NHE3 serves as a molecular tool for cAMP-mediated regulation of receptor-mediated endocytosis

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1Physiologisches Institut, University of Würzburg, Röntgenring 9, 97070 Würzburg; 2Aventis, 65926 Frankfurt, Germany; and 3Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus, Denmark

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Gekle, Michael, Oscar K. Serrano, Karina Drumm, Sigrid Mildenberger, Ruth Freudinger, Birgit Gassner, Hans Willi Jansen, and Erik I. Christensen. NHE3 serves as a molecular tool for cAMP-mediated regulation of receptor-mediated endocytosis. Am J Physiol Renal Physiol 283: F549–F558, 2002. First published April 2, 2002; 10.1152/ajprenal.00206.2001.—Receptor-mediated, clathrin-dependent endocytosis (RME) is an essential mechanism for the transport of a variety of macromolecules into cells, as well as for antigen presentation, maintenance of cell polarity, and regulation of cell-surface protein expression (27). One example of RME is the megalin/cubilin-mediated uptake of filtered proteins across the apical membrane of renal proximal tubular cells (6, 7, 10, 12, 38). An important process along the endocytic pathway is the proper pH homeostasis of endosomal compartments (17, 25, 27), because it may influence ligand-receptor dissociation, vesicle trafficking, endosomal fusion events, recycling to the plasma membrane, and coat protein formation (13, 15, 25, 27, 34). pH homeostasis is maintained, at least in part, by the vacuole-type H+ pump (31). In addition, evidence was presented for the involvement of another proton transporter, namely, Na+/H+ exchanger 3 (NHE3), in endosomal pH homeostasis (9, 20, 24). In cells expressing NHE3, this transporter cycles between the apical plasma membrane and the early endosomal compartment (19, 21). Furthermore, NHE3 is internalized through the clathrin-mediated pathway similarly to a variety of receptors serving in RME (4). In addition, Biemesderfer et al. (1) showed that the scavenger receptor megalin and NHE3 can interact specifically. Recently, our laboratory showed that inhibition of NHE3 in renal proximal tubular opossum kidney (OK) cells leads to disturbed endosomal pH homeostasis, a dramatic reduction in RME of albumin, and reduced endocytic vesicle fusion activity (10, 11). This effect could not be attributed to an inhibition of plasma membrane NHE3 but to an inhibition of endosomal membrane NHE3.

RME has been shown to be a regulated process that can be affected by different signaling systems, for example, cAMP (by means of protein kinase A [PKA]), protein kinase C, phosphatidylinositol-3 kinase, or tyrosine kinases (3, 12, 27). For instance, it has been shown that cAMP acts as a negative regulator of endocytosis in renal proximal tubule-derived OK cells. However, the precise mechanisms used by these signaling systems to regulate endocytosis are not well characterized. Recently, it has been shown that the phosphorylation state of endocytic coat proteins affects the assembly of coat complexes (33). As already mentioned above, NHE3, which is also regulated by different signaling systems, shows reduced activity due to cAMP-dependent phosphorylation (22, 28). Because
RME of albumin can be inhibited by acute blockade of endosomal NHE3 (10), we performed the present study to test the hypothesis that 1) NHE3 is important for endocytosis and 2) NHE3 serves as a molecular switch during cAMP-mediated reduction of endocytosis. If NHE3 indeed plays a role in endocytosis, the following criteria should apply: 1) NHE inhibitors should reduce endocytosis with appropriate IC50 values [this has been shown in a previous study (10)]; 2) removal of NHE3 should affect endocytosis and its cAMP sensitivity; and 3) reintroduction of NHE3 should restore endocytosis and cAMP sensitivity. The second and third predictions were tested in the present study.

We used a cell line derived from renal proximal tubule (OK cells) that shows a well-characterized apical endocytic uptake activity for albumin as well as apical expression of NHE3 but no basolateral expression of NHE (3, 12, 29). We created several NHE3-deficient clones and compared NHE3 activity with the rate of endocytosis and the cAMP sensitivity of endocytosis. Furthermore, we reintroduced human NHE3 by stable transfection and determined the impact of this procedure on the rate of endocytosis and the cAMP sensitivity of endocytosis. Our data show that in cells expressing NHE3, this transporter serves as a molecular tool for cAMP-mediated regulation of RME. Thus there is a mutual interaction of NHE3 and clathrin-mediated endocytosis. Endocytosis contributes to the regulation of apical NHE3 density, and NHE3 contributes to proper endocytosis and its regulation.

MATERIALS AND METHODS

Materials. MEM and fetal calf serum were obtained from Biochrom (Berlin, Germany). Amiloride and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) were generous gifts from Dr. Hans-Jochen Lang (Aventis, Frankfurt, Germany). All other chemicals applied were obtained from Sigma (Deisenhofen, Germany). The cDNA of human NHE3 had been cloned into the mammalian expression vector pMAMneo, as described elsewhere (32). Ringer solution was composed of (in mmol/l) 122.5 NaCl, 5.4 KCl, 1.2 CaCl2, 0.8 MgCl2, 0.8 Na2HPO4, 0.2 NaH2PO4, 5.5 glucose, and 10 HEPES.

Cell culture. OK cells were kindly provided by Dr. Jörg Biber (Department of Physiology, University of Zurich, Zurich, Switzerland). Cells were grown in plastic culture flasks (Falcon, Heidelberg, Germany) as previously described (13). Cells were used 9 days after plating (confluent monolayers). Cells were used during 15–20 rounds of subcultivation, during which the cells maintained morphology and functional characteristics.

H+-suicide technique. To knock out NHE3 from the OK cells, we applied the H+-suicide technique as described originally by Pouyssegur et al. (30). Briefly, cells in the logarithmic phase were randomly mutagenized using ethylmethanesulfonate (0.25 μM/ml, 16 h). Forty-eight hours later, cells were incubated for 120 min in LiCl-HEPES-Ringer (NaCl replaced by LiCl) for Li+-loading. Subsequently, the cells were incubated for 60 min in Na+- and Li+-free acidic Ringer solution [pH 5.5, MOPS buffered]. Finally, the cells were incubated again in MEM media. This procedure was repeated four times, followed by limited dilution cloning. Initial screening for NHE-deficient clones was performed by means of determination of propionic acid-induced and amiloride-sensitive swelling (16). In clones that were used further in the study, NHE activity was also determined by pH measurements.

Transfection. Transfection of the cells was performed with the Quiagene Effectene reagent (Quiagen, Hilden, Germany) according to the manufacturer’s instructions. Selection of transfected clones was performed with genetin (600 mg/l) and the acid stress technique, as described elsewhere (29). Clones were isolated by limited dilution cloning. Expression of NHE was assessed as described below in Determination of NHE activity.

Measurement of pH. Intracellular and endosomal pH of single cells were determined using the pH-sensitive dye 2′,7′-bis(2-carboxylethyl)-5(6)-carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR) as described elsewhere (10). Briefly, cells were loaded for 5 min with either 3 μmol/l BCECF-AM or 100 μg/ml FITC-BSA, rinsed four times with superfusion solution, and transferred to the stage of an Axiovert 100 TV microscope (Zeiss, Oberkochen, Germany). Excitation wavelengths were 460 and 495 nm and the emitted light was filtered through a band-pass filter (515–565 nm). Images were digitized on-line by using video-imaging software (Attofluor, Zeiss). One ratio every 2 s was acquired. Calibration was performed after each experiment by the nigericin technique.

Determination of cellular buffer capacity. Cellular buffer capacity-β (mmol·l−1·ΔpH−1) was determined by the sequential addition of known amounts of acid to the cells and the resulting changes in cytosolic pH in the absence of extra- cellular Na+ (replaced by N-methyl-d-glucamine) and in the presence of 1 mmol/l amiloride, 3 mmol/l Ba2+, and 50 μmol/l furosemide. These three compounds were added to avoid transport of NH4+ via Na+/H+ exchange, K+ channels, or Na+/K+-2Cl− cotransport, respectively. Cells were exposed for 5 min to 40 mmol/l NH4Cl in the Ringer solution (the concentration of NaCl was reduced accordingly). Subsequently, the NH4Cl concentration was reduced stepwise to zero, resulting in intracellular H+ delivery, because NH3 leaves the cell and NH4+ dissociates. The dependence of β on cytosolic pH in wild-type OK cells is given by β = 280 – 33·pH. For the calculation of NHE activity (see Determination of NHE activity below), the buffer capacity corresponding to the pH achieved by the acidification procedure was determined and used for the calculations, because β varies with pH. We compared the buffer capacity of wild-type cells with clones 02–2, 02–3, and 02–4 (Table 1) by the above-described method and could not detect significant differences. Thus the above equation was used for all calculations.

Determination of NHE activity. NHE activity was determined as the initial (first 60 s) Na+-dependent and amiloride-sensitive (3 mmol/l) pH recovery from an acidic load multiplied by the buffer capacity. The bath volume was ~200 μl and the flow rate was ~2 ml/min. The Ringer solution was used, and Na+ was replaced by N-methyl-D-glucamine to prepare Na+-free solutions. When NH4Cl was added, an equimolar amount of NaCl was omitted. Acidification was achieved by brief exposure to 40 mmol/l NH4Cl. On removal of NH4Cl, the cytosol acidifies because of the dissociation of NH4+. Initial experiments showed that Na+-induced pH recovery was reduced by ~95% in the presence of 3 mmol/l amiloride or 100 μmol/l EIPA, a specific NHE inhibitor. The measurement areas were set to regions of homogenous cytosolic BCECF loading to prevent interference with endosomal pH changes, although the pKa value of BCECF makes interference with acidic compartments unlikely.

Western blot analysis of NHE3 expression. Cells were washed three times with ice-cold PBS and lysed in ice-cold...
radioimmunoprecipitation assay (RIPA) lysis buffer (1% Nonidet P-40, 0.1% SDS, 0.1% Triton X-100, 5 mM EDTA, 200 mM sodium-orthovanadate, 0.1 mM phenylmethylsulfonfyl fluoride, 1 μg/ml leupeptin, 1 μM pepstatin A, 40 mg/l bestatin, 2 mg/l aprotinin in PBS) for 25 min at 4°C. Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C. Cell lysates were matched for protein, separated on SDS-PAGE, and transferred to a nitrocellulose membrane. Subsequently, membranes were blotted with a rabbit anti-NHE3 antibody (Biotrend, Köln, Germany). The primary antibody was detected using horseradish peroxidase-conjugated secondary IgG visualized by the Amersham enhanced chemiluminescence system.

**Determination of megalin distribution by direct ELISA.** For quantification of surface and total megalin as well as the distribution of megalin, we performed megalin ELISA using a modification of the method described by Versteeg et al. (35). The megalin antibody that we used has been previously characterized (26). In addition, we have shown that the megalin antibody is highly specific for megalin in OK cells and therefore suitable for ELISA applications (38). Because the antibody inhibits albumin binding and endocytosis in OK cells (38), it can be concluded that this antibody recognizes the extracellular domain of megalin. Previously, we have shown that during incubation at 4°C, proteins do not enter OK cells but bind only to the surface (14). Thus incubation of OK cells at 4°C with an antibody directed against the extracellular domain of megalin leads to surface labeling of megalin without labeling of intracellular megalin. To label total megalin (= surface + intracellular), the cells have to be permeabilized. In a previous study, it was shown that after permeabilization the antibody recognizes intracellular megalin as well (38).

For the quantification of total and surface megalin, cells were seeded in 96-well plates (Maxisorp, Nunc) and serum starved for 48 h. After incubation under the same conditions as described for the binding studies, the cells in one-half of the wells were washed at 4°C using PBS and then incubated with anti-megalin antibody (1:2,000 in PBS + 1% BSA) at 4°C for 60 min without permeabilization (= surface labeling). Subsequently, the cells were washed three times with PBS, fixed with 4% formaldehyde in PBS for 20 min at room temperature, and washed three times with PBS containing 0.1% Triton X-100. Cells were again washed three times in PBS, blocked with 1% BSA in PBS for 1 h, and incubated for 60 min with PBS + 1% BSA. After three washes with PBS, the cells were incubated with secondary antibody (peroxidase-conjugated mouse anti-sheep antibody, dilution 1:2,000) in PBS + 1% BSA for 1 h at room temperature and washed three times with PBS/0.05% Tween 20.

<table>
<thead>
<tr>
<th>Clone</th>
<th>JNH, mM/min</th>
<th>Albumin Uptake, μg/mg</th>
<th>Albumin Binding, μg/mg</th>
<th>Dextran Uptake, μg/mg</th>
<th>Albumin Uptake/Binding, %</th>
<th>cAMP-Sensitive Albumin Uptake, μg/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>13.50 ± 1.52</td>
<td>4.80 ± 0.65</td>
<td>0.38 ± 0.01</td>
<td>0.018 ± 0.001</td>
<td>12.90 ± 0.11</td>
<td>33 ± 3</td>
</tr>
<tr>
<td>Clone 15</td>
<td>10.13 ± 1.39</td>
<td>4.14 ± 0.53</td>
<td>0.37 ± 0.03</td>
<td>0.025 ± 0.002</td>
<td>11.19 ± 0.60</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Clone 14</td>
<td>9.03 ± 0.89</td>
<td>4.90 ± 0.51</td>
<td>0.35 ± 0.03</td>
<td>0.015 ± 0.001</td>
<td>13.00 ± 0.60</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Clone 16</td>
<td>8.44 ± 0.68</td>
<td>2.58 ± 0.44</td>
<td>0.36 ± 0.05</td>
<td>0.018 ± 0.001</td>
<td>8.60 ± 1.00</td>
<td>27 ± 1</td>
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<tr>
<td>Clone 17</td>
<td>7.59 ± 0.83</td>
<td>1.80 ± 0.33</td>
<td>0.35 ± 0.04</td>
<td>0.016 ± 0.001</td>
<td>7.41 ± 0.80</td>
<td>24 ± 3</td>
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<tr>
<td>Clone 09</td>
<td>5.06 ± 0.55</td>
<td>1.19 ± 0.39</td>
<td>0.28 ± 0.03</td>
<td>0.015 ± 0.001</td>
<td>4.96 ± 0.60</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Clone 19</td>
<td>5.06 ± 0.68</td>
<td>1.19 ± 0.39</td>
<td>0.28 ± 0.03</td>
<td>0.015 ± 0.001</td>
<td>4.96 ± 0.60</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Clone 02</td>
<td>4.22 ± 0.89</td>
<td>1.68 ± 0.48</td>
<td>0.31 ± 0.03</td>
<td>0.017 ± 0.001</td>
<td>5.42 ± 0.60</td>
<td>15 ± 2</td>
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<tr>
<td>Clone 06</td>
<td>3.97 ± 0.68</td>
<td>1.92 ± 0.65</td>
<td>0.30 ± 0.02</td>
<td>0.015 ± 0.002</td>
<td>5.11 ± 1.00</td>
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<tr>
<td>Clone 02-4</td>
<td>1.76 ± 0.81</td>
<td>0.98 ± 0.10</td>
<td>0.19 ± 0.02</td>
<td>0.030 ± 0.005</td>
<td>6.00 ± 0.40</td>
<td>2 ± 1</td>
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<tr>
<td>Clone 02-2</td>
<td>1.21 ± 0.68</td>
<td>0.68 ± 0.03</td>
<td>0.17 ± 0.02</td>
<td>0.020 ± 0.001</td>
<td>4.50 ± 0.16</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Clone 02-3</td>
<td>0.42 ± 0.27</td>
<td>0.23 ± 0.03</td>
<td>0.16 ± 0.04</td>
<td>0.020 ± 0.001</td>
<td>2.00 ± 0.22</td>
<td>1 ± 1</td>
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</table>

Values are means ± SE; 6 ≤ n ≤ 20. JNH, Na⁺/H⁺ exchanger, 3 activity given at intracellular pH 6.2–6.4; OK, opossum kidney.

For labeling of total megalin cells, one-half of the wells were washed at 4°C using PBS and then incubated with anti-megalin antibody (1:2,000 in PBS + 1% BSA) at 4°C for 60 min without permeabilization (= surface labeling). Subsequently, the cells were washed three times with PBS, fixed with 4% formaldehyde in PBS at 20 min at room temperature, and washed three times with PBS containing 0.1% Triton X-100 (= permeabilization). Cells were again washed three times in PBS, blocked with 1% BSA in PBS for 1 h, and incubated for 60 min with anti-megalin antibody (1:2,000 in PBS + 1% BSA; = total labeling). After three washes with PBS, the cells were incubated with secondary antibody (peroxidase-conjugated mouse anti-sheep antibody, dilution 1: 2,000) in PBS with 1% BSA for 1 h at room temperature and washed three times with PBS/0.05% Tween 20.

Subsequently, the cells were incubated with 50 μl of a solution containing 0.4 mg/ml o-phenylenediamine, 11.8 mg/ml Na₂HPO₄, 7.3 mg/ml citric acid, and 0.015% H₂O₂ for 15 min at room temperature in the dark. The resulting signal was detected at 490 nm with a multiwell, multilabel counter (Victor², Wallac, Turku, Finland). After the peroxidase reaction, the cells were washed twice with PBS and twice with demineralized water. After drying the wells for 5 min, 100 μl of trypan blue solution (0.2% in PBS) were added for 5 min at room temperature. Subsequently, the cells were washed four times with demineralized water, and 100 μl of 1% SDS solution were added and incubated on a shaker for 1 h at room temperature. Finally, the absorbance was measured at 595 nm with the above-mentioned ELISA reader. The values are expressed as megalin-specific signal (i.e., after blank subtraction) corrected for total cellular protein per well.

**Uptake and binding studies.** Uptake experiments were performed as previously described (12, 14) after the cells were cultivated for 48 h in serum-free media. After three acidic washes (pH 6.0) at 4°C, the monolayers grown on plastic petri dishes (9 days) were incubated with Ringer solution containing 10 mg/l FITC-BSA at 37°C (uptake) or 4°C (binding) for the time periods indicated. The acidic washes were performed to remove residual proteins bound to receptors at the apical membrane. Although this procedure is not necessarily required when the cells were incubated in serum-free media before the experiments, we nevertheless applied the washes to perform the same routines as previously described. We also determined whether the acidic washes influenced the differences in uptake data from wild-type cells and deficient cells, but we did not detect any effect.
Thus the acidic washes at 4°C do not affect the deficient cells to a different degree than the wild-type cells. In a previous study, our laboratory has already shown that at 4°C the substrates bind to the plasma membrane but are not internalized (13). At 37°C, albumin is taken up by RME (12–14). Less than 10% of FITC-albumin uptake is nonspecific (12). The amount of internalized substrate was determined by subtracting the portion of bound albumin from total cell-associated albumin. Unbound FITC-albumin was removed by rinsing eight times with ice-cold Ringer solution (13). Cells were disintegrated by detergent (0.1% Triton X-100 (vol/vol) in MOPS solution, which guaranteed that all fluorescence measurements were performed at pH 7.4), and the cell-associated fluorescence was measured using a multiwell fluorometer (Victor²) according to Gekle et al. (12, 13). Protein was determined as described elsewhere (23). The rate of fluid-phase endocytosis was determined by the uptake of FITC-dextran using the same protocol as for FITC-albumin uptake (13).

Calculations and statistics. Curve fitting was performed according to the least-squares method using the SigmaPlot for Windows software (Jandel Scientific). Data are presented as mean values ± SE, and n represents the number of petri dishes (for uptake) or cells studied (for pH). Cells of at least three passages were used for each experimental series. Significance of difference was tested by Student's t-test or ANOVA, as appropriate. Differences were considered significant if P < 0.05.

RESULTS

NHE3 deficiency abolishes endocytosis. To select NHE3 knockout cell clones, we screened 40 clones obtained after different rounds of the H⁺-suicide procedure (30) and limited dilution cloning. From these 40 clones, 11 were selected for further experiments. Figure 1 compares the NHE3 activity of wild-type OK cells and clone 02–3, which is deficient in NHE3 activity (Table 1) and was obtained after the last round of the H⁺-suicide procedure. The photomicrographs in Fig. 1 also show that cells of the deficient clone 02–3 have an appearance similar to the wild-type and form confluent monolayers. Absence of NHE3 was confirmed in this clone by Western blot analysis (Fig. 1).

Fig. 1. Characterization of Na⁺/H⁺ exchanger 3 (NHE3)-deficient opossum kidney (OK) cells. A: determination of NHE3 activity. The 2 tracings show the Na⁺-dependent pH recovery after an acid load by the NH₄Cl-prepulse technique in wild-type cells and NHE3-deficient cells (clone 02–3, Table 1). Although there is a fast recovery from the acid load in wild-type cells, there is virtually no Na⁺-dependent pH recovery in the deficient clone. B: Western blot analysis with anti-NHE3 was performed to determine whether the functionally deficient clone shows altered NHE3 expression. The immunoblot shows that NHE3 is no longer expressed in the deficient clone. C: to compare cell morphology of the deficient clone with wild-type cells, photomicrographs were taken. The photomicrographs show that the deficient cells still display an epithelial phenotype and do not show signs of cell damage. Bar = 20 μm.
Figure 2A shows the correlation of the NHE3 activity of the different clones obtained after different rounds of the H⁺-suicide procedure with RME. Endocytosis correlated significantly with NHE3 activity. By contrast, fluid-phase endocytosis, as determined by FITC-dextran uptake, did not depend on NHE3 activity (Fig. 2B). Binding of albumin to the apical membrane of OK cells was unchanged at an NHE3 activity ≥40% of control (Fig. 2C). However, in clones with an almost completely abolished NHE3 activity, albumin binding was reduced by ~50%. To exclude the possibility that reduced albumin uptake was simply due to changes in binding, we determined the correlation of NHE3 activity with the uptake/binding ratio. If changes in uptake
were the result of altered binding, this ratio should not change. However, as can be seen in Fig. 2D, the uptake/binding ratio also correlated significantly with NHE3 activity. Thus reduced NHE3 activity leads to reduced albumin internalization. To test whether the reduced binding rate was the result of an altered distribution of megalin between the cell surface and the cell interior, we performed megalin-specific ELISA in OK cells (for details, see MATERIALS AND METHODS). We performed megalin ELISA, because megalin anchors the megalin/cubilin complex to the cell membrane; therefore, changes in distribution should be detectable with megalin (5). We compared megalin expression in wild-type cells and in clone 02–3. As shown in Fig. 2E, the total megalin signal was not significantly different for the two cell clones. However, the percentage of surface megalin (compared with total megalin) was reduced significantly in clone 02–3. These data indicate that NHE3 deficiency does not reduce megalin expression but impairs megalin surface delivery, possibly by affecting intracellular trafficking events. These data are in good agreement with the reduced binding rates shown in Fig. 2C. In addition, we determined whether acute inhibition of NHE3 with EIPA or vesicular alkalization using 20 mmol/l NH4Cl also affected megalin distribution. As shown in Fig. 2F, this was indeed the case. Of course, we do not presently know whether the observed alteration in distribution is specific for megalin or whether the distribution of other apical membrane proteins is affected as well. This issue has to be investigated in future studies.

In the deficient clone 02–3 (Table 1), we additionally measured endosomal pH. Steady-state endosomal pH was slightly, although significantly, increased (from 6.20 ± 0.02 in control cells to 6.30 ± 0.03 in knockout cells, n = 60). Furthermore, the extent of endosomal alkalization induced by addition of the NHE inhibitor EIPA (10) was reduced dramatically in the knockout clone (ΔpH=0.11 ± 0.03, n = 60; this slight pH change results from the weak base effect of EIPA (10)) compared with control cells (ΔpH=0.50 ± 0.05, n = 60, P < 0.05). These data show that NHE3 knockout affected endosomal pH homeostasis. Cytosolic steady-state pH was slightly lower in NHE3-deficient cells (−0.15−0.20 pH units). However, cytosolic acidification in this range has been shown to leave RME unaffected under the experimental conditions of our laboratory (10).

NHE3 deficiency abolishes cAMP sensitivity of endocytosis. Figure 3 shows the effects of dibutyryl cAMP on NHE3 activity and endosomal pH. As expected, NHE3 activity is reduced by cAMP. In addition, cAMP led to an increase of endosomal pH in wild-type cells, indicating that endosomal NHE3 is also inhibited, espe-

Fig. 3. A: cAMP induces inhibition of NHE3 activity; 100 μmol/l cAMP reduced apical plasma membrane NHE3 activity by −40%. The exposure time was 10 min (n = 50). NHE3 activity was determined from the Na⁺-dependent pH recovery and the buffer capacity. B: 100 μmol/l cAMP led to an alkalization of endosomal pH (pHe). This alkalization is consistent with an inhibition of NHE3 in early endosomes, whereby the Na⁺ gradient allows NHE3 to translocate H⁺ into the organelle (n = 40). Arrow, time point of cAMP addition. C: percentage of cAMP-sensitive albumin endocytosis (i.e., the percentage of albumin endocytosis that can be inhibited by cAMP). cAMP-sensitive albumin endocytosis decreases with decreasing NHE3 activity. D: correlation of the absolute values of cAMP-sensitive albumin endocytosis with NHE3 activity (n = 6–12). The symbols for the different cell clones correspond to the legend in Fig. 2. Values are means ± SE.
cially because cAMP has been reported to leave H\textsuperscript{+}-ATPase unaffected (18). In the deficient clone 02–3 (Table 1), cAMP (100 μmol/l) did not affect endosomal pH significantly (ΔpH = +0.03 ± 0.03, n = 40; see also Fig. 4).

These data open up the possibility that cAMP modulates endocytosis by means of NHE3. In the presence of cAMP, cytosolic pH dropped slightly (−0.05 ± 0.02, n = 40, P < 0.05 vs. control). However, as shown recently, small changes in cytosolic pH do not inhibit albumin endocytosis (10). To test whether the effects of cAMP were mediated by PKA-dependent phosphorylation, we used the specific PKA inhibitor H-89 (10 μM). In the presence of H-89, the cAMP effect on RME was only 5 ± 5% (n = 6) compared with 33 ± 3% in the absence of H-89 (n = 9).

Figures 3, C and D, and Table 1 summarize the effect of cAMP on albumin endocytosis in the different cell clones. The inhibitory effect of cAMP depends on NHE3 activity. Of course, it is not surprising that the absolute activity of cAMP on albumin endocytosis in the different cell clones. The inhibitory effect of cAMP depends on NHE3 activity. Of course, it is not surprising that the absolute activity of cAMP on albumin endocytosis (10). To test whether the effects of cAMP were mediated by PKA-dependent phosphorylation, we used the specific PKA inhibitor H-89 (10 μM). In the presence of H-89, the cAMP effect on RME was only 5 ± 5% (n = 6) compared with 33 ± 3% in the absence of H-89 (n = 9).

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Restoration of NHE3 activity restores cAMP sensitivity. We selected one cell clone with almost completely abolished NHE3 activity (clone 02–3; Table 1) and stably transfected it with human NHE3 or with the empty plasmid (mock transfection). Figure 4 shows the characteristics of clone 02–3 compared with wild-type cells. Transfection with NHE3 but not with empty plasmid led to a restoration of NHE3 activity (Fig. 5 shows the data for clone B4; a summary of the transfected clones is given in Table 2). In addition, NHE3 transfection led to an increase in RME under control conditions and restored the cAMP sensitivity of RME and endosomal pH. The photomicrographs in Fig. 5 also show that the cells have a similar appearance and form confluent monolayers. These data show that NHE3 is responsible, at least in part, for cAMP sensitivity of albumin endocytosis. In Fig. 6, albumin uptake and cAMP sensitivity of six different clones transfected with human NHE3 are shown. These data

![Figure 4](http://ajprenal.physiology.org/) show the data for

<table>
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<tr>
<th>Table 2. Characterization of NHE3-deficient OK cell clones transfected with hNHE3 or with the empty vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jmax, Albumin Uptake,</td>
</tr>
<tr>
<td>mM/min</td>
</tr>
<tr>
<td>Wild-type</td>
</tr>
<tr>
<td>Clone B4</td>
</tr>
<tr>
<td>Clone D5</td>
</tr>
<tr>
<td>Clone D9</td>
</tr>
<tr>
<td>Clone E1</td>
</tr>
<tr>
<td>Clone F10</td>
</tr>
<tr>
<td>Clone K23</td>
</tr>
<tr>
<td>02-2-mock</td>
</tr>
<tr>
<td>02-3-mock</td>
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</tbody>
</table>

Values are means ± SE; 6 ≤ n ≤ 12. hNHE3, human NHE3; mock, empty vector.
Fig. 5. Direct comparison of NHE3-deficient clone 02–3 transfected with either human NHE3 (hNHE3) or the empty vector (mock). The parameters NHE3 activity (A), cAMP-induced (100 µM) endosomal alkalinization (B), albumin endocytosis (C), and percentage of cAMP-sensitive (100 µM) albumin endocytosis were increased by the expression of hNHE3, indicating that NHE3 transfection is sufficient to restore albumin endocytosis and cAMP sensitivity of albumin endocytosis. The Western blot analysis confirms the expression of hNHE3 on the protein level. The photomicrographs show that the cells still display an epithelial phenotype. Values are means ± SE. Bar = 20 µm. *P < 0.05 vs. wild-type cells (n = 6–12).

Fig. 6. Correlation of albumin endocytosis and cAMP sensitivity (100 µM) of albumin endocytosis in different cell clones obtained after stable transfection with hNHE3 or empty vector (mock). A: correlation of albumin endocytosis with NHE3 activity. B: correlation of the percentage of cAMP (100 µmol/l)-sensitive albumin endocytosis with NHE3 activity. In both cases, a significant correlation was observed, indicating that NHE3 transfection is sufficient to restore albumin endocytosis and cAMP sensitivity of albumin endocytosis. Values are means ± SE. n = 5–10.
clearly demonstrate that transfection with NHE3 restores endocytosis. Albumin binding in the NHE3-transfected clones was 70–80% of binding in wild-type OK cells (data not shown).

DISCUSSION

The data of the present study show, on one hand, the involvement of NHE3 in RME by a nonpharmacological approach and, on the other hand, that NHE3 represents one molecular target for cAMP-mediated regulation of endocytosis. Until now, the possible involvement of NHE3 in RME has been investigated using pharmacological tools, such as NHE inhibitors (10). Thereby, it has been shown that acute inhibition of NHE3 blocks RME of albumin. The data presented here show that NHE3 deficiency leads to a dramatic reduction of endocytosis. Moreover, retransfection with human NHE3 restored endocytosis. Thus NHE3 is important for early steps along the endocytic pathway because of its contribution to vesicle acidification (9, 10). Of course, we do not know at the moment whether all receptor-mediated endocytic uptake in different NHE3-expressing cells exhibit the same dependence on NHE3 activity. However, we could show that NHE3 inhibition also affects albumin endocytosis in another cell type, LLC-PK1 (10). The reduced binding rate in clones with virtually abolished NHE3 activity can be explained by impaired trafficking of megalin (2, 7, 38, 39), which depends on proper acidification of the endocytic pathway (8). Thus instead of recycling to the plasma membrane, megalin most probably is retained within the cell. Our megalin-ELISA data support this hypothesis. The overall expression of megalin was not reduced in NHE3-deficient cells. We can exclude the possibility that the reduced uptake rates in the various clones resulted from different growth states (confluence, proliferation rate). In all experiments, the cells were completely confluent (the protein content/dish was not significantly different for the various clones) and made quiescent by serum removal for 48 h. We do not presently know whether the observed alteration in distribution of megalin is specific or whether the distribution of other apical membrane proteins, e.g., Na\(^+\)/phosphate cotransporter or peptide hormone receptors, is affected as well. This issue of specificity has to be investigated in future studies to determine whether there is a general impairment of apical protein trafficking or whether only proteins that possibly interact with NHE3 are affected.

RME is a highly complex event that can be affected by different regulatory pathways (27). Because it is highly complex, it is difficult to determine the target sites for the regulatory pathways. Because NHE3 activity is important for proper endocytosis, it is conceivable that NHE3 serves as one molecular tool for regulation. Furthermore, NHE3 and albumin endocytosis are both under the negative control of cAMP (12, 22). Thus the creation of NHE3-deficient OK cells provided the opportunity to determine the importance of NHE3 for cAMP-mediated regulation of RME. Our data show that both cAMP sensitivity of endosomal pH and RME are abolished in NHE3-deficient cells. Furthermore, cAMP sensitivity is restored by NHE3 transfection but not by transfection of the empty plasmid. Because NHE3 is localized in the plasma membrane and the early endosomal compartment, and also because NHE3 activity in the plasma membrane does not affect endocytosis (10), the data presented here show for the first time that vesicular NHE3 serves as a molecular tool for cAMP-mediated regulation of RME. Meanwhile, it is well known that regulation of NHE3 by cAMP requires a multiprotein signal complex containing the regulatory factor NHE-RF (36). This regulatory factor is also expressed in OK cells (37), underlining their suitability as a model system. According to our model, the loss of NHE-RF could also lead to reduced cAMP sensitivity of endocytosis. However, in the case of our deficient clones, a loss of NHE-RF is not responsible for the reduced cAMP sensitivity, because transfection with NHE3 was sufficient to restore cAMP sensitivity. Nevertheless, future studies will have to investigate the role of NHE-RF in the regulation of endosomal pH and RME.

Taken together with previous findings (4, 10, 11, 19, 21), we propose the following model. NHE3 is recruited to clathrin-coated vesicles, which leads to the observed sorting between the early endosomal compartment and the plasma membrane (4). Because of the existing Na\(^+\) gradient across the membrane of endocytic vesicles (10), NHE3 is still functional in this compartment. Thus there is a mutual interaction between NHE3 and the early endosomal compartment; endocytosis controls apical transporter density and NHE3 contributes to vesicular pH homeostasis. This contribution of NHE3 is important, for example, during vesicle fusion (11). cAMP leads to a decrease in NHE3 activity by means of PKA-mediated phosphorylation (39). Consequently, cAMP also disturbs vesicular pH homeostasis, which is important for proper endocytosis. Thus cAMP inhibits endocytosis by means of its interaction with NHE3.

In conclusion, our data show that in NHE3-expressing cells, this transporter can be important for cAMP sensitivity of clathrin-dependent RME because of its contribution to endosomal pH homeostasis and endocytic vesicle fusion.

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