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The Role of NHERF and E3KARP in the cAMP-mediated Inhibition of NHE3*

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NHE3 is the apically located Na^+/H^+ exchanger in the gut and in the renal proximal tubule. Acute inhibition of this transporter by cAMP requires the presence of either of two NHE3-associated proteins, NHERF or E3KARP. It has been suggested that these proteins either directly regulate NHE3 activity after being phosphorylated by protein kinase A (PKA) or that they may serve as adapters that localize PKA near NHE3. We studied the role of NHERF and E3KARP in opossum kidney cells, which endogenously express NHE3, NHERF, and ezrin and display cAMP-dependent inhibition of NHE3. *In vivo* phosphorylation studies showed that NHERF is a phosphoprotein under basal conditions, but does not change its phosphorylation state after 8-bromo-cAMP treatment, and that E3KARP is not phosphorylated at all. Co-immunoprecipitation showed that NHERF and E3KARP bind both NHE3 and ezrin. Using cAMP analogs it was demonstrated that NHE3 activity, measured as sodium-dependent recovery of the intracellular pH after intracellular acidification, is inhibited by PKA type II. Because others have shown that ezrin binds PKA type II and that NHE3 is phosphorylated by PKA we suggest that NHERF and E3KARP are adapters that link NHE3 to ezrin, thereby localizing PKA near NHE3 to allow NHE3 phosphorylation.

$NHE3$[1] is the apically located Na^+/H^+ exchanger isoform that together with a Cl^-/HCO_3^- exchanger or a Na^+/HCO_3^- co-transporter, respectively, mediates the majority of NaCl or $NaHCO_3$ absorption in the ileum and proximal colon and in the proximal tubule (1, 2). Cyclic AMP is one of the major intracellular messengers mediating the inhibition of NHE3 (1, 3). Two models of how NHE3 is inhibited by cAMP have evolved: the first model proposes that NHE3 is regulated through direct phosphorylation of the transport protein. This model is based on findings that in AP1 cells treatment with 8-Br-cAMP results in the phosphorylation of NHE3 that parallels the inhibition of transport activity (4, 5). The other model proposes that regulatory proteins are required to transduce cellular signals between protein kinase A and NHE3 (6, 7). The existence of one or more regulatory proteins was suggested based on the finding that in solubilized rabbit renal brush-border a protein fraction that was required for regulation of NHE3 could be separated from NHE3 itself (6), and that in PS120 fibroblasts several signals that regulate NHE3 did not change the phosphorylation state of NHE3 (7). It was speculated that such regulatory proteins would be the substrate for protein kinases (6, 7) and that they would interact with NHE3 in a phosphorylation dependent manner, resulting in a change of NHE3 activity. It is not known whether these two mechanisms are independent processes or complementary in that both the regulatory proteins and NHE3 are phosphorylated in response to cAMP.

Two closely related regulatory proteins of NHE3, NHERF (NHE regulatory factor), and E3KARP (NHE3 kinase A regulatory protein), have recently been identified (8, 9). We previously showed that there is a requirement for the presence of NHERF or E3KARP for the cAMP-induced inhibition of NHE3 to occur (9). However, the mechanism by which these regulatory proteins facilitate cAMP inhibition of NHE3 is not understood. These regulatory proteins may induce a conformational change of NHE3 or alternatively may function to physically bring PKA near NHE3, thereby allowing phosphorylation of NHE3 to occur. In the latter mode, the regulatory proteins could act as direct anchors for PKA, known as A kinase anchoring proteins (AKAP) (10), or they could be adapters linking NHE3 to other molecules that interact with PKA. These models appear attractive because NHERF and E3KARP share their highest homology along two PDZ domains, which are modules for protein-protein interaction and which have been shown to be involved in the linking of membrane proteins to signaling complexes in other systems (11).

Here we report that NHERF and E3KARP are not substrates for PKA and therefore do not directly regulate NHE3 activity. Instead NHERF and E3KARP are shown to be adapters between the cytoskeletal protein ezrin and NHE3. Because ezrin is an AKAP we propose that the regulatory proteins indirectly localize PKA type II near NHE3 and thereby provide specificity in the PKA signaling pathway by co-localizing PKA and its substrate NHE3.

EXPERIMENTAL PROCEDURES

Expression of Recombinant Proteins—For bacterial expression the region corresponding to base pairs 41 to 1377 of E3KARP was amplified by polymerase chain reaction and cloned into pET30a (Novagen). Full-length NHERF was used as described (12). Recombinant His-tagged

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[1] The abbreviations used are: NEH, NHE regulator factor; E3KARP, NHE3 kinase A regulatory protein; MBP, maltose-binding protein; 8-Br-cAMP, 8-bromo-adenosine 3′,5′-cyclic monophosphate; 8-Pip-cAMP, 8-piperazinyl-cAMP; 8-AHA-cAMP, 8-aminohexylamino-cAMP; 6-Benz-cAMP, 6-benzoyl-cAMP; PAGE, polyacrylamide gel electrophoresis; BCECF, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; NTA, nitrilotriacetic acid; PKA, cAMP-dependent protein kinase; AKAP, A kinase anchoring protein; MAP-2, microtubule associated protein 2; AKAP, A kinase anchoring proteins; OK, opossum kidney.

proteins were expressed in *Escherichia coli* strain BL21 and purified by affinity to Ni^{2+}-NTA resin as suggested by the manufacturer (Qiagen). An expression construct of the entire C terminus of rat NHE3 (amino acids 405 to 831) as a MBP fusion protein was the generous gift of Dr. O. Moe (5). The MBP fusion protein was induced and expressed at 30 °C and partially purified on amylose resin according to the recommendations of the manufacturer (New England Biolabs). This is referred to as MBP-NHE3C.

Cell Culture and Transfection of Cells—OK cells between passage 53 and 73 were grown in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin in 95% air, 5% CO_2. For expression in OK cells, NHERF, and E3KARP were cloned into pcDNA3.1HisA and pcDNA3.1HisC (Invitrogen), respectively, resulting in pcDNA3.1His/E3KARP and pcDNA3.1His/NHERF. OK cells were transfected with pcDNA3.1His/E3KARP or pcDNA3.1His/NHERF using LipofectAMINE (Life Technologies, Inc.) and clonal cell lines expressing the recombinant fusion proteins were established by serial dilution. These cells are referred to as OK/NHERF and OK/E3KARP, respectively.

PS120 fibroblasts were grown as described previously (7, 9). For expression in PS120 fibroblasts the entire construct of His- and S-tagged NHERF or E3KARP, respectively, was subcloned from pET30a into the expression vector pMT3 (Genetics Institute), resulting in pMT3/NHERF-HS and pMT/E3KARP-HS. PS120/NHE3V fibroblasts, in which NHE3 carries a C-terminal VSVG tag (9), were co-transfected using Lipofectin (GicoLife Technologies, Inc.) with pMT3/NHERF-HS or pMT/E3KARP-HS and pPol2 for selection by hygromycin (600 units/ml). Clonal cell lines were established by serial dilution and are referred to as PS120/NHE3V/NHERF-HS and PS120/NHE3V/E3KARP-HS, respectively.

In Vivo Phosphorylation—OK cells were grown to confluence on tissue culture plates and then serum starved for 3 days. After two washes with phosphate-free media, the cells were incubated for 3 h in phosphate-free media plus 0.8 mCi/ml [^{32}P]orthophosphoric acid (2.4 mCi total per plate). After two washes with phosphate-free media, the cells were further incubated for 15 min in the presence or absence of 300 μM 8-Br-cAMP. All subsequent steps were carried out at 4 °C. Cells were lysed in 400 μl of 8 M urea, 100 mM sodium phosphate, pH 8.0 (buffer B), plus 5 mM β-mercaptoethanol and 1% Triton X-100. The lysate was cleared by centrifugation at 16,000 $\times$ *g* for 30 min. The His-tagged fusion proteins were then purified by incubation of the lysate with 20 μl of Ni^{2+}-NTA resin for 1 h followed by washing 3 times with buffer B plus 1% Triton X-100 and 3 times with buffer B titrated to pH 6.3. The fusion proteins were separated from the beads using 100 mM EDTA and 2 $\times$ Laemmli sample buffer. Proteins were separated by 8.5% SDS-PAGE, transferred to nitrocellulose, and probed by anti-sera against either NHERF (Ab-RF) (13) or E3KARP (Ab2570) (14). Relative amounts of the fusion proteins were determined by enhanced chemiluminescence (Renaissance Reagent plus, NEN Life Science Products Inc.) and at least two separate, nonsaturating exposures were quantified using a densitometer (Molecular Dynamics). [^{32}P] signals from the same membranes were then quantified using a PhosphorImager (Molecular Dynamics). The data were analyzed using ImageQuant (Molecular Dynamics). For two-dimensional phosphopeptide mapping, bands were cut out of dried SDS-PAGE gels and digested with 10 μg of chymotrypsin according to a standard method (15). Digested peptides were separated on thin layer chromatography plates and the plates were analyzed by PhosphorImager.

For isoelectric focusing, the purified material was separated from the Ni^{2+}-NTA resin and then concentrated in 8 M urea, 5% β mercaptoethanol, 0.002% bromphenol blue over a spin filter (10-kDa molecular mass cut off; Millipore): 2% Triton X-100 and 5% ampholyte 3/10 were then added to the resulting sample of 40 μl. The tube gels consisted of 4% acrylamide, 9.5 M urea, 2% Triton X-100, and 5% (v/v) ampholyte 3/10 (Bio-Rad). After pre-electrophoresis, IEF was carried out for 3.5 h at 750 V. Separation in the second dimension, immunoblotting, and autoradiography were carried out as described above.

pH_i Measurements—To study intracellular pH (pH_i) using the ratiofluorometric, pH-sensitive dye BCECF, cells were seeded on glass coverslips, grown to confluence, and then serum starved for 3 days. Cells were dye-loaded for 20 min with 6.5 μM BCECF/AM in 130 mM NaCl/pH_i buffer (20 mM HEPES, 5 mM KCl, 1 mM tetramethylammonium-PO_4, 2 mM $CaCl_2$, 1 mM $MgSO_4$). The coverslip was mounted in a perfusion cuvette heated to 30 °C as described earlier (16). Cells were acidified by ammonium prepulse in 40 mM NH_4Cl, 90 mM NaCl/pH_i buffer for 4 min and subsequently perfused with 130 mM tetramethylammonium-Cl/pH_i buffer for 200 s. 130 mM NaCl was then reintroduced and the sodium-dependent pH_i recovery was recorded as described previously (16). At the end of each experiment the fluorescence ratio was calibrated to pH_i using the high potassium/nigericin method at the extracellular pH of 6.0, 6.3, and 7.2. Slopes were calculated along the pH_i recovery by linear least square analysis over a minimum of 9 s. When indicated, 8-Br-cAMP and the cAMP analogues (Biolog) were present during the prepulse period.

R_{II} Overlay—Blot overlays for the detection of AKAP were done using His-tagged E3KARP and NHERF and MBP-NHE3C as described previously (17).

Immunoprecipitation—OK or PS120 cells were washed twice in phosphate-buffered saline, scraped, and lysed in lysis buffer, containing 50 mM Tris, 100 mM NaCl, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, pH 7.5, 1% Triton X-100, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM phenanthrolidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin). The crude lysate was spun at 16,000 $\times$ *g* for 30 min. This lysate was cleared by incubation with 30 μl of protein-A Sepharose for 1 h and the supernatant was then incubated overnight with appropriate antisera. Immunocomplexes were purified with 40 μl of protein-A-Sepharose, washed 3 times in lysis buffer and 3 times in lysis buffer without Triton X-100. Bound immuncomplexes were eluted by incubating the beads in Laemmli sample buffer for 10 min at 85 °C and were then separated by SDS-PAGE. The proteins were then transferred to nitrocellulose and immunoblotted using the indicated antibodies.

RESULTS

8-Br-cAMP Changes the pH_i Dependence of NHE3 through Activation of PKA Type II—In order to study the inhibitory effect of cAMP on the transport kinetics of Na^+/H^+ exchange, OK cells were acidified to pH_i 6.0 and the sodium-dependent pH_i recovery was recorded. Fig. 1*A* shows a typical trace and the inhibitory effect of 100 μM 8-Br-cAMP. Fig. 1*B* shows that sodium-dependent pH_i recovery was dose-dependently inhibited by 8-Br-cAMP with a maximal effect seen at 100 μM 8-Br-cAMP. Therefore all further fluorometric studies were done using 100 μM 8-Br-cAMP.

We measured the pH_i recovery rate at different intracellular pH in the absence and presence of 100 μM 8-Br-cAMP (Fig. 1*C*) and calculated the relative inhibition of the pH_i recovery by 100 μM 8-Br-cAMP at different pH_i (Fig. 1*D*). At pH_i 6.0, 100 μM 8-Br-cAMP resulted in only 15% inhibition of sodium-dependent pH_i recovery, but at more alkaline pH_i a stronger inhibition (50% at pH_i 7.0, 70% at pH_i 7.3) became apparent (Fig. 1*D*). Thus our data indicate that 8-Br-cAMP changed the pH_i dependence of the transporter; the small inhibition even at the most acidic pH_i studied suggests that a small change in V_{max} may also be present.

The participation of NHE1, which is regulated by changes in its pH_i dependence (16, 18), in the sodium-dependent pH_i recovery, was ruled out by applying 20 μM Hoe694 during the pH_i recovery. Twenty μM Hoe694, which blocks NHE1 but not NHE3 (18), resulted in less than 10% inhibition (data not shown). This is consistent with previous reports that NHE1 is absent from OK cells (19, 20). Any influence of 100 μM 8-Br-cAMP on another, as yet unidentified, sodium-dependent proton-exporting mechanism was also ruled out, because in the presence of 1 mM amiloride, which inhibited sodium-dependent pH_i recovery by 80–95%, 100 μM 8-Br-cAMP had no additional inhibitory effect (data not shown).

Two types of PKA, type I (PKA I) and type II (PKA II), display different biochemical properties due to differences in their regulatory (R) subunits (10). Each R subunit contains two cAMP-binding sites to which cAMP bind cooperatively (10). Until recently it was believed that only PKA II is membrane bound and affects proteins in or near membranes (10) but more recently examples of membrane bound PKA I have been described (21, 22). In pilot experiments we found that OK cells express both PKA isoforms, making it necessary to determine which isoform is responsible for the regulation of NHE3. We therefore used combinations of site-specific cAMP analogs to

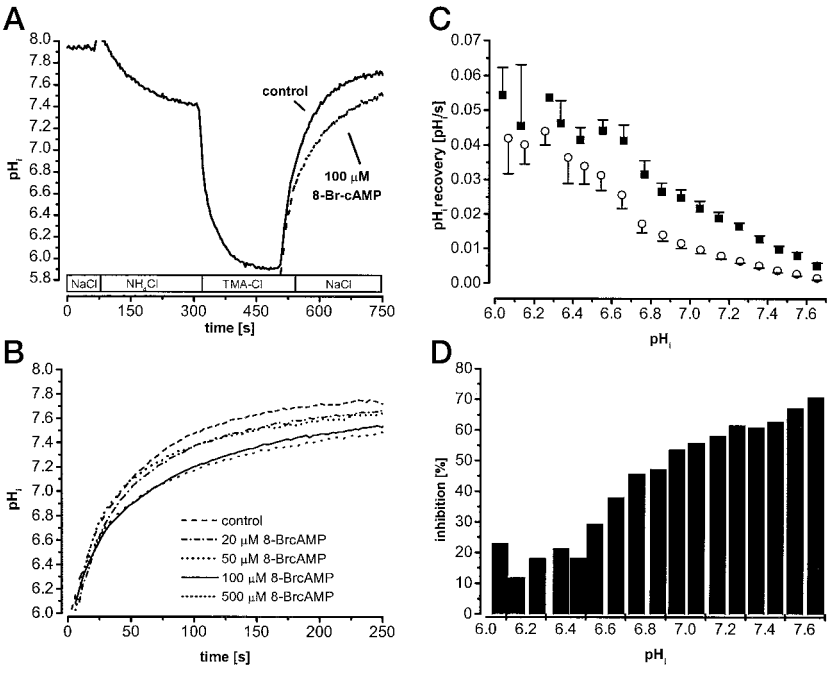

FIG. 1. **8-Br-cAMP inhibits Na^+/H^+ exchange dose dependently through a change of the pH_i dependence of NHE3.** *A,* representative trace of cAMP-dependent inhibition of sodium-dependent pH_i recovery. OK cells were acidified by ammonium prepulse (40 mM) and subsequent perfusion with tetramethylammonium-Cl. pH_i recovery was facilitated by re-addition of NaCl (130 mM) (*solid line*). When indicated 100 μM 8-Br-cAMP was present during the ammonium prepulse (*dotted line*). *B,* dose response to 8-Br-cAMP. Different concentrations of 8-Br-cAMP were applied during the period of the ammonium prepulse and the effect on sodium-dependent pH_i recovery was recorded. *C,* pH_i dependence of the 8-Br-cAMP effect. pH_i recovery rates ($\Delta pH_i/\Delta t$) were calculated over short time intervals during the pH_i recovery under control conditions (■) and after treatment with 100 μM 8-Br-cAMP (○). The recovery rates ($\Delta pH_i/\Delta t$) are plotted at different intracellular pH. *D,* from the data shown in *panel C*, the inhibition induced by 100 μM 8-Br-cAMP at different pH_i was calculated.

TABLE I
Relative affinities of cAMP analogs used in this study

The values indicate the binding affinity of cAMP analogs for the two cAMP-bindings sites (site A and site B) in the regulatory subunits of both PKA isoforms (type I and type II), all compared to cAMP (24). The combination of 8-Pip-cAMP and 8-AHA-cAMP was used to preferentially acitvate PKA I and the combination of 8-Pip-cAMP and 6-Benz-cAMP was used to preferentially activate PKA II.

cAMP analog	PKA type I		PKA type II	
	Site A	Site B	Site A	Site B
8-AHA-cAMP	0.11	1.6	0.021	0.29
6-Benz-cAMP	3.5	0.18	4.1	0.034
8-Pip-cAMP	2.3	0.065	0.046	3.2

preferentially activate either of the two isoforms (Table I). These cell-permeable, relatively phosphodiesterase-resistant drugs bind with different affinities to the two cAMP-binding sites of each PKA regulatory subunit. Therefore combinations of cAMP analogs can be chosen, that synergistically activate either PKA I or II, although at high concentrations these analogs activate both PKA isoforms (23, 24). 8-Pip-cAMP was used as the common agent at a "priming" concentration of 10 μM, which alone had no effect on sodium-dependent pH_i recovery (Fig. 2). To preferentially activate PKA I or PKA II, 8-Pip-cAMP was combined with either 8-AHA-cAMP or 6-Benz-cAMP, respectively. Consistent with the inhibition by 8-Br-cAMP, application of these combinations inhibited the sodium-dependent pH_i recovery also by a change in the pH_i dependence (data not shown). For quantification, the extent of inhibition of the pH_i recovery by the cAMP analogs was compared with that by 100 μM 8-Br-cAMP at pH_i 7.0. As shown in Fig. 2, the combination of 8-Pip-cAMP and 6-Benz-cAMP, directed at PKA II, resulted in a larger extent of inhibition than the combination of 8-Pip-cAMP and 8-AHA-cAMP, which is directed at PKA I. The inhibitory effect of the PKA I directed combination is probably due to some "cross-activation" of PKA II (see Table I). 8-Pip-cAMP and 6-Benz-cAMP also displayed cooperativity in that addition of 8-Pip-cAMP at a priming concentration of 10 μM significantly increased the effect of 6-Benz-cAMP. A smaller degree of cooperativity was also seen with the combination of 8-Pip-cAMP and 8-AHA-cAMP, but it did not achieve statistical

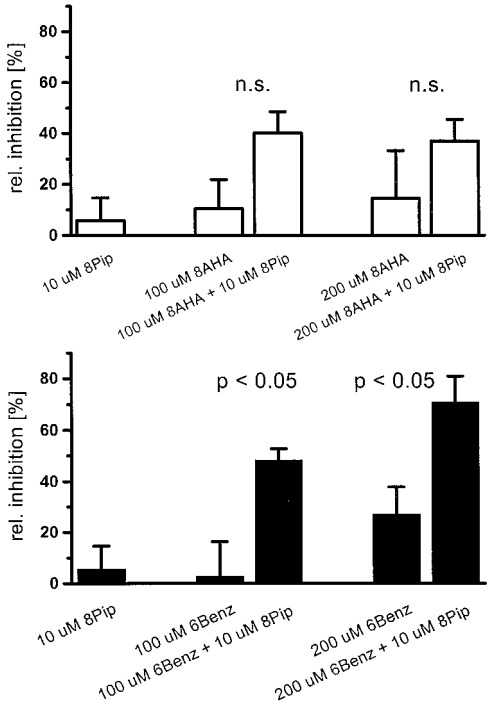

FIG. 2. **cAMP inhibits NHE3 through PKA type II.** OK cells were treated with the indicated concentrations of 8-Pip-cAMP, 8-AHA-cAMP, and 6-Benz-cAMP either individually or in combinations known to activate preferentially PKA I (8-Pip-cAMP plus 8-AHA-cAMP, *upper panel*) or PKA II (8-Pip-cAMP plus 6-Benz-cAMP, *lower panel*). To quantitatively compare the inhibition of sodium-dependent pH_i recovery induced by these compounds to the inhibition induced by 100 μM 8-Br-cAMP pH_i recovery rates were calculated at pH_i 7.0. n = 3–15. Values were compared by unpaired t test.

significance. These data suggest that the inhibition of NHE3 is mediated by PKA II.

In Vivo Phosphorylation of NHERF and E3KARP—We next determined whether the cAMP mediated inhibition of NHE3 involves changes in the phosphorylation state of NHERF and E3KARP *in vivo*. Because NHERF and E3KARP have approximately the same molecular mass as IgG, thereby rendering

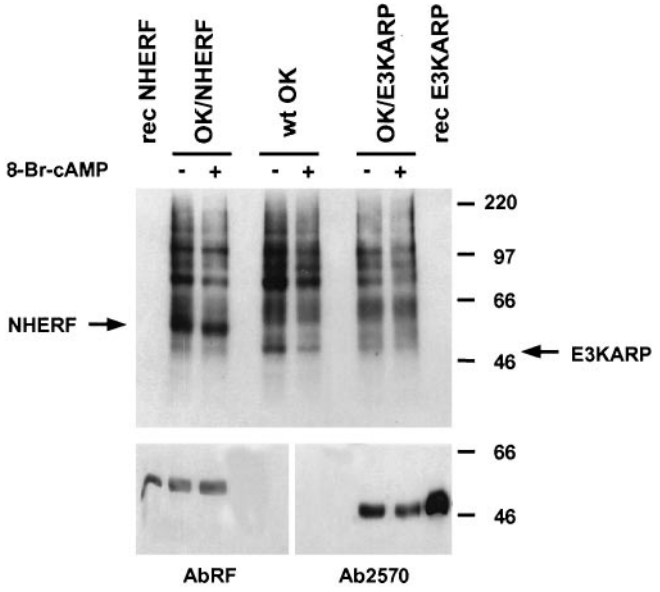

FIG. 3. ***In vivo* phosphorylation of NHERF.** Wild type OK, OK/NHERF, and OK/E3KARP cells were ^{32}P-labeled *in vivo*. Where indicated the cells were treated with 300 μM 8-Br-cAMP for 10 min at the end of the labeling period. NHERF or E3KARP were purified as His-tagged fusion proteins on Ni^{2+}-NTA resins. Untransfected wild type OK cells served as a control for the specificity of purification. Purified proteins were separated on SDS-PAGE, blotted onto nitrocellulose, and the autoradiogram was obtained (*upper panel*). The respective parts of the membrane were then probed by AbRF or Ab2570 to determine the amount of purified protein in the presence or absence of cAMP (compare *lane 2* with *lane 3* and *lane 6* with *lane 7*) (*lower panel*). Recombinant NHERF and E3KARP (*lanes 1* and *8*) served as size controls for the Western blot. The *arrows* indicate the molecular mass of NHERF and E3KARP. Representatives of five experiments are shown.

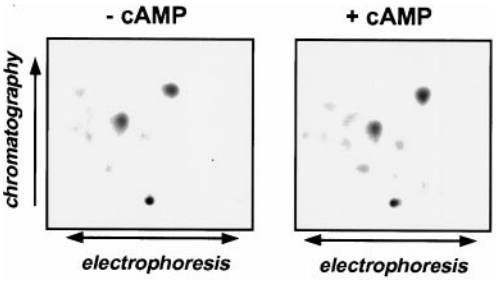

FIG. 4. **Two-dimensional phosphopeptide mapping of NHERF.** The bands corresponding to NHERF purified from OK/NHERF cells under basal conditions (−cAMP) or after treatment with 300 μM 8-Br-cAMP were cut out of the nitrocellulose membranes and digested with chymotrypsin. The phosphopeptides were spotted on thin-layer chromatography plates and separated by electrophoresis and chromatography. Representative autoradiograms of four experiments are shown.

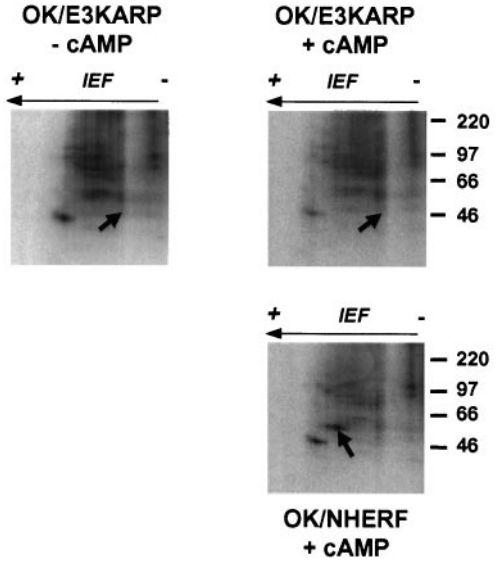

FIG. 5. **Two-dimensional PAGE of proteins purified by Nickel agarose from OK/E3KARP cells.** E3KARP was purified by Ni^{2+}-NTA resins under basal conditions or after treatment with 300 μM 8-Br-cAMP for 10 min of OK/E3KARP cells labeled *in vivo* with ^{32}P. The purified proteins were separated first by isoelectric focusing (*IEF*) and then by conventional SDS-PAGE and finally transferred to nitrocellulose for autoradiography and subsequent Western blot (*upper panel*). Representative autoradiograms are shown. OK/NHERF cells served as a positive control (*lower panel*). The *arrows* indicate the location where E3KARP or NHERF were detected by Western blot.

protein quantification by Western blot following immunoprecipitation difficult, the regulatory proteins were expressed as His-tagged fusion proteins to allow affinity purification on Ni^{2+}-NTA resins. OK cells were labeled with ^{32}P *in vivo* and the recombinant His-tagged regulatory proteins were purified using Ni^{2+}-NTA resins. The amount of ^{32}P incorporated was determined by a PhosphorImager and was corrected for the amount of protein as determined by Western immunoblot. Fig. 3 shows the autoradiogram of phosphoproteins purified from labeled wild type OK, OK/NHERF, and OK/E3KARP cells and the corresponding Western blots. The autoradiogram in Fig. 3 shows that His-tagged NHERF was purified from OK/NHERF as a phosphoprotein under basal conditions. Following treatment with 8-Br-cAMP, the amount of ^{32}P incorporated into NHERF did not change significantly (0.99 ± 0.03-fold relative to untreated cells). Normalization of the ^{32}P radioactivity to the amount of NHERF purified also revealed that the phosphorylation level of NHERF did not change in response to 8-Br-cAMP (1.05 ± 0.03-fold relative to untreated cells).

Changes in the phosphorylation state may be masked either by a high degree of basal phosphorylation or by an increase in phosphorylation at one site and a concomitant decrease at another site. To address this possibility, the NHERF bands were cut out, digested with chymotrypsin, and two-dimensional phosphopeptide mapping was performed (Fig. 4). In four independent experiments two major and one minor spot were detected and all had identical intensities under basal conditions and after 8-Br-cAMP treatment. This rules out the possibility of masked changes in the phosphorylation state of NHERF.

E3KARP migrated in the SDS-PAGE gels together with a phosphoprotein that was co-purified as a contaminant from wild type OK cells (compare *lanes 3* and *4* to *lanes 5* and *6* of the autoradiogram in Fig. 3) making it difficult to judge its phosphorylation state by the one-dimensional approach. Therefore proteins purified from OK/E3KARP cells were separated by two-dimensional PAGE. No phosphoprotein signal was detected by PhosphorImager at the location were E3KARP was detected by Western blot under basal conditions or after 8-Br-cAMP treatment (Fig. 5). By contrast, NHERF, which was run as a positive control, again showed a phosphoprotein spot at the location where the protein was detected by Western. This indicates that E3KARP is neither constitutively phosphorylated nor phosphorylated after 8-Br-cAMP treatment.

NHERF and E3KARP Are Not AKAPs—Based on our finding that NHE3 is inhibited by cAMP through an action of PKA II but NHERF and E3KARP are not direct targets for phosphorylation, we determined if NHERF and E3KARP function as AKAPs. AKAPs are defined by their ability to bind the regulatory subunit (R_{II}) of PKA II (17). His-tagged NHERF and E3KARP transferred onto nitrocellulose were probed with ^{32}P-labeled R_{II}. Fig. 6 shows that NHERF and E3KARP both yielded positive signals although at much weaker intensity

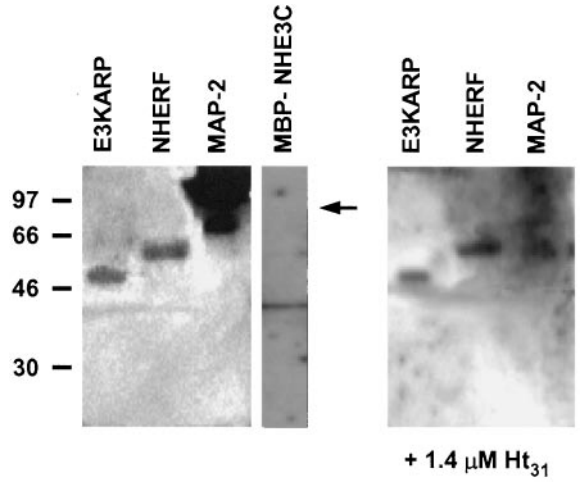

FIG. 6. **[^{32}P] R_{II} overlay assay on recombinant NHERF, E3KARP, and NHE3C.** His- and S-tagged E3KARP and NHERF and MBP-NHE3C were separated on PAGE and blotted onto nitrocellulose. The membranes were probed with ^{32}P-labeled R_{II} in an overlay assay to detect AKAP. Where indicated the inhibitory peptide Ht31, which disrupts the R_{II} AKAP interaction, was present. MAP-2 served as a positive control. The *arrow* denotes the molecular mass of MBP-NHE3C.

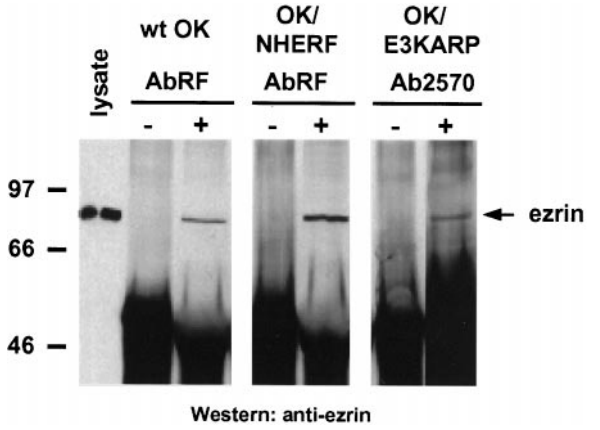

FIG. 7. **Co-immunoprecipitation of ezrin with NHERF and E3KARP in OK cells.** Lysates from wild type OKs, OK/NHERF, and OK/E3KARP cells were immunoprecipitated with AbRF or Ab2570 (+). Preimmune serum was used as a negative control (−). The precipitated immuncomplexes were probed for the presence of ezrin by anti-ezrin antibody. Representatives of four experiments are shown.

than microtubule-associated protein 2 (MAP-2), which was used as a positive control (25). By contrast, ^{32}P-labeled R_{II} did not bind to MBP-NHEC3. To test whether the signals from NHERF and E3KARP are specific, the R_{II} overlay assay was performed in the presence of 1.4 μM Ht31, a peptide which specifically disrupts the binding of R_{II} to AKAP (26). In the presence of Ht31, the binding of ^{32}P-labeled R_{II} to MAP-2 was almost completely blocked whereas the weak binding to NHERF and E3KARP remained unchanged (Fig. 6). This indicates that the R_{II} subunit nonspecifically bound to E3KARP and NHERF. We conclude from these data that neither NHERF/E3KARP nor the cytoplasmic tail of NHE3 functions as AKAP.

NHERF and E3KARP Bind Ezrin and NHE3—In placental brush-border, human NHERF (called EBP50) has recently been identified as a protein that binds to the cytoskeletal protein ezrin (27). In parietal cells ezrin functions as an AKAP (28). We therefore tested whether NHERF and E3KARP interact with both ezrin and NHE3. NHERF and E3KARP were immunoprecipitated and immuncomplexes were tested for the presence of NHE3 or ezrin by Western analysis.

Fig. 7 shows that ezrin was co-immunoprecipitated with NHERF from both wild type OK and OK/NHERF cells. However, the amount of ezrin was clearly higher in the immunoprecipitate from OK/NHERF cells, which is consistent with the larger amount of NHERF expressed in the transfected cells. Similarly, ezrin was co-immunoprecipitated with E3KARP from OK/E3KARP cells, demonstrating that E3KARP also binds ezrin. However, we could not clearly determine whether NHE3 also co-immunoprecipitated with NHERF or E3KARP in OK cells. This is mainly because both the antibody against OK NHE3 (20) and the antibodies against NHERF and E3KARP are raised in the same species, *i.e.* rabbit, which led to considerable background.

To overcome this limitation, we used PS120 cells transfected with NHE3V and either NHERF or E3KARP. In these cells co-immunoprecipitated NHE3 was detected by a monoclonal antibody against the VSVG tag. Fig. 8 shows that NHE3V was co-immunoprecipitated with NHERF and E3KARP from PS120/NHE3V/NHERF-HS and PS120/NHE3V/E3KARP-HS cells, respectively. Fig. 8 shows that ezrin was also co-immunoprecipitated with NHERF and E3KARP from these cells.

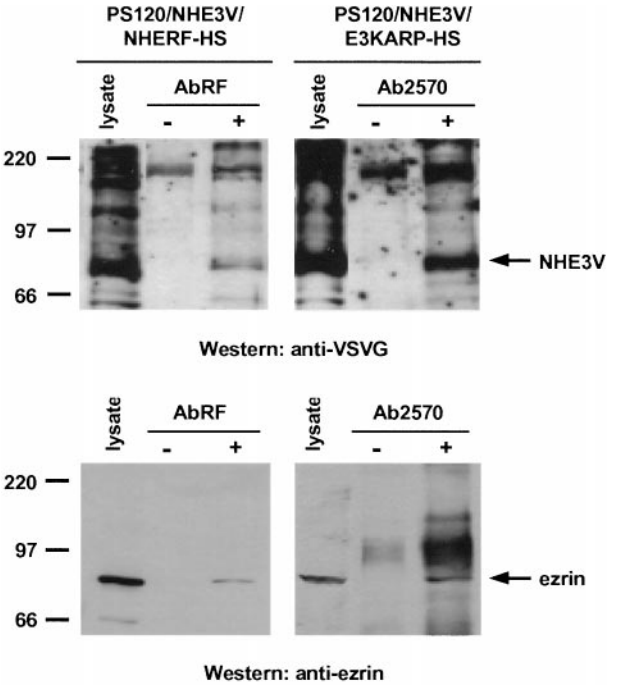

FIG. 8. **Co-immunoprecipitation of NHE3 and ezrin with NHERF and E3KARP in PS120 fibroblasts.** Lysates from PS120/NHE3V/NHERF-HS and PS120/NHE3V/E3KARP-HS fibroblasts were immunoprecipitated with AbRF (*left panels*) or Ab2570 (*right panels*), respectively (+). Preimmune serum was used as a negative control (−). The isolated immuncomplexes were probed for the presence of NHE3 by monoclonal anti-VSVG (*upper panels*) or anti-ezrin antibody (*lower panels*).

DISCUSSION

OK Cells as a Model to Study the Role of NHERF and E3KARP in the cAMP-mediated Inhibition of NHE3—The molecular mechanisms involved in the regulation of NHE3 have been studied in a number of different models (3). In our and other laboratories (4, 5, 9, 16) a number of studies have been conducted in non-epithelial cells devoid of the ubiquitous NHE1 and transfected with NHE3. This approach carries the potential problem that proteins required for the regulation of NHE3, such as parts of the signaling cascade, may be missing or not appropriately located in these non-polarized cells. For example, PS120 fibroblasts were shown to lack the regulatory

proteins NHERF and E3KARP that are required for the cAMP-dependent inhibition of NHE3 (9).

For the present study we have chosen opossum kidney (OK) cells, which are derived from the renal proximal tubule, for the following reasons: (*a*) OK cells are a polarized epithelial cell line (19), that expresses only the NHE3 isoform of the known Na^+/H^+ exchangers (19, 20). (*b*) NHE3 is inhibited by cAMP in these cells (29), indicating that the machinery mediating this process, although not fully understood, is present. (*c*) OK cells express NHERF (30). (*d*) OK cells also endogenously express ezrin just like proximal tubule cells (31). These characteristics of OK cells make them an ideal physiological model to study the role of NHERF in the cAMP-mediated inhibition of NHE3. In order to study the role of E3KARP in this model we transfected E3KARP into OK cells. Of note, mRNA for E3KARP is expressed in kidney (9), suggesting that E3KARP has a physiological role in the regulation of renal NHE3 as well.

Protein Kinase A Inhibits NHE3 by a Change of the pH_i Dependence of the Transporter—Na^+/H^+ exchange mediated by all NHE isoforms is allosterically modified by the intracellular pH (16). Second messenger-mediated regulation of NHE1 has been shown to affect this pH_i dependence, while regulation of NHE2 and NHE3 in transfected PS120 fibroblasts by protein kinase C, calmodulin/CaM kinase II, serum, and fibroblast growth factor changes the V_{max} of the transporters without affecting their pH_i dependence (16, 32). By contrast, we found that in OK cells NHE3 is predominantly inhibited by cAMP through a change in its pH_i dependence. Because the initial pH_i recovery was very fast and the buffering capacity declined considerably between pH 6.0 and 6.5 we could not reliably fit the data to an allosteric Hill kinetic model. Instead pH_i recovery rates were calculated at different pH_i along the pH_i recovery and the values from cells treated with 8-Br-cAMP were then compared with the values of control cells. If inhibition occurred by a change in V_{max}, the relative inhibition should be the same at every given pH_i. By contrast, if the pH_i dependence was changed, this should be reflected in differing degrees of inhibition at different pH_i. Our data indicate that NHE3 is predominantly inhibited by a change in its pH_i dependence, but we cannot rule out a small decrease in V_{max}. The change in pH_i dependence is in agreement with the recent findings in NHE3-transfected AP1 cells (4, 33) and with the earlier report by Miller and Pollack (34), which showed the effect of 8-Br-cAMP on the amiloride inhibitable $^{22}Na^+$ uptake into OK cells clamped to different pH_i.

As the next step, we determined which PKA isoform mediates this process. For this we employed combinations of cAMP analogs, that preferentially activate either PKA I or PKA II (Table I) (23). We found that PKA II mediates the cAMP dependent inhibition of NHE3. The functional importance of PKA II in the regulation of NHE3 is supported by the finding that expression of a dominant negative regulatory subunit type II abolishes cAMP regulation (35) and by identification of PKA II in the apical membrane of proximal tubule cells (36).

The Phosphorylation State of NHERF and E3KARP Does Not Change upon 8-Br-cAMP Treatment—Two lines of evidence have suggested that not NHE3 itself but one or more associated regulatory proteins of NHE3 may be the substrates for PKA regulating NHE3. (*a*) Regulation of NHE3 in transfected PS120 cells by fibroblast growth factor or protein kinase C does not involve direct phosphorylation of the transporter (7). (*b*) In renal brush-border membranes a protein fraction that is necessary for cAMP inhibition of NHE3 can be separated from NHE3 itself (6). NHERF could be such a substrate for PKA, since it is necessary for cAMP-mediated inhibition of NHE3 (6, 9), contains at least one putative PKA concensus phosphorylation site (8), and has also been isolated as a phosphoprotein (6). On the other hand, E3KARP which also mediates cAMP inhibition of NHE3 in PS120 cells, does not have a putative PKA phosphorylation site (9). In addition, the recent finding of cAMP-dependent phosphorylation of NHE3 at S605 in AP-1 cells (4) raises the question of whether cAMP-dependent phosphorylation of the regulatory proteins is necessary for the regulation of NHE3. Using *in vivo* phosphorylation, we found that neither protein is a substrate for PKA. NHERF is a phosphoprotein under basal conditions, consistent with a recent report (12). But more importantly its phosphorylation state does not change after treatment with 8-Br-cAMP. E3KARP is neither a phosphoprotein under basal condition nor after treatment with 8-Br-cAMP. These data show that regulation of NHE3 does not occur through a change in phosphorylation of the regulatory proteins. If then, how do these regulatory proteins function in the regulation of NHE3?

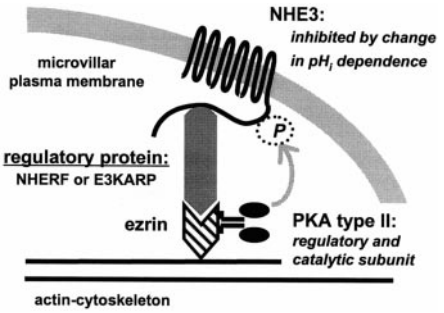

FIG. 9. **Proposed model for the role of NHERF and E3KARP in the cAMP-mediated inhibition of NHE3.** The regulatory proteins work as adapters between NHE3 and ezrin. Ezrin is an AKAP. Upon binding of cAMP to the regulatory subunit of PKA II, the catalytic subunit is released and phosphorylates NHE3.

NHERF and E3KARP Are Not AKAPs but Are Linkers between NHE3 and the AKAP Ezrin—Our data discussed so far show that NHERF and E3KARP are not substrates for PKA but are involved in the inhibition of NHE3 by PKA II (9). It is therefore plausible that the regulatory proteins function to physically localize PKA II near NHE3. In many systems, AKAPs localize PKAII in close proximity of its substrates (10), but our data clearly indicate that NHERF and E3KARP are not AKAPs and also that the C-terminal tail of NHE3 does not function as an AKAP either. These negative results do not rule out that the regulatory proteins may bind to yet another molecule that would function as an AKAP.

During the course of this study, an ezrin-binding protein of 50-kDa molecular mass, EBP50, was identified in placental brush border (27). Cloning revealed that EBP50 is the human homologue of NHERF. One function of ezrin is to link the actin based cytoskeleton to the plasma membrane (27, 31), but so far only three transmembrane proteins, CD44, CD43, and ICAM-2, binding to ezrin have been identified (37). Therefore EBP50 has been suggested to be an intermediate between ezrin and one or several as yet unidentified transmembrane proteins (27). In the present study we demonstrate that ezrin co-immunoprecipitates with NHERF and E3KARP in OK cells and in PS120 fibroblasts. NHE3 also co-immunoprecipitates with the regulatory proteins in PS120 fibroblasts. These results for the first time demonstrate NHE3 as a transmembrane protein that is linked to ezrin through one of the regulatory proteins (NHERF/EBP50 or E3KARP). The biochemical details of the interaction of NHERF (38) and E3KARP (14) with ezrin have recently been described.

Ezrin has been identified as an AKAP in parietal cells (28). We have confirmed this finding and also found that ezrin, based on the R_{II} overlay assay, is a weak AKAP (data not

shown). Nevertheless we could co-immunoprecipitate R_{II} with NHERF (data not shown). One interpretation is that those ezrin molecules that interact *in vivo* with the regulatory proteins also bind R_{II} at the same time.

Taking the findings in the present study together with the report by Kurashima *et al.* (4), we propose the following model for the role of NHERF and E3KARP in the regulation of NHE3 (Fig. 9). The regulatory proteins link NHE3 to ezrin thereby adding physical support to the microvillar structure. Furthermore, ezrin functions as an AKAP, thereby placing PKA II into close proximity of NHE3. Upon stimulation by cAMP the catalytic subunit of PKA dissociates from the regulatory subunit and phosphorylates NHE3 probably at the serine residue corresponding Ser-605 of rat NHE3. This phosphorylation results in inhibition of transport activity by change in the pH_i dependence of the transporter. In this model the function of the regulatory proteins is to co-localize NHE3 and PKA II.

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