectin pattern should be indicative of the underlying protein distribution pattern. The lack of mobility of the lectin binding sites as well as their temporal and spatial stability is consistent with many recent observations about the organization of cell membranes into 'corrals' (Wier & Edidin, 1988; Edidin, Kuo & Sheetz,

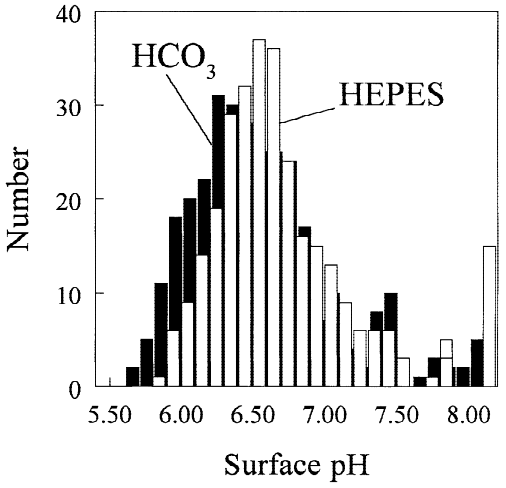

Fig. 8. Distribution of pH values measured in patches from young cells stained with WGA-fluorescein in HEPES (open bars) or HCO_3 buffered perfusates. The HEPES data show 305 patches in six monolayers and the bicarbonate data are from 314 patches in 2 monolayers. The mean values are not significantly different.

1991; Simson et al., 1998). It is reasonable to assume that the membrane proteins and, hence, their glycosylation groups are fixed in position in the cell membrane by cytoskeletal elements (Jacobson & Dietrich, 1999). We speculate that such fixed protein clusters are associated with chemically distinct membrane lipid domains, as described in the membrane 'raft' hypothesis (Jacobson & Dietrich, 1999). The lack of mobility of the glycoproteins is then consistent with anchoring of such rafts to the cytoskeleton creating a kind of mosaic of lipid-protein-sugars associated by functional requirements. Such membrane regions also alter the local pH or electrostatic microenvironment on the extracellular cell surface both by the nature of the sugar moieties expressed in the glycocalyx as well as by the transport of charged solutes, possibly optimizing extracellular conditions for their function. The return of fluorescent labeling to the same locations on the apical membrane after neuraminidase treatment indicates that glycosylated proteins are targeted to fixed locations on specific domains of the apical membrane, a result consistent with recent reports about the distribution of clathrin-coated pits (Gaidarov et al., 1999). Young, flattened but confluent MDCK cells may prove to be a useful tool for the production of a detailed map of these domains in the apical membrane.

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