Ezrin binding domain-deficient NHERF attenuates cAMP-mediated inhibition of Na\(^+\)/H\(^+\) exchange in OK cells

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Weinman, Edward J., Deborah Steplock, James B. Wade, and Shirish Shenolikar. Ezrin binding domain-deficient NHERF attenuates cAMP-mediated inhibition of Na\(^+\)/H\(^+\) exchange in OK cells. Am J Physiol Renal Physiol 281: F374–F380, 2001.—Na\(^+\)/H\(^+\) exchanger regulatory factor (NHERF), an essential protein cofactor in cAMP-mediated inhibition of Na\(^+\)/H\(^+\) exchange transporter 3 (NHE3), facilitates the formation of a signal complex of proteins that includes NHE3, NHERF, and ezrin. This model for NHE3 regulation was developed in fibroblasts and its applicability to epithelial cells remains to be established. Opossum kidney (OK) cells were transfected with either empty vector (control), full-length mouse (m) NHERF(1–355), or a truncated mNHERF(1–325) that lacked ezrin binding and had been demonstrated in fibroblasts to bind NHE3 but not mediate its cAMP-associated inhibition. 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP) at 10\(^{–4}\) M inhibited Na\(^+\)/H\(^+\) exchange activity in control and OK cells expressing wild-type mNHERF(1–355) by >60% but by <10% in cells expressing mNHERF(1–325). NHE3 coimmunoprecipitated with mNHERF(1–325), but cAMP phosphorylation of NHE3 was impaired in cells expressing mNHERF(1–325). The inhibitory effect of hyperosmolality on NHE3 activity and the uptake of 3-O-methyl-D-glucose was the same in all three cell lines. Cell surface expression of NHE3 was not changed by cAMP in any of the cells lines. These data indicate that disruption of the NHERF-ezrin signal complex attenuates the inhibitory effect of cAMP on NHE3 activity in OK cells and provides evidence supporting the proposed model of protein kinase A regulation of NHE3 in epithelial cells.

renal electrolyte transport; PSD-95/Dlg/ZO proteins; protein kinase A; sodium/hydrogen exchange transporter 3; acid-base physiology; opossum kidney cells

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METHODS

OK cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in DMEM/F-12 medium (1:1) supplemented with 10% (vol/vol) FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown to confluence in serum, split, and regrown to sub-confluence in the presence of serum. mNHERF1 (1–355) and mNHERF1 (1–325) cDNAs were cloned into pcDNA3.1/Hygro vectors and transfected into OK cells using Lipofectin (GIBCO-BRL) (17, 21, 22). Cells transfected with the pcDNA3.1/Hygro vector alone served as the control. Cells resistant to 600 U/ml hygromycin were selected through eight passages before study. Transfected cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in DMEM/F-12 medium (1:1) supplemented with 10% (vol/vol) FCS, penicillin (100 U/ml), streptomycin (100 mg/ml), and 600 U/ml hygromycin.

Na⁺/H⁺ exchange activity was assayed in cells grown on 40-mm glass coverslips using the pH-sensitive fluorescent dye, the acetoxymethyl ester of 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) (Molecular Probes) (17, 21, 22). Cells were serum deprived for 24–36 h and then loaded into OK cells using Lipofectin (GIBCO-BRL) (17, 21, 22).

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abundance of OK NHERF was not altered by the over-expression of mNHERF(1–355) or mNHERF(1–325). As seen in lanes 2 and 3, the NHERF antibody recognized an additional band not present in lane 1 representing mNHERF(1–355) and mNHERF(1–325). Immunoprecipitates of anti-OK cell NHE3 were probed with anti-NHERF antibody and, as seen in Fig. 2, only the smaller OK cell NHERF was recovered from control cells. By contrast, anti-NHE3 immunoprecipitates from cells expressing mNHERF(1–355) showed a broader band that reflected both mNHERF and OK NHERF. Surprisingly, the larger mNHERF was the predominant species recovered with the anti-NHE3 immunoprecipitates from the mNHERF(1–325)-expressing cells. Figure 3 shows representative tracings of the initial sodium-dependent pH recovery in the absence and presence of cAMP in the three cell lines. Table 1 summarizes the measurements of the rate of Na+/H+ exchange and the effect of cAMP. Control OK cells transfected with the pcDNA vector alone had a rate of sodium-dependent pH recovery (ΔpH/min) from an acidifying stimulus of 0.035 ± 0.004 in the absence of cAMP and 0.010 ± 0.002 in the presence of 10^{-4} M 8-BrcAMP (P < 0.01). OK cells expressing mNHERF(1–355) had a rate of 0.040 ± 0.006 ΔpH/min in the absence of cAMP and 0.010 ± 0.002 in the presence of cAMP (P < 0.01). OK cells expressing mNHERF(1–325) had a rate of 0.045 ± 0.003 ΔpH/min in the absence of cAMP and 0.043 ± 0.002 in the presence of cAMP [P = not significant (NS)].

We next analyzed the covalent modification of NHE3 in OK cells using an in vitro back-phosphorylation assay of immunoprecipitated NHE3. In control cells treated with cAMP, the amount of ^32P incorporated in vitro determined by autoradiography and normalized for the amount of NHE3 by Western immunoblot was decreased by 37.1 ± 7.1% (n = 3, P < 0.05) compared with cells not treated with cAMP (Fig. 4). This suggested the prior phosphorylation of this fraction of NHE3 in OK cells in response to cAMP. In cells expressing mNHERF(1–355), cAMP treatment de-
Table 1. The effect of cAMP on Na+/H+ exchange in OK cells expressing mouse wild-type NHERF or an ezrin binding domain-deficient NHERF

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Basal</th>
<th>cAMP</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
<td>0.035 ± 0.004</td>
<td>0.010 ± 0.002*</td>
<td>67.5 ± 8.3</td>
</tr>
<tr>
<td>Mouse NHERF  (1–355) (n = 6)</td>
<td>0.040 ± 0.006</td>
<td>0.010 ± 0.002*</td>
<td>75.0 ± 4.7</td>
</tr>
<tr>
<td>Mouse NHERF (1–325) (n = 6)</td>
<td>0.045 ± 0.003</td>
<td>0.043 ± 0.002</td>
<td>3.7 ± 4.2</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE and represent the initial rate of sodium-dependent intracellular pH (pHi) recovery (ΔpHi/min) after the cell was acidified to pH 6.0. No. in parentheses indicates the no. of measurements. Na+/H+ exchange transport was measured in opossum kidney (OK) cells using [2,7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF] fluorescence in the absence or presence of 10^{-4} M 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP) for 15 min. OK cells were transfected with an empty pcDNA vector (control), Na+/H+ exchanger regulatory factor (NHERF)(1–355) representing full-length mouse NHERF, or NHERF(1–325) representing the ezrin binding domain-deficient NHERF truncation. *P < 0.01 (vs. control).

Table 2. The effect of hyperosmolality on Na+/H+ exchange in OK cells expressing mouse wild-type NHERF or an ezrin binding domain-deficient NHERF

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Normal</th>
<th>Hyperosmolality</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>0.051 ± 0.009</td>
<td>0.034 ± 0.006*</td>
<td>32.4 ± 2.5</td>
</tr>
<tr>
<td>Mouse NHERF  (1–355) (n = 6)</td>
<td>0.035 ± 0.003</td>
<td>0.022 ± 0.002*</td>
<td>34.8 ± 5.6</td>
</tr>
<tr>
<td>Mouse NHERF (1–325) (n = 6)</td>
<td>0.047 ± 0.005</td>
<td>0.027 ± 0.004*</td>
<td>42.1 ± 5.5</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE and represent the initial rate of sodium-dependent pHi recovery (ΔpHi/min) after acidifying the cell to pH 6.0. Na+/H+ exchange transport was measured using BCECF fluorescence in OK cells incubated in control (normal) or media made hypertonic (hyperosmolality) by addition of 80 mM mannitol for 60 min. No. in parentheses indicates the no. of measurements. OK cells were transfected with an empty pcDNA vector (control), NHERF(1–355) representing full-length mouse NHERF, or NHERF(1–325) representing the ezrin binding domain-deficient NHERF truncation. *P < 0.05 (vs. control).

Table 3. The effect of cAMP on sodium-dependent 3-O-methyl-d-glucose uptake in OK cells expressing mouse wild-type NHERF or an ezrin binding domain-deficient NHERF

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Basal</th>
<th>cAMP</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>710.0 ± 17.3</td>
<td>738.3 ± 15.5</td>
<td>8.2 ± 0.8</td>
</tr>
<tr>
<td>Mouse NHERF  (1–355) (n = 6)</td>
<td>703.3 ± 76.0</td>
<td>730.0 ± 44.7</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>Mouse NHERF (1–325) (n = 6)</td>
<td>678.8 ± 75.6</td>
<td>683.7 ± 117.4</td>
<td>1.6 ± 0.1</td>
</tr>
</tbody>
</table>

Results are means ± SE expressed as cpm/μg protein where cpm is counts/min. No. in parentheses indicates the no. of measurements. The sodium-dependent uptake of 3-O-methyl-d-glucose was measured in OK cells in the absence or presence of 10^{-4} M 8-BrcAMP for 15 min. OK cells were transfected with an empty pcDNA vector (control), NHERF(1–355) representing full-length mouse NHERF, or NHERF(1–325) representing the ezrin binding domain-deficient NHERF truncation.
with $10^{-4}$ M 8-BrcAMP for 15 min or in the mNHERF(1–355)- and mNHERF(1–325)-expressing cells after 60 min of treatment with cAMP.

**DISCUSSION**

Recent experiments have indicated that NHERF is a necessary cofactor in cAMP-mediated inhibition of NHE3 (16–20). Biochemical and cell studies have advanced evidence that NHERF binds to both NHE3 and ezrin and is required for cAMP to phosphorylate NHE3 (17, 22). In this model, ezrin is considered to be the PKA anchor protein. NHERF is envisioned to function as an adaptor that facilitates formation of a multiprotein complex that mediates the rapid phosphorylation of NHE3 by bringing the PKA-ezrin complex into proximity with NHE3 (18). The subsequent phosphorylation of NHE3 results in the acute downregulation of its activity. This model of the signal complex regulation of NHE3 activity was developed exclusively from studies performed in PS-120 cell fibroblasts. An important shortcoming of the proposed model, however, has been the lack of its validation in an epithelial cell.

The present studies derive from recent experiments from our laboratory indicating that a construct of NHERF that lacked the putative ezrin binding domain [mNHERF(1–325)] did not support PKA-mediated phosphorylation of NHE3 or inhibition of transport activity, but did bind to NHE3 in vivo (17). Accordingly, we considered that if the ezrin binding domain-deficient NHERF could be expressed in an epithelial cell line, it might displace native NHERF and, by virtue of its inability to bind ezrin, disrupt cAMP regulation of NHE3 activity.

OK cells are a proximal tubule cell line that expresses endogenous NHE3 and NHERF. OK cell NHERF has a lower apparent molecular mass on SDS-PAGE than mNHERF, and this size differential permitted identification of both the native OK cell NHERF and expressed mNHERF using the same antibody. Stable expression of mNHERF(1–355) or mNHERF(1–325) did not affect the abundance of OK cell NHERF. The association of NHE3 with the specific NHERF proteins was examined by immunoprecipitation of NHE3. In control cells, only the smaller OK cell NHERF was recovered in association with NHE3. Anti-NHE3 immunoprecipitates from cells expressing mNHERF(1–355) showed a broader band that reflected both mNHERF and OK NHERF. Surprisingly, the larger mNHERF was the predominant form recovered with the anti-NHE3 immunoprecipitates from the mNHERF(1–325)-expressing cells. As the expression of NHERF in both cell lines was equivalent, this suggests that mNHERF(1–325) may have a higher affinity for OK NHE3 than mNHERF(1–355). It is also possible that the association of NHERF(1–355) with ezrin reduces its affinity for NHE3. Whatever the correct explanation, the data indicate an in vivo association between both the wild type and truncated form of NHERF with NHE3.

In control OK cells transfected with the pcDNA vector alone and in cells transfected with full-length mNHERF(1–355), 15-min exposure to cAMP inhibited Na$^+/H^+$ exchange activity by $>60\%$. By contrast, the mNHERF(1–325) cells behaved as NHERF dominant-negative cells, and the effect of cAMP to acutely downregulate Na$^+/H^+$ exchange activity was markedly attenuated. In PS-120 cells, NHERF is required for cAMP-mediated phosphorylation of NHE3 (22). The relationship between NHE3 phosphorylation and NHERF also appears to be present in OK cells. In control cells and in cells expressing mNHERF(1–355), cAMP resulted in the phosphorylation of NHE3. On the other hand, in cells expressing mNHERF(1–325), the amount of $^{32}$P incorporated in vitro into the immuno-

![Fig. 5. Cell surface expression of NHE3 in OK cells transfected with mNHERF(1–355) (A–C), mNHERF-(1–325) (D–F), or with an empty pcDNA vector (G and H) in the absence (–cAMP, A, D, G) or presence (+cAMP, B, C, E, F, H) of $10^{-4}$ M 8-BrcAMP for 15 min or 60 min. Cells were stained with a polyclonal antibody against rabbit NHE3. Bar, 10 μm.](http://ajprenal.physiology.org/)

**F378 NHERF AND PKA INHIBITION OF Na$^+/H^+$ EXCHANGE TRANSPORT IN OK CELLS**

**AJP-Renal Physiol • VOL 281 • AUGUST 2001 • www.ajprenal.org**
precipitated NHE3 was essentially the same whether the cells were previously treated with cAMP. This indicates that mNHERF(1–325) disrupted the cellular mechanism that promotes NHE3 phosphorylation in vivo in response to cAMP and provides a biochemical explanation for the failure of cAMP to inhibit NHE3 activity in these cells. The specificity of the effect observed in the mNHERF(1–325) cells was examined in two additional sets of experiments. Acute increases in the osmolality of the incubation media have previously been demonstrated to inhibit NHE3 activity in OK cells by a mechanism presumed to be independent of cAMP (1). In the present studies, incubation of the three cell lines in media containing 80 mM of added mannitol for 60 min resulted in an acute decrease in the rate of Na\(^{+}/\mathrm{H}^{+}\) exchange transport compared with cells studied in the normal media. We also examined the sodium-dependent uptake of the nonmetabolized sugar 3-O-methyl-D-glucose to establish the integrity of the sodium gradient across the cell and to rule out nonspecific effects of the transfections on cell function. The sodium-dependent uptake of the sugar was the same in all three cell lines, and cAMP treatment had no effect on this transport process.

We have recently reported that NHERF, NHE3, and ezrin colocalize in the apical membrane of the rat proximal tubule and that NHERF and ezrin communoprecipitate from OK cells (12, 15). Although consistent with the presence of an association among the proposed components of the signal complex, the above studies did not establish a physiological role for the multiprotein complex in control of Na\(^{+}/\mathrm{H}^{+}\) exchange transport activity in renal tissue or kidney-derived cell lines. The demonstration herein that the effect of cAMP on Na\(^{+}/\mathrm{H}^{+}\) exchange transport in the mNHERF(1–325)-expressing cells is blunted, however, provides the required functional and biochemical data to support the above studies (12, 15). When considered together, the results indicate that PKA regulation of NHE3 activity in an epithelial cell is mediated by a signal complex of proteins that includes NHERF.

A growing literature indicates that NHERF is involved in the trafficking and recycling of transport proteins and receptors (3, 8, 14). Prior studies in renal tissue suggested that parathyroid hormone caused a redistribution of Na\(^{+}/\mathrm{H}^{+}\) exchange activity and NHE3 protein from the cell surface to internal membrane pools (5, 11). Studies of Fan et al. (5) in the rat proximal tubules showed that the acute inhibitory effect of parathyroid hormone (PTH) on NHE3 activity resulted from phosphorylation of the antiporter, whereas over a longer time course of ∼3 h, PTH facilitated the endocytic removal of NHE3 from the plasma membrane (5). In more recent studies, they established a similar dual mode of regulation of NHE3 in OK cells in response to PTH, with a significant decrease in cell surface NHE3 being evident after only 30 min of hormone treatment (4). Optical sectioning of the OK cells used in the present experiments indicated that the distribution of ezrin, concentrated at the apical surface, was similar in all cell lines and did not change in response to cAMP.

In comparable optical sections, there was no difference in cell surface expression of NHE3 between the three cell lines either before or after 15-min exposure to cAMP. Cell surface expression of NHE3 was also not different after 60 min of treatment with cAMP in the mNHERF(1–355)- and mNHERF(1–325)-expressing cells. In OK cells, PTH has been demonstrated to use multiple second messenger pathways (10). Thus the present findings, when considered with the results of Fan et al. (5), suggest that the effect of PTH on the cellular redistribution of NHE3 is transduced by a non-cAMP-mediated mechanism. We would conclude that the acute regulation of NHE3 by cAMP analyzed in the current experiments most likely represents PKA phosphorylation of COOH-terminal residues in NHE3 mediated by the NHERF-ezrin signal complex (17, 22).

In summary, the present studies indicate that a truncated form of NHERF deficient in ezrin binding functioned as a dominant-negative reagent when expressed in OK cells. Although mNHERF(1–325) had little effect on basal Na\(^{+}/\mathrm{H}^{+}\) exchange activity, it markedly blunted the acute inhibitory response to cAMP. These studies, then, provide the first functional validation for the signal-complex model of NHE3 regulation in renal epithelial cells. Recent experiments have established an association between NHERF and two other cAMP-regulated transporters in the renal proximal tubule. The sodium bicarbonate cotransporter (NBC) is inhibited by cAMP by a process that requires NHERF (2). Gisler et al. (7) recently showed that the COOH-terminus of the type IIa sodium phosphate cotransporter associated with NHERF, although the physiological relevance of this association was not established. The present experiments suggest that the expression of the dominant-negative NHERF(1–325) in appropriate epithelial cells may help elucidate the role of the NHERF-ezrin complex in the hormonal control of NBC and other renal transporters.

Dr. Mark Knepper kindly provided a polyclonal antibody to rat NHE3 (L546) (6), and Dr. Orson Moe provided a polyclonal antibody to OK cell NHE3. The authors acknowledge the technical support of Christine Evangelista, Min-Zhi Liu, and Jie Liu.

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