Activation of Protein Kinase A Acutely Inhibits and Phosphorylates Na/H Exchanger NHE-3

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Abstract
In the mammalian renal proximal tubule, protein kinase A (PKA) plays an important role in mediating hormonal regulation of apical membrane Na/H exchanger activity. This exchanger is likely encoded by NHE-3. The present studies examined regulation of NHE-3 by PKA. NHE-3 was stably expressed in Na/H exchanger-deficient fibroblasts (AP-1/NHE-3 cells). PKA activation (0.1 mM 8-Br-cAMP x 20 min) inhibited NHE-3 activity by 39% (P < 0.01) with no change in NHE-3 protein abundance in the plasma membrane. To define the structural requirements for PKA-mediated inhibition, full-length NHE-3 and a cytoplasmic domain-truncated mutant (NHE-3Δcyto) were expressed in Xenopus laevis oocytes. 8-Br-cAMP inhibited NHE-3 activity by 27% (P < 0.05), an effect that was blocked by 10−7 M PKA inhibitor peptide. NHE-3Δcyto had baseline activity similar to that of full-length NHE-3 but its activity was not regulated by 8-Br-cAMP. The purified recombinant cytoplasmic domain of NHE-3 was phosphorylated in vitro by the catalytic subunit of PKA on serine residues. In AP-1/NHE-3 cells, NHE-3 was immunoprecipitated as a ~87-kD phosphoprotein. Addition of 0.1 mM 8-Br-cAMP increased the phosphocontent of NHE-3 by threefold. In summary, acute activation of PKA inhibits NHE-3 activity, an effect that is likely mediated by phosphorylation of its cytoplasmic domain. (J. Clin. Invest. 1995; 96:2187–2194.) Key words: Na transport • hydrogen ion transport • hormonal regulation

Introduction
In eukaryotic cells, Na/H exchangers are ubiquitous plasma membrane proteins that use inwardly directed Na gradients to extrude protons in a 1:1 stoichiometry (1–3). Na/H exchangers perform a variety of cell functions including cell volume regulation, cell pH defense, and transmembrane signal transduction (1–3). In transporting epithelia, apical membrane Na/H exchangers are critical for mediating transepithelial Na and H+ equivalent transport (1, 2). These diverse cellular functions are served by different members of the Na/H exchanger multi-gene family. To date, five distinct molecular isoforms (NHE-1 to 5) have been cloned (4–10). Of the five members, the most likely candidate for the epithelial apical membrane Na/H exchanger is NHE-3, although other isoforms may also play a role. The presumption of a major role of NHE-3 is based on inhibitor pharmacokinetics (11, 12), transcript distribution (7, 8), ontogenic pattern (13), regulation by glucocorticoids (14, 15), and immunohistochemistry (16, 17).

Hormonal regulation of NaCl and NaHCO3 transport in renal and gastrointestinal epithelia is mediated at least in part via modulation of apical membrane Na/H exchanger activity (1–3). In the mammalian renal proximal tubule, a number of hormones that regulate transepithelial NaCl and NaHCO3 transport alter cellular cyclic adenosine monophosphate (cAMP) levels (15–20). Maneuvers that increase cAMP levels have been shown to acutely decrease Na/H exchange activity in renal cortical apical membrane vesicles (21, 22) and in cultured proximal tubule cell lines (23–25). The present studies demonstrate that acute protein kinase A (PKA)1 activation inhibits the activity and induces the phosphorylation of NHE-3.

Methods

Generation and culture of stable NHE-3-expressing cell lines (AP-1/NHE-3 cells). Na/H exchanger-deficient Chinese hamster ovary AP-1 cells (gift from Dr. Sergio Grinstein, Toronto, Canada) (26) were maintained in Eagle α-modified complete minimum essential medium (Sigma Immunochemicals, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin. Full-length rat NHE-3 cDNA (nucleotides 50–4980) (provided by Dr. John Orlovski, Montreal, Canada and Dr. Gary Shull, Cincinnati, OH) (7) was cloned into the mammalian expression plasmid pcMV-5 (gift from Dr. David Russell, Dallas, TX). AP-1 cells were cotransfected by CaPO4 coprecipitation (27) with the NHE-3-expressing plasmid (15 μg/100-mm dish) along with Ch110/β-gal (6 μg/100-mm dish) to monitor transfection efficiency and pSV40/neo (6 μg/100-mm dish) to provide a selectable marker (AP-1/NHE-3 cells). Cells treated in an identical fashion except that the pcMV5 expression vector did not bear the NHE-3 insert were used as controls (AP-1/pCMV5 cells). Transfectants were selected with 400 μg/ml G418 (Gibco) 48 h after transfection and neo+ survivors were maintained in 200 μg/ml G418. 2 wk after transfection, neo+ cells were

1. Abbreviations used in this paper: CSU, catalytic subunit; MBP, maltose-binding protein; PKA, protein kinase A; RSU, regulatory subunit.
enriched for a neo+/NHE-3 phenotype by acid selection as described by Franchi et al. (28). Acid selection was repeated every 2–3 d for 2 wk. Transfectants selected in this fashion showed much higher and more consistent Na/H exchange activity compared with G418 selection alone. An identical round of acid selection was repeated every 3–4 mo. Clones of AP-1/NHE-3 cells were obtained by limiting dilution on 96-well plates. Studies were performed with single clones as well as pooled stable transfectants. G418 was removed from medium 1 wk and serum was removed 48 h before all experiments. PKA was acutely activated by incubating cells with 0.1–0.2 mM 8-BrcAMP or chloro-phenyl-thiocAMP for the stated period of time.

**Measurement of NHE-3 mRNA, immunoreactive protein, and activity in cultured cells.** Poly(A)+ RNA was harvested from monolayers by guanidium thiocyanate/phenol chloroform extraction and oligo-dT chromatography, size-fractionated with formaldehyde gel electrophoresis, and transferred to nylon membranes. NHE isoform–specific transcripts were detected by hybridization to uniformly 32P-labeled cDNAs (NHE-1, 1.9-kb BamHI fragment; NHE-2, 1.85-kb Aval fragment; NHE-3, 1.2-kb PSII fragment; NHE-4, 0.2-kb Nsi-l-BstEI and 0.63-kb BglII-BstEI fragments; NHE-5, 0.8-kb EcoRI-BglII fragments; NHE-6, 0.4-kb EcoRI-NsiI fragments; NHE-7, 0.2-kb HindIII-SmaI fragments). Antisense 1314 was directed against a chimeric protein composed of maltose-binding protein (MBP) fused to the whole cytoplasmic domain (amino acids [aa] 405–831) of rat NHE-3 (NHE-3s) (29). In addition, antipeptide antisera were generated to YGRNLPNEDEKQ (aa 633–646; antisense #1566) and DSFLQADGPEEQLQN (vol/vol) #1568 (7). For immunodetection of NHE-3, cells were lysed in membrane buffer (mM: 150 NaCl, 50 Tris-HCl, pH 7.5, 2.5 EDTA; 100 PMSF, 4 aprotinin, 4 leupeptin) at 4°C by Dounce homogenization. To obtain the plasma membrane fraction, whole cell lysate was centrifuged at 55,000 g (Beckman TLX: TLA100.3 rotor, 35,000 rpm, 15 min, 2°C; Beckman Instruments, Inc., Fullerton, CA). Membrane pellets were reconstituted in SDS buffer (1% [wt/vol] SDS, 2% [vol/vol] 2-mercaptoethanol, 20% [vol/vol] glycerol), size-fractionated by SDS-PAGE, and transferred to nitrocellulose filters. Filters were sequentially incubated with the primary antibody (1:500 to 1:750) and peroxidase-coupled goat anti–rabbit secondary antibody (1:2,000). Labeling was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL). NHE-3 activity was measured by BCECF fluorescence as Na-dependent intracellular pH recovery after acid loading with the K/H ionophore nigericin (29). Calibration and buffer capacities were performed exactly as described previously (29). Statistical significance was established by the two-tailed Student’s t test.

**Expression in Xenopus oocytes.** Oocytes dissected from Xenopus laevis (Nasco, Fort Atkinson, WI) were defollicularized with collagen-ase D (2 mg/ml) (Boehringer Mannheim Corp., Indianapolis, IN) in ORI (mM: 82.5 NaCl, 2 KCl, 1 MgCl2, 10 Hepes/Tris, pH 7.5) and stored in modified Barth’s solution (mM: 88 NaCl, 1 KCl, 0.82 MgSO4, 0.41 CaCl2, 0.33 Ca(NO3)2, 2.4 NaHCO3, 10 Hepes/Tris, pH 7.40, 3 mM Hepes-Tris buffered at 2188°C). After transformation into Echerichia coli, the MBP/NHE-3 fusion protein was induced by 0.3 mM IPTG at 20°C and purified by anion exchange column chromatography. Two bands were consistently seen on Coomasie-stained gels from different preparations representing the full-length MBP/NHE-3s fusion protein plus a truncated product (see Results).

In vitro phosphorylation and phosphoamino acid analysis. Rat NHE-3s was cloned into the isopropylthio-β-D-galactoside (IPTG)–inducible bacterial expression vector pMAL-c2 (New England Biolab, Beverly, MA) 3’ to and in frame with the coding sequence of MBP. After transformation into Echerichia coli, the MBP/NHE-3s fusion protein was induced by 0.3 mM IPTG at 20°C and purified by anion exchange column chromatography. Two bands were consistently seen on Coomasie-stained gels from different preparations representing the full-length MBP/NHE-3s fusion protein plus a truncated product (see Results).

In vitro phosphorylation was performed by incubating 100 ng of the fusion protein in a buffer containing (mM): 50 Hepes/Tris, pH 7.0, 3 DTT, 5 MgCl2, 1 EGTA, 0.1 ATP, 30 μCi [γ-32P]ATP, 25 U of the catalytic subunit of PKA (Pharmacia, Uppsala, Sweden). This antiserum was directed against the regulatory subunit of PKA (PKA-RSU). Reaction was initiated by addition of 75 μM cAMP. Phosphorylation was terminated at the indicated times by adding aliquots from the reaction and boiling the sample in SDS buffer. 32P-labeled phosphoproteins were resolved by SDS-PAGE, dried, and exposed to film.

Phosphoamino acids were analyzed by two-dimensional electrophoresis (10). The 32P-labeled phosphoprotein was eluted from dried polyacrylamide with 50 mM NH4HCO3, TCA-precipitated, and hydrolyzed by boiling in 6 M HCl. 32P-labeled phosphoamino acids along with standards (P-Ser, P-Thr, P-Tyr) were spotted on TLC plates and electrophoretically resolved (first dimension: 2.2% formic acid, 7.8% acetic acid, pH 1.9; second dimension: 5% acetic acid, 0.5% pyridine, pH 3.5) with a Hunter thin layer electrophoresis unit (HYTELE: 7000 CBS Scientific, Del Mar, CA). Phosphoamino acids were identified by autoradiography and identified by alignment with ninhydrin-stained standards.

**Immunoprecipitation and in vivo phosphorylation.** For experiments characterizing the immunoprecipitation, AP-1/NHE-3 cells were solubilized with RIPA buffer (containing [mM]: 150 NaCl, 80 NaF, 50 Tris-HCl, pH 8.0, 5 EDTA, 1 EGTA, 25 Na pyrophosphate, 1 Na orthovanadate; NP-40 1%[vol/vol], deoxycholate 0.5%[wt/vol], SDS 0.1%[vol/vol]) at 4°C and the solubilized proteins were separated from the membranes as a 100,000 g supernatant at 4°C. Membranes (80 μg per lane, 55,000 rpm, 30 min, 2°C). After preclearing with normal rabbit serum and protein A–Sepharose, polyonal anti–NHE-3 fusion protein antibody (#1314) was added at a 1:50 dilution and the mixture was incubated for 1 h at 4°C. The Ag–Ab complex was precipitated by incubation with protein A–Sepharose, washed with RIPA buffer, eluted with SDS buffer, resolved on SDS-PAGE, and electrotransferred to nitrocellulose membranes. The identity of the precipitated protein was checked by immunoblots using an anti–fusion protein antiserum #1314, or antipeptide antiserum #1565 or #1568. To examine the specificity of labeling by antiserum #1565 and #1568, 50 μg/ml of the appropriate immunogenic peptide was precoprecipitated with the antiserum before labeling of the nitrocellulose membranes. To examine the specificity of the immunoprecipitation, the immunoprecipitating antiserum #1314 was first saturated with 1 μg/ml of the purified bacterial fusion protein before exposure to solubilized AP-1/NHE-3 membranes containing the mammalian-expressed NHE-3.

To quantify in vivo phosphorylation of NHE-3, AP-1/NHE-3 cells were phosphate-depleted in nominally phosphate-free medium for 1 h at 37°C and then labeled with phosphate-free medium containing 330 μCi/ml 32P-orthophosphate for 3 h. After PKA activation with 0.1 mM 8-BrcAMP, cells were cooled to 4°C and NHE-3 was immunoprecipitated and size-fractionated as described above. The 32P-content of NHE-3 was quantified by overnight autoradiography. The amount of NHE-3 protein was subsequently determined by immunoblotting the same nitrocellulose membranes with anti–NHE-3 antibody as described.
above. Changes in phosphorylation status were expressed as changes in the $^{32}$P signal normalized to the antigenic signal.

**Results**

**AP-1 cells: NHE-3 activity is inhibited by acute activation of PKA.** We first examined the effect of PKA on NHE-3 in mammalian cells. To secure that the reconstituted Na/H exchanger phenotype was indeed due to NHE-3 expression, we showed that NHE-3 mRNA, protein, and Na/H exchange activity was expressed only in the AP-1/NHE-3 cells and not in the wild-type AP-1 or AP-1/pCMV5 controls (Fig. 1). RNA blots of AP-1/NHE-3 cells for NHE-1, 2, and 4 transcripts were negative (data not shown).

Fig. 2 shows the effect of PKA activation on NHE-3 activity in pooled AP-1/NHE-3 cells. When these cells were incubated with 8-Br-cAMP for 20 min, NHE-3 activity was inhibited by 39% (control: $0.65 \pm 0.05$ pH U/min, cAMP: $0.40 \pm 0.03$ pH U/min, $n = 12$, $P < 0.01$) 8-Br-cAMP had no effect on buffer capacity (control: $20.0 \pm 2.9$ mM/pH U, cAMP $18.9 \pm 3.4$ mM/pH U, $n = 4$, NS), resting cell pH (control: $7.45 \pm 0.06$ vs. cAMP: $7.41 \pm 0.08$, $n = 10$, NS), or the trough pH before Na addition (control: $6.80 \pm 0.09$ vs. cAMP: $6.77 \pm 0.08$, $n = 10$, NS). Fig. 3 shows that this change in activity was not associated with changes in NHE-3 protein abundance in whole cell lysate or plasma membrane fraction. These results indicate that degradation of existing NHE-3 is unlikely responsible for the inhibition. However, these findings are compatible with CAMP-induced covalent modification of NHE-3 leading to either internalization or inhibition of intrinsic transporter activity.

**Xenopus oocytes: inhibition of NHE-3 by acute PKA activation requires an intact cytoplasmic domain.** To confirm that PKA activation inhibits NHE-3, a second expression system, *Xenopus* oocytes were used to examine the structural requirements for acute PKA regulation. As shown in Fig. 4, oocytes treated with 8-Br-cAMP were compared with control cells (con). Molecular mass standards are shown on the left. Three independent experiments showed similar results.

**Figure 1.** Characterization of AP-1/NHE-3 cells. NHE-3 expression was studied in AP-1 cells stably transfected with the rat NHE-3 cDNA (NHE-3) and compared with wild-type AP-1 cells (WT) and control cells transfected with only pCMV5 and the neo selection marker gene (pCMV). Typical RNA blot [5 μg poly(A)$^+$ RNA/lane], immunoblot (20 μg/lane), and fluorometric functional assay (Na-dependent cell alkalization after acid load) of NHE-3 activity are shown for the three cell types.

**Figure 2.** NHE-3 activity is inhibited by PKA activation. NHE activity (pH unit/min) was assayed fluorometrically as Na-dependent cell pH recovery after acidification in AP-1/NHE-3 cells. PKA was activated by incubating cells with 8-Br-cAMP for 20 min before assay. Data represent mean $\pm$ SE ($n = 12$). $*P < 0.01$ by t test.

**Figure 3.** PKA activation does not alter abundance of NHE-3 immunoreactive protein. Immunoblots were performed with an anti-NHE-3 fusion protein antibody (#1314). Lanes 1 and 2 were from whole cell lysates. Lanes 3 and 4 were from 55,000 g plasma membrane pellets. Cyclic AMP- treated cells (cAMP) were compared with control cells (con). Molecular mass standards are shown on the left. Three independent experiments showed similar results.

**Figure 4.** NHE-3 activity is inhibited by PKA. *Xenopus* oocytes were injected with 5 ng of full-length NHE-3 cRNA and NHE activity was measured by $^{22}$Na flux 4 d later. Water-injected controls are shown on the left. For inhibitor studies, the inhibitor peptide (TYADPI-ASGRTGRRNAl) was injected into oocytes 6 h before the experiments were performed. PKA activation was achieved by incubation of oocytes with 0.1 mM 8-Br-cAMP for 20 min before uptake. Data points are means $\pm$ SD from 15–20 oocytes. Cyclic AMP-treated cells (filled bars) were compared with controls (open bars). $*P < 0.05$ by t test. Three independent sets of experiments showed similar results.
injected with the full-length NHE-3 cRNA exhibited a fivefold increase in $^{22}$Na uptake compared with water-injected controls. Addition of 0.1 mM 8-BrcAMP for 20 min caused a 20% inhibition of $^{22}$Na uptake. When oocytes were first injected with the PKA inhibitor peptide, subsequent cAMP addition did not lead to inhibition of Na uptake (Fig. 4). There was a tendency for baseline NHE-3 activity to be higher in inhibitor peptide–injected oocytes, but this finding was not consistent in every experiment.

Fig. 5 compares the activity of the full-length 831-aa NHE-3 protein with the NHE-3$_{cyto}$ 474-aa protein. The transmembrane domain of the protein alone was sufficient to induce Na uptake to levels comparable with the full-length protein. However, while acute cAMP addition inhibited Na uptake in the full-length transporter, the activity of the truncated protein was unaltered by cAMP addition (Fig. 5). These studies suggest that the cytoplasmic domain is necessary for PKA to exert its acute effect on NHE-3 activity.

NHE-3$_{cyto}$ is a substrate for PKA phosphorylation in vitro.

A possible mechanism for the above effects is phosphorylation of the NHE-3 cytoplasmic domain by PKA. We tested this hypothesis first by examining the ability of PKA-CSU to phosphorylate the cytoplasmic domain of NHE-3 in vitro. The purified fusion protein was comprised of two bands (see Fig. 8, Coomassie blue stain). Antigenically, antisera #1566 (epitope: aa 633–646) recognized both bands, whereas antisera #1568 (epitope: aa 809–822) recognized only the faster migrating band (17). This suggests that the faster migrating band is a truncated product of the full-length protein. This result was consistent over several preparations and purifications, independent of protease inhibitors, and likely represents a product of systematic premature bacterial translational termination. When the fusion protein was exposed to equimolar amounts of PKA-CSU and PKA-RSU, no phosphorylation was observed over 10 min (Fig. 6). When cAMP was then added to the reaction, phosphorylation was initiated in a time-dependent manner (Fig. 6). To demonstrate that phosphorylation of the fusion protein occurred on the NHE-3$_{cyto}$ domain of the fusion protein and not MBP, we subjected a mixture of the MBP/NHE-3$_{cyto}$ fusion protein with pure MBP to PKA-CSU in vitro (Fig. 7). Even when the fusion protein was phosphorylated to saturation, no phosphorylation was observed on MBP alone (Fig. 7). Phosphoamino acid analysis of the phosphorylated NHE-3$_{cyto}$ revealed only phosphoserines with no detectable counts on threonine or tyrosines (negative 1-wk exposure) as one would expect for the vast majority of PKA substrates (Fig. 8) (31).

NHE-3 phosphorylation is stimulated in vivo by PKA activation. Partial denaturation of proteins in solution can expose cryptic phosphorylation sites that may not be relevant in the in vivo environment. Therefore, in vitro phosphorylation by purified PKA does not establish a priori that NHE-3 is a substrate for phosphorylation by PKA in vivo. To investigate in vivo phosphorylation, we next examined the effect of cAMP on the phosphorylation status of NHE-3 in intact cells. We documented the specificity of the immunoprecipitation of NHE-3 from AP-1/NHE-3 cells in several ways. First, the anti–fusion protein antisera #1314 precipitated a single 87-kD protein which has the same mobility as NHE-3 and was labeled by the same antisera on immunoblot (Fig. 9, lane 2). Second, preimmune serum from the same animal (Fig. 9, lanes 1, 4, and 7) or nonimmune serum from control rabbits (data not shown) did not precipitate any proteins that were recognized by any of the anti–NHE-3 antisera. Third, when the immunoprecipitating antisera 1314 was first saturated with bacterially expressed MBP/NHE-3$_{cyto}$ fusion protein before it was added to solubilized membrane protein from AP-1/NHE-3 cells, the only proteins bound to the immunoglobulin was the fusion protein (Fig. 9, lane 3). Incubation with MBP did not block the precipitation (data not shown). Fourth, the identity of the precipitated protein was further established antigenically by labeling with two anti–peptide antisera (#1565 and #1568) directed against two different NHE-3 cytoplasmic epitopes (Fig. 9, lanes 5 and 8). Labeling by either antisera was blocked by preincubation with the appropriate peptide (Fig. 9, lanes 6 and 9).

We next pulsed AP-1/NHE-3 cells with $^{32}$PO$_4$ and examined
immunoprecipitated NHE-3. Control cells were compared with cells treated with permeable cAMP analogues for 20 min. Fig. 10 shows that NHE-3 exists as a phosphoprotein under baseline conditions in vivo and that activation of PKA increased the phosphocontent of NHE-3 by threefold without a change in its protein abundance.

Discussion

In the renal proximal tubule, adrenergic agonists (32–34), para-thyroid hormone (PTH) (21, 23, 35), dopamine (20, 36), angiotensin II (37–39), and endothelin (40, 41) all regulate proximal tubule NaCl and/or NaHCO₃ absorption, in part via modulation of apical membrane Na/H exchanger activity. For hormones such as dopamine and PTH, PKA activation is presumed to play a major role in mediating inhibition of Na/H exchanger activity (21–23, 35, 42). Similarly, inhibition of PKA has been postulated by some to mediate Na/H exchanger stimulation by angiotensin II (37–39) and catecholamines (32–34). Although more than one NHE isoforms may be involved, current evidence (see Introduction) suggests that NHE-3 is the predominant isoform responsible for proximal tubule apical membrane NaCl and NaHCO₃ transport. We therefore studied the effect of PKA activation on the NHE-3 isoform.

We showed functional inhibition of NHE-3 by activation of PKA in two eukaryotic expression systems. In contrast, when rabbit NHE-3 was expressed in PS120 cells, an Na/H exchanger–deficient cell line derived from Chinese hamster lung fibroblasts, cAMP addition had no effect on NHE-3 activity (43). Rabbit NHE-3 has several consensus PKA sites that are conserved with rat and opossum NHE-3 (7–9). Although both PS120 and AP-1 (Chinese hamster ovary–derived) cells are both fairly poorly differentiated cell lines, phenotypic differences between the two cell types are likely to exist and may account for the disparate results.

In Xenopus oocytes, the transmembrane domain of NHE-3 alone is sufficient to sustain Na transport. Although the cytoplasmic domain was not obligatory for Na transport, it was crucial for mediating the effect of PKA on NHE-3 activity. The 474aa NHE-3crop tended to have higher basal levels of Na uptake although this finding was not consistent in every experiment. This may reflect a variable degree of baseline PKA activity in oocytes. This modular design of respective transport and regulatory roles for the transmembrane and cytoplasmic domains has been observed in the NHE-1 isoform which shares the same predicted secondary structure with all the members of the NHE gene family (4–10). Wakabayashi et al. (44) showed that cytoplasmic domain–truncated NHE-1 mutants retained the ability to perform Na/H exchange but lost their ability to be regulated acutely by growth factors. Winkel et al. (45) blocked the endothelin and α-thrombin–induced stimulation of native Na/H exchanger activity in Chinese hamster ovary cells (likely NHE-1) by microinjecting cells with anti–NHE-1 antibodies directed against cytoplasmic domain epitopes. A piscine homologue of mammalian NHE-1 (βNHE) which shares the same predicted topology with the mammalian NHE family, is acutely stimulated by cAMP (46). A βNHE mutant with two-thirds of its cytoplasmic domain truncated retained its transport function, but was no longer activated by PKA (46, 47). In addition, human NHE-1 which is PKA insensitive can be rendered PKA sensitive if its cytoplasmic domain is replaced by the βNHE cytoplasmic domain (48).

In the present study, differences in AP-1/NHE-3 plasma membrane NHE-3 protein abundance cannot account for the 40% inhibition of NHE-3 activity induced by cAMP. This finding is consistent with the hypothesis of inhibition of existing NHE-3 transporters by acute phosphorylation. Alternatively, acute phosphorylation can lead to endocytosis of surface NHE-3. The present data cannot definitively distinguish these two possibilities. We showed that recombinant rat NHE-3 cytoplasmic domain was a direct substrate for purified PKA in vitro. Rat NHE-3 cytoplasmic domain contains numerous putative PKA consensus motifs. Three of these sites at Ser275, Ser409, and Ser640 are conserved in rat, rabbit, and opossum NHE-3 (7–9). Neither the presence of consensus sequences nor direct in vitro PKA phosphorylation per se proves unequivocally that NHE-3 is a direct substrate for PKA in vivo. However, collectively they are highly suggestive of phosphorylation of NHE-3 by PKA in vivo. In the Na-K-ATPase α subunit, GLUT-4 glucose transporter, and CFTR, most putative PKA consensus sites are phosphorylated in vitro by purified PKA as well as in vivo by cAMP addition (49–53). We demonstrated that NHE-3 exists as a phosphoprotein and PKA activation increased its net

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**Figure 7.** PKA phosphorylates NHE-3crop and not MBP. A mixture of MBP/NHE-3crop fusion protein and purified MBP was subjected to phosphorylation by PKA-CSU for 30 min and resolved by SDS-PAGE. The left lane shows the Coomassie-stained proteins and the right lane shows the autoradiograph.

**Figure 8.** PKA phosphorylates NHE-3 in vitro on serine residues. MBP/NHE3crop fusion protein was phosphorylated by PKA-CSU in vitro, digested with HCl and 32P-labeled phosphoamino acids were resolved by two-dimensional electrophoresis as described in Methods. Migration positions of phosphoamino acid standards are indicated. Three independent experiments showed similar results.

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phosphorylation status in intact cells. The in vivo data do not distinguish whether NHE-3 is a direct substrate for PKA in vivo, whether NHE-3 is phosphorylated via activation of other kinases downstream from PKA, or both.

Increase in NHE-3 phosphocontent paralleled inhibition of NHE-3 activity with application of cAMP. This is suggestive, but by itself does not sustain the conclusion that phosphorylation is the sole and direct cause for inhibition. One sees a clear-cut situation in the α subunit of Na-K-ATPase where mutation of the highly conserved PKA-phosphorylated serine residue seemed to entirely abate functional regulation of pump activity by PKA (51). In other instances, the situation is more complex. Although serum induces acute phosphorylation of NHE-1 (54, 55), the identities of the phosphorylated residues are still unknown. Cytoplasmic deletion from amino acid 635 onwards in NHE-1 removed all major growth factor–induced phosphorylation sites but only led to a partial reduction of growth factor response (44). Conversely, internal deletion of amino acids 567–635 appeared to abolish growth factor regulation but did not affect phosphopeptide patterns (56). Point mutation of two PKA consensus sites in β(NHE only partially abrogated PKA sensitivity, while total PKA insensitivity was achieved with a further internal deletion of a cytoplasmic fragment (48). When all 10 PKA consensus sites of CFTR were empirically point-mutated, PKA regulation remained intact (57). Total abolition of PKA stimulation of CFTR Cl channel activity was accomplished in a quadruple mutant of the four in vivo PKA sites of CFTR using a combination of point mutations and internal deletions (58).

We speculate that acute PKA regulation of NHE-3 will likely require intact PKA consensus sites as well as other regulatory regions of NHE-3. Weinman et al. (59, 60) had purified a 42–55-kD phosphoprotein from renal cortical brush border membranes and postulated that it is a cofactor for PKA regulation of brush border Na/H exchanger activity. When renal corti-

Figure 9. Characterization of immunoprecipitated NHE-3. AP-1/ NHE-3 cells were solubilized and NHE-3 was immunoprecipitated by the antiserum indicated in the bottom of the figure and the precipitated protein was analyzed by immunoblot by antiserum indicated on the top of the figure. Mobility of the bacterially expressed MBP/NHE-3 fusion protein and the mammalian-expressed NHE-3 are indicated on the left hand side. 1314 + fusion protein, antiserum 1314 saturated with 2 μg/ml of bacterial fusion protein; 1566 + peptide and 1568 + peptide, antiserum preincubated with 50 μg/ml of the corresponding immunopeptide.

Figure 10. PKA induces NHE-3 phosphorylation in AP-1 cells. AP-1/ NHE-3 cells were labeled with 32P-orthophosphate and PKA was activated by 0.1 mM 8-BrcAMP. NHE-3 was immunoprecipitated with antiserum 1314, resolved on SDS-PAGE, and blotted to nitrocellulose filters. After quantitation of phosphocontent by autoradiography (32P AUTORAD), the same membrane was probed with anti–NHE-3 antiserum 1568 to quantitate immunoreactive NHE-3 (IMMUNOBLOT). Three independent experiments showed similar results.
cal apical membranes were immunodepleted of this cofactor, in vitro activation of PKA no longer inhibited apical membrane NHE activity (59–61). Since our data do not prove direct phosphorylation of NHE-3 by PKA in vivo, our findings do not contradict those of Weimann et al. (59–61). Regulation of NHE-3 by PKA may require phosphorylation of NHE-3 in addition to binding and/or phosphorylation of regulatory cofactors. Although the regulatory cofactor described by Weimann tends to have an epithelial distribution, it is possible that other related regulatory factors are present in AP-1 cells and oocytes.

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