Role of dipeptidyl peptidase IV in regulating activity of Na\(^+\)/H\(^+\) exchanger isoform NHE3 in proximal tubule cells

Adriana C. C. Girardi, Felix Knauf, Hans-Ulrich Demuth, and Peter S. Aronson

Department of Internal Medicine and Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520-8029; and Probiodrug AG, Halle (Saale) D-06120, Germany

Submitted 12 April 2004; accepted in final form 21 June 2004

Girardi, Adriana C. C., Felix Knauf, Hans-Ulrich Demuth, and Peter S. Aronson. Role of dipeptidyl peptidase IV in regulating activity of Na\(^+\)/H\(^+\) exchanger isoform NHE3 in proximal tubule cells. Am J Physiol Cell Physiol 287: C1238–C1245, 2004.—We recently reported that NHE3 exists in multimeric complexes with dipeptidyl peptidase IV (DPPIV) in renal brush-border membranes. To examine the possible role of DPPIV in modulating NHE3 activity, we evaluated whether specific competitive inhibitors that bind to the active site of DPPIV affect NHE3 activity in the OKP line of opossum kidney proximal tubule cells. The DPPIV inhibitors diprotin A and P32/98 significantly reduced NHE3 activity, whereas the inactive isomer P34/98 had no effect. DPPIV inhibitors did not reduce the activity of another brush-border transport process, Na-phosphate co-transport. Effects of DPPIV inhibitors on NHE3 activity were not associated with detectable changes in amount or apparent molecular weight of NHE3 or in NHE3 surface expression. To investigate the signaling mechanisms involved in modulation of NHE3 activity by DPPIV, we used inhibitors of protein kinase pathways known to regulate NHE3. Whereas the PKA inhibitor H-89 failed to block the effect of DPPIV inhibitors, the tyrosine kinase inhibitor genistein alone caused a decrement in NHE3 activity similar in magnitude to that caused by P32/98. We also found that the effects of genistein and P32/98 on NHE3 activity were not additive. In contrast, forskolin/IBMX and P32/98 had additive inhibitory effects on NHE3 activity. These findings suggested that the effect of DPPIV inhibitors to reduce NHE3 activity results from inhibition of a tyrosine kinase signaling pathway rather than by activation of PKA. We conclude that DPPIV plays an unexpected role in modulating Na\(^+\)/H\(^+\) exchange mediated by NHE3 in proximal tubule cells.

sodium/hydrogen exchange; diprotin A; P32/98; tyrosine kinase

**NA\(^+\)/H\(^+\)** _exchangers_ (NHEs) are integral membrane proteins that mediate the electroneutral exchange of intracellular protons for extracellular sodium (50). The transport activity of this protein is crucial to regulation of intracellular pH and cellular volume. In polarized epithelia, Na\(^+\)/H\(^+\) exchangers are also involved in transepithelial NaHCO\(_3\) and NaCl transport. Na\(^+\)/H\(^+\) exchange is inhibited by amiloride and its analogs, although the inhibitory potency of these drugs varies significantly among the isoforms (15, 37).

To date, five Na\(^+\)/H\(^+\) exchanger isoforms (NHE1, NHE2, NHE3, NHE4, and NHE8) have been identified to be expressed on the plasma membrane of renal tubular cells (5, 6, 9, 10, 19). Of these, NHE3 is expressed on the apical membrane of cells in the proximal tubule and the loop of Henle (3, 5, 7). Studies using inhibitors and knockout mice have demonstrated that NHE3 is responsible for the majority of apical membrane Na\(^+\)/H\(^+\) exchange activity and transepithelial NaHCO\(_3\) and volume reabsorption in the proximal tubule (12, 30, 48, 51, 52, 55). The exquisite regulation of NHE3 activity is therefore essential for the maintenance of sodium, acid-base, and fluid homeostasis.

C1238

0363-6143/04 $5.00 Copyright © 2004 the American Physiological Society

http://www.ajpcell.org

METHODS

Materials. Reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified. OKP cells, a clonal subline of the opossum kidney cell line originally described by Cole et al. (13), were provided by Dr. Orson Moe (University of Texas Southwestern Medical School, Dallas, TX). Dulbecco’s modified Eagle’s medium (DMEM), heat-inactivated fetal bovine serum, sodium pyruvate, and penicillin/streptomycin were purchased from Life Technologies (GIBCO BRL, Gaithersburg, MD). 22Na and [32P]phosphoric acid were obtained from NEN Life Science (Boston, MA). EZ-Link sulfo-NHS-SS-biotin as well as immunopure immobilized streptavidin were purchased from Pierce (Rockford, IL). Monoclonal antibodies directed to opossum NHE3 (3H3) and megalin (6A6) were generously provided by Dr. Daniel Biemesderfer (Yale School of Medicine, New Haven, CT). A polyclonal antibody (rabbit IgG) to DPPIV/CD26 was purchased from Santa Cruz Biotechnology (Lake Placid, NY). DPPIV inhibitors were obtained as follows: diprotin A (Ile-Pro-Ile) (46) was purchased from Bachem (Philadelphi, PA); P32/98 (isoleucyl thiazolidine fumarate) (38) and its inactive isomer, P34/98 (o-isoleucyl thiazolidine fumarate), were obtained from Probiodrug (Halle (Saale), Germany).

Cell culture. OKP cells were maintained in 75-cm² tissue culture flasks in DMEM containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were incubated at 37°C in a humidified 5% CO₂-95% air atmosphere. Cells were subcultured using Ca²⁺/Mg²⁺-free phosphate-buffered saline and 0.25% trypsin-EDTA. The medium was replaced every 2 days. For experiments, cells were seeded onto tissue culture plates and were used 2 days after reaching 100% confluence.

Immunoprecipitation. OKP cells grown in six-well plates were solubilized at 4°C in Tris-buffered saline (TBS) buffer (pH 7.4) containing 1% Triton X-100 and the protease inhibitors pepstatin A (0.7 µg/ml), leupeptin (0.5 µg/ml), and PMSF (40 µg/ml). The samples were subjected to centrifugation (15,000g for 10 min) using a table-top centrifuge (Hermle model Z230M; National Labnet, Woodbridge, NJ). Primary antibodies (50 µg) were added to the supernatants, and the samples were incubated at 4°C for 1 h. Immune complexes were collected using 5 µg/sample of protein G-Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ). The beads were washed five times in solubilization buffer and then prepared for SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblotting. Protein samples were solubilized in SDS sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.1% bromophenol blue, and 50 mM Tris, pH 6.8), and proteins were separated by SDS-PAGE using 7.5% polyacrylamide gels according to the method reported by Laemmli (27). Immunoblotting was performed as described previously (18).

DPPIV enzymatic assay. DPPIV activity was assayed in OKP cells grown in 24-well plates by measuring the release of p-nitroaniline resulting from the hydrolysis of glycyrlprolyl-p-nitroanilide tosylate (21). Cells were incubated with 2 mM glycyrlprolyl-p-nitroanilide tosylate in phosphate-buffered saline (PBS) buffer (pH 7.4) for 30 min at 37°C. Reaction was terminated by the addition of 1 M acetate buffer (pH 4.2). Determination of p-nitroaniline liberated enzymatically was based on measuring absorbance at 380 nm. One unit of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 µM p-nitroaniline per minute under the conditions described.

Sodium uptake assays. 22Na uptake assays were performed in 24-well plates in which OKP cells were preincubated for 20 min at room temperature in NH₄⁺ loading buffer containing 30 mM NH₄Cl, 90 mM choline chloride, 5 mM HEPES-Tris, pH 7.4. The NH₄⁺ loading buffer was then removed, and cells were incubated for 5 min at room temperature with an NH₄⁺-free solution containing 1 µCi/ml 22Na and 1 mM NH₄Cl, 120 mM choline chloride, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, and 20 mM HEPES-Tris, pH 7.4. The NH₄⁺ loading buffer was then removed, and cells were incubated for 5 min at room temperature with an NH₄⁺-free solution containing 1 µCi/ml 22Na and 1 mM NH₄Cl, 120 mM choline chloride, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, and 20 mM HEPES-Tris, pH 7.4. Uptake was terminated by washing cells three times with ice-cold radiouclide-free NH₄⁺-free buffer (pH 7.4). The cell monolayers were solubilized in 0.2 ml of 0.2 M NaOH and neutralized by adding 0.2 ml of 0.2 M HCl. Aliquots from each well were aspirated into a scintillation vial, and 22Na content was analyzed by liquid scintillation spectroscopy. Nonspecific retention (time 0 value) of 22Na uptake was determined and subtracted from the values for the incubated samples. All test compounds used in our studies were present during both the 20-min preincubation period and the 5-min Na uptake period. When the PKA inhibitor N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide (H-89) was used, it was preincubated with cells for an additional 30-min period before NH₄⁺ loading.

Phosphate uptake assays. Sodium-dependent uptake of phosphate was measured in OKP cells grown to confluence on 24-well plates as described by Reshkin et al. (40). Briefly, cells were preincubated with a sodium-free medium (137 mM tetramethylammonium chloride, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgSO₄, 0.1 mM KH₂PO₄, and 10 mM HEPES-Tris, pH 7.4) at room temperature for 20 min. Measurement of phosphate uptake was initiated by adding transport medium containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgSO₄, 0.1 mM KH₂PO₄, 10 mM HEPES-Tris, pH 7.4, and [32P]phosphoric acid (2 µCi/ml) at room temperature. Uptake was terminated after 10 min by aspirating the transport medium and washing the cells with ice-cold sodium-free medium. Radioisotopic activity was determined by liquid scintillation spectroscopy. Nonspecific retention of 32PO₄⁻ was determined and subtracted from the values for the incubated samples.

Cell surface biotinylation. OKP cells grown to confluence in six-well plates were serum starved for 24–48 h and subsequently incubated for 30 min at 37°C with either DPPIV inhibitors or diluent. All of the following manipulations were performed at 4°C. Cells were washed twice with PBS containing 0.1 mM CaCl₂ and 1.0 mM MgCl₂ (PBS-Ca-Mg). The surface membrane proteins were then biotinylated by incubating the cells twice for 5 min with 2 ml of biotinylation buffer (150 mM NaCl, 10 mM triethanolamine, 2 mM CaCl₂, and 2 mg/ml EZ-Link sulfo-NHS-SS-biotin). The cells were washed twice for 20 min with a quenching buffer (PBS-Ca-Mg/100 mM glycine) and then solubilized for 1 h by adding a buffer containing 125 mM potassium acetate, 25 mM HEPES, 15 mM sodium pyrophosphate, 1% Triton X-100, 0.7 µg/ml pepstatin A, 0.5 µg/ml leupeptin, and 40 µg/ml PMSF, pH 7.4. The samples were centrifuged at 15,000 g for 10 min, and 50 µl of streptavidin-coupled agarose were added to the supernatants. After 1 h of incubation, the beads were washed five times in solubilization buffer and then prepared for SDS-PAGE and immunoblotting.

Analysis of protein tyrosine phosphorylation. Tyrosine kinase activity was measured in the OKP-DPPIV immune complex by Western blotting using an anti-phosphotyrosine antibody (17). OKP cells grown to confluence in six-well plates were incubated at 37°C for 20 min with NH₄⁺ loading buffer. Cells were solubilized in 1 ml of ice-cold buffer containing 20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM diethiothreitol, 1 mM EDTA, 100 mM sodium vanadate, 0.7 µg/ml pepstatin A, 0.5 µg/ml leupeptin, and 40 µg/ml PMSF. Immunoprecipitation with anti-DPPIV was then performed as described above. The immune complexes were solubilized by addition of 100 µl of hot Laemmli sample buffer and boiled for 5 min. Samples were resolved by SDS-PAGE using 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and then blocked with TBS buffer containing 5% albumin. Immunoblotting was performed by an overnight incubation with anti-phosphotyrosine monoclonal antibody (4G10) diluted 1:2,000.

Statistical analysis. All results are reported as means ± SE. Comparisons between two groups were performed with unpaired t-tests. Differences among multiple groups were evaluated by analysis of variance with post hoc Tukey’s test. A P value <0.05 was considered significant.
RESULTS

Modulation of NHE3 activity by DPPIV inhibitors in OKP cells. We previously demonstrated that the brush border Na\(^+\)/H\(^+\) exchanger NHE3 associates with DPPIV in rabbit renal proximal tubule (18). DPPIV, also known as CD26, is a multifunctional protein that, in addition to its proteolytic properties, acts as a binding protein and signaling molecule. The emerging evidence for regulation of transporters by proteases led us to investigate whether DPPIV modulates NHE3.

For our experiments we used OKP cells, a line of opossum proximal tubule cells that has transport properties very similar to those of the native mammalian proximal tubule (13). We first verified whether NHE3 exists in protein complexes with DPPIV in OKP cells. Figure 1 shows the results of an experiment in which solubilized OKP cell proteins were immunoprecipitated with a commercial antibody to DPPIV (42). The immune complexes were prepared for immunoblotting and probed with antibodies against DPPIV, NHE3, and megalin. All three proteins were detected in the OKP cell lysate. DPPIV and NHE3 were coprecipitated by the anti-DPPIV antibody. In contrast, anti-DPPIV did not coprecipitate megalin, confirming the specificity of the NHE3-DPPIV interaction. This observation is in agreement with our previous studies performed in rabbit proximal tubule that revealed that the pools of NHE3 complexed with DPPIV and megalin are largely distinct (18).

To examine the possible role of DPPIV in modulating NHE3, we evaluated whether specific inhibitors that bind to the active site of DPPIV affect NHE3 activity in OKP cells. The following inhibitors were used: diprotin A (H-Ile-Pro-Ile-OH), P32/98, and its inactive optical isomer, P34/98, as a negative control. Table 1 shows the concentration dependence for inhibition of DPPIV enzymatic activity in OKP cells. We found that maximal (>99%) inhibition of DPPIV activity in OKP cells was achieved by 1 mM diprotin A and 10 \(\mu\)M P32/98. As expected, P34/98 did not affect DPPIV catalytic ability.

We next evaluated whether the concentration of each inhibitor that maximally affected DPPIV enzymatic activity would affect NHE3 transport activity. NHE3 activity was assayed as the effect of DPPIV inhibitors on EIPA-sensitive component of \(^{22}\)Na uptake. Each assay was performed in triplicate, and values are means ± SE calculated from 6 assays. *\(P < 0.05\) vs. control.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Specific Activity, U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>387±30</td>
</tr>
<tr>
<td>Diprotin A</td>
<td>141±12*</td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>28±4*</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>2.1±0.5*</td>
</tr>
<tr>
<td>10 mM</td>
<td>1.9±0.3*</td>
</tr>
<tr>
<td>P32/98</td>
<td>27±5*</td>
</tr>
<tr>
<td>10 (\mu)M</td>
<td>0.8±0.3*</td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>0.5±0.1*</td>
</tr>
<tr>
<td>P34/98</td>
<td>399±33</td>
</tr>
<tr>
<td>10 (\mu)M</td>
<td>365±29</td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>345±42</td>
</tr>
</tbody>
</table>

Values for specific activity are means ± SE from 3 experiments. OKP cell monolayers were incubated with increasing concentrations of dipeptidyl phosphatase IV (DPPIV) inhibitors for 30 min at 37°C. DPPIV activity was assayed in triplicate. *\(P < 0.05\) compared with control.
22Na uptake in the absence or presence of 1 mM diprotin A, 10 μM P32/98, and 10 μM P34/98 after OKP cells were acid loaded using the ammonium prepulse technique. As shown in Fig. 2A, the control rate of 22Na uptake measured under these conditions was inhibited 90% by 100 μM 5-(N-ethyl-N-isopropyl)amiloride (EIPA), consistent with NHE3 activity. Both diprotin A and P32/98 significantly inhibited 22Na uptake. In contrast, the inactive isomer P34/98 did not affect 22Na influx. None of the inhibitors affected the small component of 22Na uptake that was EIPA insensitive. The effects of the inhibitors on the EIPA-sensitive component of 22Na uptake are shown in Fig. 2B, which indicates that both diprotin A and P32/98 inhibited NHE3 activity by ~40%.

We then examined the concentration dependence of the effects of the DPPIV inhibitors on NHE3 activity to allow correlation with inhibition of DPPIV enzymatic activity. The effects of increasing concentrations of diprotin A, P32/98, and P34/98 on EIPA-sensitive uptake of 22Na after an acid load are shown in Fig. 3. The dose-response curves for inhibition of NHE3 activity correlate with the effects of the inhibitors on DPPIV catalytic activity in that P32/98 is ~100-fold more potent than diprotin A for inhibiting both DPPIV activity and NHE3 activity. However, there were two striking differences between the dose-response curves for inhibition of DPPIV activity and NHE3 activity. First, maximum inhibition of NHE3 activity reached a plateau of ~45%, whereas DPPIV activity could be completely inhibited. This finding indicates that only a fraction of NHE3 activity is dependent on DPPIV activity. Second, the dose-response curves for inhibition of NHE3 activity by diprotin A and P32/98 are shifted to the right by a factor of 10 compared with those for inhibition of DPPIV activity. For example, concentrations of diprotin A (1 μM) and P32/98 (0.01 μM) that caused >30% inhibition of DPPIV activity caused no detectable inhibition of NHE3 activity. These findings indicate that only a small fraction of residual DPPIV activity is required to fully stimulate the component of NHE3 activity that is dependent on DPPIV activity. Thus near maximal inhibition of DPPIV activity is required to reduce NHE3 activity. This is qualitatively analogous to the dependence of proximal tubule bicarbonate reabsorption on carbonic anhydrase activity, in which case enzyme activity must be inhibited >99% to observe a significant physiological effect (32).

To verify the specificity of the effect of DPPIV inhibitors on NHE3 activity, we evaluated whether these agents would affect the activity of another brush-border membrane sodium-dependent transporter, the Na-Pi cotransporter, which we had previously found not to be associated with DPPIV (18). Na-Pi cotransporter activity was measured as 32P-radiolabeled phosphate uptake in the presence of sodium. As shown in Fig. 4A,
phosphate uptake was significantly stimulated by the presence of sodium, consistent with Na-Pi cotransporter activity. Neither diprotin A nor P32/98 inhibited phosphate uptake measured in either the presence or absence of sodium. As shown in Fig. 4B, no detectable difference was observed in the sodium-dependent component of radiolabeled phosphate uptake in the presence of DPPIV inhibitors. Taken together, the results shown in Figs. 2, 3 and 4 indicate that DPPIV inhibitors specifically affect the activity of NHE3 in OKP cells and that the catalytic site of DPPIV is directly involved in NHE3 regulation.

Effect of DPPIV inhibitors on stability of the NHE3-DPPIV complex. As the first approach to investigate the mechanism by which DPPIV inhibitors affect NHE3 activity, we sought to examine whether binding of inhibitors to the DPPIV catalytic site would affect the stability of the NHE3-DPPIV complex. To test this, we treated OKP cells with the high-affinity DPPIV inhibitor P32/98 (10 μM), its inactive optical isomer P34/98 (10 μM), or vehicle. Cells were then immunoprecipitated with an anti-DPPIV antibody, and the immune complexes as well as samples of starting material were analyzed for the presence of NHE3 by Western blotting. As shown in Fig. 5, the abundance of NHE3 that was coprecipitated by anti-DPPIV was essentially the same in the presence of the DPPIV inhibitor P32/98 compared with control. The P32/98 inactive isomer P34/98 also had no effect on NHE3 abundance. This result indicates that binding of inhibitors to the DPPIV catalytic site does not affect its association with NHE3. Therefore, DPPIV inhibitors reduce NHE3 activity by a mechanism that does not involve disruption of the NHE3-DPPIV complex.

Effect of DPPIV inhibitors on NHE3 abundance and subcellular distribution. We next investigated whether the effect of DPPIV inhibitors to reduce NHE3 activity is due to a change in either total or surface NHE3 expression. Figure 6A displays the results of a typical Western blot analysis of total cell lysate. Total NHE3 protein expression did not change when these cells were treated with 10 μM P32/98. Figure 6A also shows that DPPIV does not seem to be involved in processing of NHE3, because there was no detectable shift in apparent molecular weight of NHE3 when cells were treated with DPPIV inhibitor.

In OKP cells, it has been well documented that NHE3 is regulated by trafficking between intracellular pools and the apical surface (14). Following protocols for surface biotinylation of NHE3 that have been successfully adapted to OKP cells (2, 56), we examined whether NHE3 surface expression would be altered by DPPIV inhibition. Surface proteins were biotinylated and precipitated with streptavidin. The precipitate was then probed for NHE3 by immunoblotting. As shown in Fig. 6B, DPPIV inhibition did not change NHE3 surface expression. On the basis of the results shown in Fig. 6, we conclude that DPPIV inhibitors do not alter either the total amount or apparent molecular weight of NHE3 or change NHE3 surface expression.

Role of protein kinases in mediating the effect of DPPIV inhibitors on NHE3 activity. The signal transduction cascade mediating the acute effect of NHE3 agonists and antagonists involves multiple pathways. Given that cAMP is one of the major intracellular messengers mediating inhibition of NHE3 in OKP cells (8, 54), we examined whether NHE3 modulation by DPPIV inhibitors would occur through activation of this second messenger system. The involvement of a cAMP/PKA-mediated pathway in the regulation of NHE3 by DPPIV inhibitors was examined by pretreating OKP cells for 30 min with the specific PKA inhibitor H-89. OKP cells were then incubated with 100 μM forskolin/1.0 mM IBMX, 10 μM P32/98, or vehicle. NHE3 transport activity was then measured. As expected, exposure to 10 μM H-89 completely blocked the inhibitory effect of forskolin/IBMX on Na+/H+ exchange (Fig. 7). However, 10 μM H-89 did not attenuate the effects of DPPIV inhibitors on NHE3 activity in OKP cells, indicating that PKA does not mediate the effect of DPPIV inhibitors.

Several studies have demonstrated that tyrosine kinase signaling pathways can stimulate NHE3 activity (26, 45, 53). We
then considered the possibility that the effect of DPPIV inhibitors to reduce NHE3 activity in OKP cells might arise from inhibition of tyrosine kinase signaling. To address this issue, we tested whether the tyrosine kinase inhibitor genistein would reduce NHE3 activity in OKP cells and, if so, whether its effect would be additive to that of the DPPIV inhibitor P32/98. As shown in Fig. 8, genistein caused a decrement in NHE3 activity very similar in magnitude to that caused by P32/98, and the effects of P32/98 and genistein were not additive. In contrast, as also shown in Fig. 8, P32/98 and forskolin/IBMX produced an additive inhibitory effect on NHE3 activity, consistent with our previous conclusion that DPPIV inhibitors inhibit NHE3 activity via a PKA-independent mechanism.

To further investigate whether DPPIV inhibitors actually decrease cell tyrosine kinase activity in OKP cells and thereby mimic the effect of genistein, we analyzed the content of tyrosine-phosphorylated proteins present in the DPPIV immune complex from OKP cells. Cells incubated in presence of genistein, P32/98, or vehicle were solubilized, and the resulting supernatant was immunoprecipitated with anti-DPPIV. The phosphorylation on tyrosine residues of DPPIV-associated proteins was analyzed by immunoblotting with an anti-phospho-tyrosine MAb. As shown in Fig. 9, genistein and P32/98 each induced a significant decrease in the tyrosine phosphate residues of a single protein with a high apparent molecular mass (~212 kDa). As expected, addition of the phosphatase inhibitor pervanadate enhanced the levels of tyrosine phosphorylation of this protein compared with control. The findings shown in Fig. 9 provide direct evidence that DPPIV inhibitors affect tyrosine kinase signaling in OKP cells. Finally, it may be noted that tyrosine phosphorylation of NHE3 itself was not detected in the anti-DPPIV immunoprecipitate.

DISCUSSION

We have used two highly specific DPPIV inhibitors to examine the role of this peptidase in regulating the activity of NHE3 in OKP cells. As mentioned earlier, DPPIV is a serine protease that cleaves NH₂-terminal dipeptides from proteins with a penultimate proline or alanine residue (16, 25). Diprotin A (Ile-Pro-Ile) is a tripeptide substrate that binds in the active site of DPPIV but is only slowly cleaved (39, 43). Based on the
structural similarity of thiazolidine to proline, P32/98 (isoleucine-thiazolidine) is a dipeptide product analog with high affinity for binding to the active site and inhibiting DPPIV (38, 41). Dpiprotin A and P32/98 are respectively 10 and 80 times more potent for inhibiting DPPIV than for inhibiting the functionally related proline-specific protease DPPII (29, 41).

We now report that these two DPPIV inhibitors significantly decrease NHE3 activity in OKP cells, suggesting that the catalytic site of this peptidase has a role in modulating NHE3. Previously documented mechanisms that mediate the acute regulation of NHE3, including alteration of surface expression and phosphorylation by PKA, do not appear to be involved in the downregulation of NHE3 by DPPIV inhibitors. In addition, we could not detect any change in the apparent molecular weight of NHE3 resulting from possible DPPIV-mediated proteolysis of the transporter. However, we have found that the decrement in NHE3 activity induced by the DPPIV inhibitor P32/98 is not additive with that caused by the tyrosine kinase inhibitor genistein, suggesting that DPPIV inhibitors may affect NHE3 by a tyrosine kinase signaling pathway.

It is therefore of interest that DPPIV (CD26) regulation of cell proliferation and cytokine production in lymphocytes has been associated with changes in tyrosine kinase signaling (20, 22–24, 36). Indeed, it has been specifically demonstrated that DPPIV inhibitors affect tyrosine phosphorylation of multiple proteins in lymphocytes (23, 24). Similarly, we found that the DPPIV inhibitor P32/98 decreased tyrosine phosphorylation of a high apparent molecular mass protein (>212 kDa) that coprecipitates with DPPIV, indicating a role for DPPIV in regulating tyrosine kinase signaling in OKP cells.

The molecular mechanisms by which DPPIV inhibitors affect protein tyrosine phosphorylation in lymphocytes or other cell types are not known. The cDNA sequence of DPPIV predicts a type II membrane protein, which is anchored to the cell surface by a single hydrophobic segment and has a short cytoplasmic region consisting of only six amino acids (42). Therefore, DPPIV is very unlikely to signal by itself. One possibility is that binding of DPPIV inhibitors to the catalytic site results in a conformational change that, in turn, affects tyrosine kinase signaling mediated by associated proteins. For instance, in T lymphocytes, DPPIV interacts with CD45 (22, 44), a tyrosine phosphatase that plays an important role in T cell activation. Another possibility is that DPPIV inhibitors affect cell signaling by blocking DPPIV catalytic activity. Known substrates for DPPIV include cytokines, chemokines, growth factors, and hormones (28). Processing by DPPIV activity can lead to either activation of proforms or peptide degradation. Thus DPPIV catalytic site inhibitors could alter cell signaling and protein phosphorylation by blocking DPPIV-mediated activation or inactivation of peptide ligands that bind to cell surface receptors.

In summary, we have found that DPPIV inhibitors significantly decrease NHE3 activity in OKP cells, most likely by inhibiting a tyrosine kinase signaling pathway. Thus our studies reveal an unexpected role for DPPIV in modulating NHE3 activity in proximal tubule cells.

REFERENCES
19. Hegen M, Kameoka J, Dong RP, Schlossman SF, and Morimoto C. Cross-linking of CD26 by antibody induces tyrosine phosphorylation and

GRANTS
This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-33793 and DK-17433 (to P. S. Aronson) and a Postdoctoral Research Fellowship from the American Heart Association (to A. C. C. Girardi).


