Subcellular Redistribution Is Involved in Acute Regulation of the Brush Border Na\(^+/H^+\) Exchanger Isoform 3 in Human Colon Adenocarcinoma Cell Line Caco-2

PROTEIN KINASE C-MEDIATED INHIBITION OF THE EXCHANGER*

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Na\(^+/H^+\) exchanger isoform 3 (NHE3), an epithelial brush border isoform of the Na\(^+/H^+\) exchanger gene family, plays an important role in reabsorption of Na\(^+\) in the small intestine, the colon, and the kidney. In several cell types, phorbol 12-myristate 13-acetate (PMA) acutely inhibits NHE3 activity by changes in \(V_{\text{max}}\) but the mechanism of this inhibition is unknown. We investigated the role of subcellular redistribution of NHE3 in the PMA-induced inhibition of endogenous brush border NHE3 in a model human colon adenocarcinoma cell line, Caco-2. Subcellular localization of NHE3 was examined by confocal morphometric analysis complemented with cell surface biotinylation and compared with NHE3 activity evaluated by fluorometric measurement of intracellular pH. PMA inhibited NHE3 activity by 28% (\(p<0.01\)), which was associated with a decrease of the ratio of the brush border/subapical cytoplasmic compartment of NHE3 from ~4.3 to ~2.4. This translocation resulted in 10–15% of the total cell NHE3 being shifted from the brush border pool to the cytoplasmic pool. These effects were mediated by protein kinase C, since they were blocked by the protein kinase C inhibitor H7. We conclude that inhibition of NHE3 by protein kinase C in Caco-2 cells involves redistribution of the exchanger from brush border into a subapical cytoplasmic compartment, and that this mechanism contributes ~50% to the overall protein kinase C-induced inhibition of the exchanger.

Of the five isoforms of mammalian Na\(^+/H^+\) exchangers (NHEs) fully cloned to date, the two characterized in most detail are NHE1 and NHE3. NHE1 is present in nearly all mammalian cells and is involved in regulation of cytoplasmic pH, volume, and perhaps cell proliferation (1, 2). In most native mammalian epithelia, NHE1 is found in the basolateral membrane domain. The other well characterized isoform, NHE3, has been found in some segments of small and large intestine and renal tubules, where it has been localized exclusively to the apical membrane domain (3–5). NHE3 plays a role in net NaCl, HCO\(_3^-\), and probably NH\(_3\) reabsorption in renal tubules and in neutral NaCl absorption in the intestine. In dogs, NHE3 accounts for almost all ileal Na\(^+\) absorption and the neurohormonal-induced increase in ileal Na\(^+\) absorption that occurs after meals (6, 7). In the rabbit ileal brush border (BB), NHE3 contributes approximately 50% of the basal Na\(^+/H^+\) exchange, the rest being contributed by NHE2 (8).

Regulation of NHE1 is generally modulated by changes in K\(^+/H^+\) (i.e. the exchanger’s affinity for intracellular H\(^+\) ions) (9, 10). In contrast, the activity of NHE3 is regulated mainly by changes in maximal velocity (\(V_{\text{max}}\)) of the exchanger. Growth factors, serum, okadaic acid, and several calmodulin blockers all increase \(V_{\text{max}}\) of NHE3, whereas agents that increase protein kinase C (PKC) activity (phorbol ester, carbachol) decrease \(V_{\text{max}}\) (11–13). Phorbol 12-myristate 13-acetate (PMA) and/or PKC have been shown to inhibit endogenous as well as transfected NHE3 activity in a variety of cells including rabbit gallbladder epithelium (14), the opossum kidney (OK) cell line (15, 16), Chinese hamster ovary (AP-1) cell line (17, 18), and Chinese hamster lung fibroblastic cell line PS120 (11, 13). Since the inhibition occurs via a decrease in \(V_{\text{max}}\) and is observed within minutes, this type of control might theoretically be achieved by a decrease in the number of active molecules at the BB (a redistribution-dependent mechanism and/or rapid degradation), by changes in the turnover number of individual exchanger molecules, or both.

Recently, multiple plasma membrane transport proteins have been shown to be regulated, at least in part, by cellular redistribution (endocytic retrieval from and/or exocytic insertion into the plasma membrane). Well characterized examples include glucose transporters GLUT1 and GLUT4 in adipocytes (19), the K\(^+/H^+\)-ATPase pump in gastric parietal cells (20), the water channel aquaporin 2 in the kidney (21), the renal NaPi-2 cotransporter (22), the Na\(^+\)/glucose cotransporter SGLT1 (23), the renal Na\(^+\)-K\(^+\)-ATPase pump (24), and the cystic fibrosis transmembrane conductance regulator (CFTR) (25). Much less is known, however, about the mechanisms of short term regulation of glucose transporters (26).
lation of NHE3. The acute regulation, via growth factors and protein kinases, may be important in rapid adjustments of changing intraluminal sodium concentration in renal tubules and in altering Na\(^+\) absorption in the intestine and the kidney. Some evidence indicates that translocation from BB into the cytoplasm might be involved in the short term inhibition of NHE3 in renal proximal tubules, although the specific nature of the cytoplasmic vesicular compartment containing the internalized NHE3 has not been defined (26, 27). Until now, no results have addressed the possible contribution of the redistribution-based mechanism in the regulation of intestinal NHE3.

The results presented in this study indicate that acute inhibition of NHE3 activity by PKC in the human colon adenocarcinoma cell line Caco-2, clone PF-11, was characterized previously (28) and was recently found to express endogenous NHE3 (29). NHE3 is present exclusively at the BB, and its activity peaks at 17–22 days postconfluency. At that time, NHE3 constitutes 85–90% of total BB Na\(^+/\)H\(^-\) exchange with only marginal contribution of NHE1. For all experiments described in this report, Caco-2 cells were plated at a density 5–8 \(\times\) 10\(^5\) cells/cm\(^2\) on a 0.45-\(\mu\)m pore size, HD Falcon PET membranes (Becton Dickinson Labware, Franklin Lakes, NJ) and grown in Dulbecco’s modified Eagle’s medium supplemented with 0.1 mM nonessential amino acids, 1 mM pyruvate, penicillin (50 IU/mL), streptomycin (50 \(\mu\)g/mL), and 10% fetal bovine serum, in a 10% CO\(_2\) humidified incubator at 37 \(^\circ\)C. For evaluation of Na\(^+/\)H\(^-\) exchange activity by fluorometry, cells were grown on small apertures in plastic coverslips (“filterslips”) as described in detail elsewhere (30, 31).

**Experimental Procedures**

**Cell Culture**—Human colon adenocarcinoma cell line Caco-2, clone PF-11, was characterized previously (28) and was recently found to express endogenous NHE3 (29). NHE3 is present exclusively at the BB, and its activity peaks at 17–22 days postconfluency. At that time, NHE3 constitutes 85–90% of total BB Na\(^+/\)H\(^-\) exchange with only marginal contribution of NHE1. For all experiments described in this report, Caco-2 cells were plated at a density 5–8 \(\times\) 10\(^5\) cells/cm\(^2\) on a 0.45-\(\mu\)m pore size, HD Falcon PET membranes (Becton Dickinson Labware, Franklin Lakes, NJ) and grown in Dulbecco's modified Eagle's medium supplemented with 0.1 mM nonessential amino acids, 1 mM pyruvate, penicillin (50 IU/mL), streptomycin (50 \(\mu\)g/mL), and 10% fetal bovine serum, in a 10% CO\(_2\) humidified incubator at 37 \(^\circ\)C. For evaluation of Na\(^+/\)H\(^-\) exchange activity by fluorometry, cells were grown on small apertures in plastic coverslips (“filterslips”) as described previously (30). In all experiments, the monolayers were investigated at 17–22 days postconfluency.

**Measurement of Na\(^+/\)H\(^-\) Exchange Rate**—For functional evaluation of apical (AP) and basolateral (BL) Na\(^+/\)H\(^-\) exchange in Caco-2 cells, intracellular pH (pHi) was measured by a fluorometric method based on cytosolic loading with a pH-sensitive fluorophore, acetoxymethyl ester of 2',7'-bis(carboxyethyl)5(6)-carboxyfluorescein (BCECF) (Molecular Probes, Eugene, OR), as described in detail elsewhere (30, 31). Briefly, monolayers grown on filterslips were serum-starved for 8–12 h and incubated with BCECF-AM (5 \(\mu\)M) in the presence of 50 mM NH\(_4\)Cl (to inhibit Na\(^+/\)H\(^-\) exchange) to allow BCECF to enter the cells. After a 30-min incubation, monolayers were rinsed twice with PBS, and NHE3 was immunolabeled as described above to confirm polarization of Na\(^+/\)H\(^-\) exchange in Caco-2 cells. Then, monolayers were rinsed with PBS and incubated with FITC-conjugated phallloidin (FITC/phallloidin; 0.6 \(\mu\)g/mL; Sigma) for 20 min at room temperature. Monolayers were rinsed twice with PBS, and NHE3 was immunolabeled as described above. The rationale for this approach was based on the fact that nearly all of the actin within the apical zone of polarized absorptive epithelial cells is located within the microvilli, terminal web, and tight junctional complexes.

All monolayers were examined in xz and yz planes using a Zeiss LSM410 confocal fluorescence microscope as described below.

**Morphometric Confocal Analysis of Intracellular Distribution of NHE3**—For morphometric confocal analysis, serum-starved Caco-2 monolayers were exposed to FITC (1 \(\mu\)g/mL) or control medium for 20 min at 37 \(^\circ\)C. Cells were then cooled rapidly to 4 \(^\circ\)C, fixed with cold 3% paraformaldehyde, and stained as above. Using the confocal microscope, serial optical sections were obtained parallel to the monolayer surface (xy plane) at 0.4- \(\mu\)m steps. FITC/PHA and Cy3-labeled NHE3 were excited by separate lasers at 488 nm (argon laser) and 543 nm (HeNe laser), respectively. Emissions from FITC (505–530 nm) and Cy3 (580–660 nm) were collected sequentially (to avoid detection of FITC in the Cy3 channel), and the images were stored on an optical disc.

As discussed below, not all cells had adequate Cy3 fluorescence due to heterogeneity of NHE3 expression. Therefore, only cells with significant Cy3 signal (up to 70% of all cells; defined to be adequate when exceeding the average background fluorescence intensity by more than 4 times) were randomly chosen for analysis. Images of these cells were analyzed using MetaMorph software (Universal Imaging Corp.) as follows. Four separate areas of 5 \(\times\) 5 pixels were randomly selected at the surface of each analyzed cell, and pixel density in these areas was determined in consecutive optical sections along the apical-basal (zx) axis of the cell, separately for FITC and Cy3. Obtained values were then averaged against the optical section number. The curves obtained for Cy3 (representing distribution of NHE3) were divided into the part overlapping the BB (defined by the distribution of PHA-E; see below) and the part corresponding to the subapical cytoplasmic compartment (SAC). SAC was defined as an apical cytoplasmic area containing Cy3 fluorescence.
cent signal and located between the BB and the background fluorescence of the cytoplasm. Finally, the total surface area under the Cy3 curve corresponding to BB plus SAC was calculated (after subtracting the background fluorescence), and the results were expressed as SAC/(BB + SAC) × 100. The obtained values represented the relative amount of immunolabeled NHE3 located in SAC as a percentage of total apical NHE3 amount (BB + SAC) and were, therefore, independent of differences in longitudinal BB dimensions and intensity of Cy3 fluorescence among the cells examined.

The apical and intracellular BB limits were defined as the positions at which the BB signal decreased most rapidly during scanning along the apical-basal axis. This was determined using an algorithm to localize the maximum values of the second derivative of the FITC fluorescent signal (BB-bound lectin or, in some experiments, phalloidin-labeled cortical actin) at consecutive optical sections, after smoothing data with the Savitzky-Golay method to decrease random noise (Microcal Origin version 4.1, Microcal Software). The maximum values of the second derivative curve indicated the maximal changes in the slopes of the FITC curve and were assumed to correspond to the apical and intracellular BB limits.

Since the definition of intracellular limit of the BB was critical for the subsequent calculations, we used another way to verify the results of the derivative approach. The corresponding FITC/PHA and Cy3/NHE3 curves were normalized to 100% maximal pixel density, and the ratio of FITC versus Cy3 was calculated for each optical section. The point at which the Cy3 values diverged from the intracellular portion of the FITC curve (ratio of Cy3 versus FITC significantly higher than 1.0) was calculated for each cell. This approach determined the point of separation of the NHE3-related signal within the cytoplasm from the BB-defining signal.

**Evaluation of Redistribution of NHE3 from BB to the Cytoplasm by Reversible Surface Biotinylation**—Reversible surface biotinylation was used to complement the data obtained by confocal analysis, since the results of biotinylation do not rely on calculated limits of the BB. A modification of the procedure described by Le Bivic et al. (37) was used, with omission of the immunoprecipitation step. Serum-starved Caco-2 monolayers grown on 25-mm Falcon culture inserts were collected and cooled rapidly to 4 °C to inhibit endocytosis (an abbreviated flow chart of the procedure is shown in Fig. 1). The entire subsequent procedure was performed at 4 °C unless otherwise indicated. The apical monolayer surface was rinsed with PBS and exposed to Sulfo-NHS-SS-Biotin (0.5 mg/ml; Pierce) in borate buffer (10 mM H3BO3, 154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl2, pH 9.0) for 1 h, followed by extensive washing. Next, groups of 4–6 monolayers were warmed to 37 °C and incubated in serum-free DMEM containing PMA (1 μM; Fig. 1, group 3) or in DMEM only (control group; Fig. 1, group 4) for 20 min. Separate groups were incubated as above but in the presence of 65 μM H7 (also present during the preceding hour of biotinylation). Monolayers were cooled to 4 °C and exposed to 50 mM glutathione (free acid; Sigma) in 75 mM NaCl supplemented with 10% fetal bovine serum (pH 9.0) for 40 min, followed by quenching of the free NHS-SS-Biotin with Tris buffer (20 mM Tris, 120 mM NaCl, pH 7.4) for 10 min. In one group of monolayers studied to estimate the total initial amount of biotinylated NHE3 at the BB (Fig. 1, group 1), cells were exposed to control solution lacking glutathione. Also, a separate group was biotinylated and then exposed to glutathione with omission of the step at 37 °C to evaluate the efficiency of the stripping procedure (Fig. 1, group 5). Finally, in a separate group of monolayers a possible loss of surface biotinylated NHE3 during the 20 min of incubation at 37 °C was evaluated (Fig. 1, group 2). If such a loss was significant, it should be taken into account when calculating the amount of NHE3 retrieved from BB during incubation at 37 °C. These monolayers were biotinylated at 4 °C and incubated with 1 μM PMA at 37 °C for 20 min, and the cells were lysed with omission of the glutathione stripping step. The calculated total biotinylated NHE3 (BB + intracellular) was then compared with the initial (i.e. prior to incubation at 37 °C) amount of biotinylated NHE3 at the BB, using Western

![Fig. 1. An abbreviated flow chart showing the major steps of reversible cell surface biotinylation used to quantify the PKC-induced internalization of BB NHE3 in Caco-2 cells.](image-url)
PKC-mediated Subcellular Redistribution of NHE3

**RESULTS**

PKC Inhibits Endogenous NHE3 Activity in Caco-2 Cells—In control Caco-2 monolayers, the average Na\(^+\)/H\(^+\) exchange rate in the presence of 130 mM Na\(^+\) (at the AP surface) was 360 ± 55 μM/s (mean ± S.D.; at pH\(_6.4\)) (Table I). Preincubation of monolayers with 1 μM PMA inhibited the NHE3 activity (Fig. 2 and Table I). The maximal effect of PMA was noted at 10 min of exposure and did not change significantly for up to 40 min of incubation with PMA (Fig. 2). At all time points, preincubation of the cells with H7 (65 μM) resulted in a complete reversal of the PMA effect (Fig. 2).

Since stable inhibition of NHE3 activity was observed between 10 and 40 min of cell exposure to PMA, we chose a 20-min incubation with PMA in all subsequent experiments. Twenty minutes of exposure of the monolayers to 1 μM PMA resulted in a significant inhibition of the rate of intracellular alkalinization to 259 ± 29 μM/s (p < 0.01), i.e. by approximately 28% of the control values (Table I). Preincubation of the cells with H7 completely abolished the effects of PMA but had no effect on NHE3 activity in control monolayers (Table I).

**Endogenous NHE3 Is Present at the Brush Border as Well as in the Subapical Cytoplasmic Compartment**—Between 17 and 22 days postconfluency, approximately ~70% of all Caco-2 cells exhibited a strong labeling of NHE3 at the apical surface. At that time, confocal microscopy in the xy plane revealed heterogeneous expression of NHE3 at the apical surface of adjacent cells (Fig. 3), which resembled the “mosaic” pattern of distribution of several BB hydrolases described previously (38, 39). In contrast to the monolayer as a whole, the distribution of immunolabeled NHE3 throughout the apical surface of cells expressing NHE3 was very homogenous, and no significant differences were found among the NHE3 distribution patterns in any of the four 5 × 5-pixel areas analyzed in each cell.

Analysis of the images in the xz plane revealed the presence of NHE3 in a relatively wide apical zone of Caco-2 cells. To better define the cellular distribution of NHE3, we labeled the BB with FITC/PHA before the permeabilization and incubation of the monolayers with anti-NHE3 antibody. As shown in Fig. 4A, the lectin labeled a smaller apical region of the cells compared with NHE3-Ab (Fig. 4B). These results suggested the presence of NHE3 in both the BB and a closely associated SAC (Fig. 4B). Immunoreactive NHE3 was not detected at the basolateral membrane domain in any of the monolayers examined between 3 and 22 days postconfluency.

In all of the cells examined, a speckled pattern of weak Cy3 fluorescence was observed within the cytoplasm below the 4–6-μm-wide zone of SAC. For the purpose of confocal analysis, this staining was considered to represent background fluorescence.
since it did not differ significantly from the pattern observed in control monolayers in which Ab 1380 was replaced by preimmune rabbit serum (Fig. 4C).

Quantitative Comparison of BB and NHE3 Staining Patterns—Assuming that lectin labeled exclusively the BB, analyses were developed to assign the edges of the BB detected by this probe. Analysis of derivative curves obtained from 30 cells in which the BB was labeled with FITC/PHA (both control and exposed to PMA) revealed remarkable similarity of the slopes, with the apical BB limit defined at 30 ± 5%, and the intracellular BB limit at 50 ± 7% of the maximal amplitude of BB fluorescence (Fig. 5). These values were used to define the BB limits in all subsequent calculations. Since the maximal pixel density of FITC/PHA was normalized to 100% for the calculations of BB limits, differences in the density of the BB fluorescence observed among the cells examined did not affect the calculated BB limits, the latter depending on the slope of the distribution curves rather than on their amplitudes.

A similar general pattern of distribution of the BB marker was observed in cells in which the BB actin was labeled with FITC/phalloidin (Fig. 6). In these cells, the apical and intracellular BB limits, obtained by the derivative method, were not significantly different from those obtained using FITC/PHA (34 ± 5% for apical limit and 46 ± 7% for intracellular limit (n = 20)). The slight shift of the intracytoplasmic BB limit observed in the FITC/phalloidin method might result from labeling of actin within the terminal web. However, this difference was not statistically significant.

The rationale for the derivative method was significantly supported by the observation that, in all cells examined, the BB-marking FITC curve diverged from the NHE3-marking Cy3 curve at the upper boundary of the intracellular portion of the distribution curves (Fig. 7). Analysis of patterns obtained from 40 cells in which the BB was labeled with FITC/PHA revealed...
that the intracellular BB limit defined by the derivative method was also the "point of divergence" (47 ± 6 versus 50 ± 7% of maximal pixel density for the point of divergence versus derivative method, respectively (not significant)). This validated the derivative approach as a means to estimate a physiologically relevant edge of the BB.

PKC Causes Partial Redistribution of NHE3 from BB to SAC—The effect of PMA on the intracellular distribution of NHE3 was examined utilizing two independent but complementary methods: confocal morphometric analysis and reversible cell surface biotinylation. By confocal analysis, 18.7 ± 2.8% (mean ± S.D.) of total (BB + SAC) NHE3 was localized in SAC of the control cells (Table I and Fig. 7). Preincubation of the cells with PMA resulted in a significant increase of the SAC—BB of control cells, SAC of PMA-treated cells, and BB content of NHE3 by 12.9% (calculated as ((100 - SAC/BBC) - (100 - SAC/PMA))/BBC × 100, where SAC, SAC/PMA, and BB/C represent the relative abundance of NHE3 in SAC of control cells, SAC of PMA-treated cells, and BB of control cells, respectively) (Table I and Fig. 7). Pretreatment with H7 resulted in a complete inhibition of the PMA-dependent redistribution of NHE3, whereas no significant effect of H7 alone was observed in control monolayers (Table I). Moreover, PMA treatment did not significantly change the width of the BB (Table II). In preliminary experiments, Caco-2 cells were incubated in 50 mM NH₄Cl with or without PMA and then briefly exposed to 130 mM Na⁺ before fixation, as described under "Experimental Procedures" for studies of NHE3 activity. This procedure, performed to acidify the cell interior prior to fixation, did not result in any significant change in distribution of NHE3 in control or PMA-treated cells (data not shown) and, therefore, was not performed in subsequent experiments.

Possible incomplete penetration of FITC/PHA into the intermicrovillar domains of the BB was not a significant factor affecting the calculated distribution of NHE3 by confocal morphometric analysis. As shown in Table II, neither the BB dimensions nor the calculated NHE3 distribution were significantly different when either FITC/PHA (labeling the BB from outside the cell) or FITC/phalloidin (labeling the cortical actin cytoskeleton within the BB) were used to mark the BB of Caco-2 cells. Thus, labeling the BB of Caco-2 cells with FITC/phalloidin is comparable with the FITC/PHA method. However, the method was also the “point of divergence” (47 ± 6 versus 50 ± 7% of maximal pixel density for the point of divergence versus derivative method, respectively (not significant)). This validated the derivative approach as a means to estimate a physiologically relevant edge of the BB.

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ever, possible alterations of the amounts of F-actin versus G-actin caused by protein kinase agonists and/or growth factors makes the first method a less optimal approach to label the BB.

The data obtained by confocal analysis were confirmed by the results of reversible surface biotinylation. In control monolayers, 12.9 ± 3.7% (mean ± S.D.; calculated after subtraction of the poststripping background) of the total initial BB content of biotinylated NHE3 was found within the cytoplasm after 20 min of incubation at 37 °C, the results most probably representing the net effect of apical membrane recycling (Fig. 8).

Incubation of the cells with PMA for 20 min at 37 °C resulted in presenting the net effect of apical membrane recycling (Fig. 8). The intracellular pool of NHE3, whereas no significant effect of H7 incubation with H7 abolished the PMA-induced increase in the number of F-actin confounding factors in the biotinylation experiments, the possibility that a significant amount of residual cytosolic proteins in damaged cells were initially biotinylated and then contributed to the pool assumed to represent endocytosed, biotinylated surface molecules. As evident from the data shown in Fig. 8, the amount of biotin left at the BB after stripping step was very low (3.3 ± 1.9% of the initial BB amount) and, most importantly, significantly lower than the cytoplasmic, internalized pool. To investigate the second possible source of error mentioned above, the biotinylated material was separated on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and Western analysis was performed with anti-actin monoclonal antibody. As expected, a protein band corresponding to actin standard preparation was found in the cell lysate obtained from biotinylated Caco-2 monolayers and not incubated with avidin-agarose beads. In contrast, no detectable actin was found in the fractions recovered from the Avidin beads, indicating that the intracellular biotinylated NHE3 is derived only from the biotinylated pool of the surface molecules (Fig. 9).

**TABLE II**

|                        | Apical-cytoplasmic BB dimension a | NHE3 in SAC b |
|------------------------|----------------------------------|---------------|
|                        | Control                           | PMA           |
| PHA-E                  | 3.8 ± 0.5                         | 3.5 ± 0.4     |
| Phalloidin             | 4.0 ± 0.7                         | 3.8 ± 0.8     |

a. n = 30 cells.  
b. Values below show NHE3 in SAC (as a percentage of BB + SAC).  

In monolayers incubated with PMA at 37 °C with omission of the glutathione stripping step, the total (BB + intracellular) amount of biotinylated NHE3 was 96 ± 8% of that found initially at the BB (n = 3, NS). This indicated that no significant amount of the initially biotinylated NHE3 was lost from the BB during the 20-min incubation with PMA and allowed us to calculate the PMA-induced decrease of the BB content of NHE3 to be 15.2% (calculated as ((TOT PMA − TOT STRIP) − (TOT CONTR − TOT STRIP)), where TOT PMA, TOT CONTR, and TOT STRIP represent total amounts of biotinylated NHE3 in monolayers treated with PMA at 37 °C and stripped, control monolayers warmed to 37 °C and stripped, and control monolayers that were stripped without warming, respectively. Incubation with H7 abolished the PMA-induced increase in the intracellular pool of NHE3, whereas no significant effect of H7 was observed in monolayers incubated at 37 °C in control medium (Fig. 8, bottom panel).

Control experiments were carried out to rule out two potentially confounding factors in the biotinylation experiments, namely the possibility that a significant amount of residual biotin was left at the BB after the glutathione stripping step and/or that cytosolic proteins in damaged cells were initially biotinylated and then contributed to the pool assumed to represent endocytosed, biotinylated surface molecules. As evident from the data shown in Fig. 8, the amount of biotin left at the BB after stripping step was very low (3.3 ± 1.9% of the initial BB amount) and, most importantly, significantly lower than the cytoplasmic, internalized pool. To investigate the second possible source of error mentioned above, the biotinylated material was separated on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and Western analysis was performed with anti-actin monoclonal antibody. As expected, a protein band corresponding to actin standard preparation was found in the cell lysate obtained from biotinylated Caco-2 monolayers and not incubated with avidin-agarose beads. In contrast, no detectable actin was found in the fractions recovered from the Avidin beads, indicating that the intracellular biotinylated NHE3 is derived only from the biotinylated pool of the surface molecules (Fig. 9).

**DISCUSSION**

In this study, we have shown that the PMA-induced acute inhibition of endogenous NHE3 in Caco-2 cells is mediated by PKC and that it involves redistribution of the exchanger molecules from the BB to SAC. This conclusion is drawn from the results of two complementary methods: a novel morphometric method based on semiquantitative analysis of intracellular distribution of immunoreactive NHE3 in Caco-2 cells by confocal microscopy and reversible cell surface biotinylation. Since PMA caused approximately 28% decrease in NHE3 activity but...
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FIG. 9. Degree of contamination with actin (used as a marker of cytosolic proteins) of various preparations obtained from cell surface biotinylation of Caco-2 cells. Monolayers were surface-biotinylated and exposed to PMA (1 μM) at 37 °C for 20 min. Following surface biotin stripping and incubation of cell lysates with avidin-agarose, the biotinylated material was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with anti-actin antibody. Standard actin is shown in lane 1 (ACTIN). The cell lysate obtained after surface biotinylation is shown in lane 2 (CELL LYSATE). The fraction of cell lysate bound to avidin-agarose is shown in lane 3 (BIOTINYL BB), and the material obtained after exposure to PMA, stripping with glutathione, and incubation with avidin-agarose is shown in lane 4 (BIOTINYL SAC). The absence of any detectable actin in lane 3 and 4 suggests a negligible amount of biotinylated cytosolic proteins in these two preparations. Material separated on lanes 2–4 was obtained from a comparable number of cells.

only 13–15% decrease in the BB amount of the exchanger, we further conclude that the internalization of the BB NHE3 molecules is responsible for approximately 50% of the observed PMA effect. Other mechanisms, probably involving decrease in the turnover number of residual BB NHE3 molecules, are likely to account for the remainder of the inhibition.

Until now, quantitative evaluation of internalization of plasma membrane proteins was most commonly performed by analysis of cellular vesicular fractions obtained by density gradient centrifugation and a counter-current phase partitioning technique (26, 27, 40). The major limitation of this approach results from difficulties in isolating well defined and homogeneous populations of vesicles that represent specific intracellular membranous compartments. Moreover, recognition of internalized molecules is partially based on their functional properties, which could be altered following endocytosis. In contrast, confocal analysis does not depend on the activity of the internalized molecules. Moreover, the analysis can be performed on a small number of cells in native as well as cultured epithelia, as opposed to a relatively large quantity of material needed for biochemical analyses. Finally, confocal analysis combines semiquantitation of the internalized molecules with respect to their BB content with their intracellular localization, although the latter may require colocalization studies with markers of specific intracellular compartments. In this report, we did not define the exact nature of SAC. However, we have reasons to believe that it represents the early endosomal compartment and not the trans-Golgi network (see below).

One aspect of confocal analysis requires comment. The obtained numerical values reflecting the abundance of NHE3 in SAC are relative, and they depend on the definition of the BB limits. However, due to the limits of confocal resolution and a convoluted shape of the physical edges of the BB, the observed curves were bell-shaped, with upper and lower limits difficult to localize by visual inspection (Fig. 5). To resolve this problem, we developed an algorithm based on the assumption that the BB limits correspond to points at which the BB marker changes most steeply. Three lines of evidence support this rationale. First, comparison of multiple BB-defining curves resulted in a remarkably similar location of the apical and intracellular points of maximal deflections relative to the maximal pixel density of every tracing. Moreover, these values were not significantly different when the BB was marked by staining intracellular actin, which speaks against artifacts resulting from possible incomplete penetration of the FITC/PHA into the intermicrovillar domains of the BB. Second, an alternative method of defining the lower BB limit (based on the location of the “point of divergence”) yielded results virtually identical to those obtained by the second derivative approach. Finally, values of BB/SAC distribution of NHE3 obtained from confocal analysis closely resembled those obtained from surface biotinylation experiments, the latter method not relying on the definition of the BB limits. Interestingly, the BB/SAC distribution of NHE3 in control Caco-2 cells obtained by confocal analysis (81% at the BB versus 19% in the cytoplasm) closely resembles that reported for rat proximal tubule NHE3 (75% at the BB versus 25% in the cytoplasm, respectively), the latter obtained by a differential gradient centrifugation method (40).

In contrast to our data obtained from Caco-2 cells, only ~27% of the total cellular NHE3 content was found at the plasma membrane of NHE3-transfected PS120 fibroblasts (as quantified by standard surface biotinylation technique) (13). It is not clear whether the significantly higher plasma membrane/cytosol ratio of NHE3 abundance in epithelial cells versus non epithelial PS120 cells results from an overexpression of NHE3 in the transfected cells in comparison with endogenous levels in Caco-2 cells or is due to cell type-specific differences in the exchanger’s intracellular processing and plasma membrane recycling mechanism.

In this report, we have shown that PMA significantly inhibited the activity of NHE3 in Caco-2 cells and that maximum inhibition was observed as early as 10 min. The fact that the PKC blocker H7 inhibited the PMA effects indicates that the effects were mediated via increased activity of PKC. Both PMA and/or PKC have been shown to inhibit NHE3 activity in a variety of cell types, with the magnitude of the effects similar to that described here. Thus, in rabbit gallbladder epithelium PMA inhibited NHE3-dependent sodium transport by approximately 30%, an effect reversed by the PKC inhibitor calphostin C (14). In opossum kidney cells, PMA inhibited the apical NHE3 by approximately 50–65% (15, 16), and similar effects were reported for NHE3 transected into AP-1 cells (17). In NHE3-transfected PS120 fibroblasts, PMA decreased the V_{max} of the exchanger by approximately 64% of control, an effect partially blocked by PKC inhibitor H7 (10). None of these reports, however, addressed the possibility that retrieval of NHE3 from the plasma membrane could be involved in the observed, PKC-mediated inhibition of the exchanger.

Initial evidence that endocytic retrieval of NHE3 may occur during the inhibition of the exchanger was reported by Hensley et al., in 1989 (26). In rat kidney proximal tubule epithelium, PTH inhibited BB Na^{+}/H^{+} exchange that was accompanied by an increase of the exchanger activity in an intracellular population of vesicles, thus suggesting internalization of the BB NHE3. Although PTH may stimulate both PKC- and PKA-associated signal transduction pathways in renal epithelium, it is believed that at physiological conditions it acts via stimulation of PKC (16). More recently, inhibition of renal NHE3 by both endocytic retrieval and decrease in turnover number has been reported in acutely hypertensive rats, although the nature of the second messenger(s) involved in this process was not investigated (27).

The results of confocal analysis presented here demonstrate a PKC-dependent redistribution of NHE3 from BB to SAC in Caco-2 cells. Two major processes that could lead to such redistribution are the stimulation of endocytic retrieval of NHE3 from the BB, and/or inhibition of the exocytic insertion of the exchanger into the BB. By complementing confocal analysis with cell surface biotinylation, we were able to rule out two
additional mechanisms that theoretically could be involved, namely increased degradation of NHE3 at the BB and increased delivery of newly synthesized molecules to the intracellular pool of the exchanger obtained by both methods suggests that degradation of the exchanger and/or delivery of newly synthesized molecules to the intracellular pool do not play any significant role during the 20 min of exposure to PMA. Recently, PKC has been reported to generally stimulate apical, but not basolateral, endocytosis in Caco-2 cells (41, 42). Moreover, the degree of stimulation was remarkably similar to that reported here. Also, apical endocytosis of NHE3 was observed during rapid inhibition of the exchanger in kidney proximal tubule epithelium in acutely hypertensive rats (27). Based on these data, we suggest that endocytotic retrieval was, at least in part, responsible for the PMA-induced increase in the intracellular pool of NHE3 in Caco-2 cells reported here.

The degree of PMA-induced redistribution of NHE3 does not fully explain the overall inhibitory effect of PMA on the exchanger’s activity, however. The calculated decrease of NHE3 amount at the BB was ~14%, whereas the observed inhibition of the NHE3 activity was ~28%. Theoretically, three mechanisms could explain this discrepancy: 1) a PMA-stimulated increase in NHE3 degradation at the BB; 2) a selective retrieval from the BB of NHE3 molecules with the highest Na+/H+ exchange activity; and 3) PMA-induced changes in turnover number of the residual NHE3 molecules at the BB. Results of biotinylation experiments presented in this report argue against the first of the possibilities mentioned above. The second mechanism is an interesting possibility. However, such a selective retrieval of NHE3 from plasma membrane of any cell type has yet to be demonstrated. Thus, our working hypothesis is that PKC inhibits NHE3 in Caco-2 cells by both stimulating translocation of the exchanger molecules from BB into SAC as well and changing the turnover number of the residual BB molecules. Simultaneous occurrence of both mechanisms has been suggested for multiple plasma membrane transport proteins including retinal taurine transporter (43), glucose transporters GLUT1 and GLUT4 (44), and Na+,K+-ATPase (24, 27). It will be important to further define the relative contribution of each of these two mechanisms in the acute regulation of NHE3 in both epithelial and nonepithelial cells.

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