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Activation of dopamine D₁-like receptors induces acute internalization of the renal Na⁺/phosphate cotransporter NaPi-IIa in mouse kidney and OK cells

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Abstract

The Na⁺/phosphate cotransporter NaPi-IIa (SLC34A1) is the major transporter mediating the reabsorption of P_i in the proximal tubule. Expression and activity of NaPi-IIa is regulated by several factors, including parathyroid hormone, dopamine, metabolic acidosis, and dietary P_i intake. Dopamine induces natriuresis and phosphaturia in vivo, and its actions on several Na⁺-transporting systems such as NHE3 and Na⁺-K⁺-ATPase have been investigated in detail. Using freshly isolated mouse kidney slices, perfused proximal tubules, and cultured renal epithelial cells, we examined the acute effects of dopamine on NaPi-IIa expression and localization. Incubation of isolated kidney slices with the selective D₁-like receptor agonists fenoldopam (10 μM) and SKF-38393 (10 μM) for 1 h induced NaPi-IIa internalization and reduced expression of NaPi-IIa in the brush border membrane (BBM). The D₂-like selective agonist quinpirole (1 μM) had no effect. The D₁ and D₂ agonists did not affect the renal Na⁺/sulfate cotransporter NaSi in the BBM of the proximal tubule. Studies with isolated perfused proximal tubules demonstrated that activation of luminal, but not basolateral, D₁-like receptors caused NaPi-IIa internalization. In kidney slices, inhibition of PKC (1 μM chelerythrine) or ERK1/2 (20 μM PD-098089) pathways did not prevent the fenoldopam-induced internalization. Inhibition with the PKA blocker H-89 (10 μM) abolished the effect of fenoldopam. Immunoblot demonstrated a reduction of NaPi-IIa protein in BBMs from kidney slices treated with fenoldopam. Incubation of opossum kidney cells transfected with NaPi-IIa-green fluorescent protein chimera shifted fluorescence from the apical membrane to an intracellular pool. In summary, dopamine induces internalization of NaPi-IIa by activation of luminal D₁-like receptors, an effect that is mediated by PKA.

Keywords

protein kinase A; proximal tubule; brush border membrane

Phosphate reabsorption in the renal proximal tubule is a major mechanism contributing to the maintenance of overall P_i homeostasis. Two renal Na^+ /phosphate cotransporters, NaPi-IIa (SLC34A1, also named Npt2) and NaPi-IIc, are expressed on the brush border membrane (BBM) of the proximal tubule, mediating the reabsorption of filtered phosphate from urine (28–30, 34). The massive phosphaturia observed in NaPi-IIa-deficient mice supports the notion that NaPi-IIa is the major Na^+ /phosphate cotransporter (9, 29).

Renal P_i reabsorption is regulated by a variety of factors, including dietary P_i intake, metabolic acidosis, and several hormones, such as parathyroid hormone (PTH), growth factors, and dopamine (1, 6, 18, 21, 22, 29, 30, 33). PTH represents one of the most powerful phosphaturic hormones, and its action and signaling pathways leading to NaPi-IIa internalization and subsequent lysosomal degradation have been investigated in some detail (6, 30, 33, 36, 37).

Several studies have demonstrated the potent natriuretic and phosphaturic effect of dopamine and its analogs in vivo and in cell culture models (13, 17, 31). Dopamine induces natriuresis by inhibiting Na^+ transport in the proximal tubule and in the thick ascending limb on NaCl reabsorption and in cells derived from the collecting duct by inhibition of Na^+ - K^+ -ATPase activity (8, 19, 35). The phosphaturic effect of dopamine and its interaction with dietary phosphorus and PTH have been documented extensively in different animal models (10, 13, 15, 17, 18, 21, 22, 24). In these experiments, phosphaturia was elicited by dopamine and by D_1 -like receptor agonists and prevented by D_1 -like receptor antagonists. In addition, experiments using the proximal tubule cell-like opossum kidney (OK) cell culture model have demonstrated that dopamine and D_1 -like receptor agonists inhibit Na^+ /phosphate cotransport activity, most likely through a cAMP- and PKA-dependent pathway (7, 25, 32). Thus available data suggest that dopamine reduces proximal tubular Na^+ -dependent P_i reabsorption via D_1 -like receptors and that this effect may be mediated via the cAMP-PKA pathway.

The aim of the present study was to examine the effect of dopamine on the expression and localization of the major Na^+ /phosphate cotransporter NaPi-IIa. We used freshly isolated mouse kidney slices to demonstrate that D_1 -like receptor agonists induce acute internalization of NaPi-IIa from the BBM, as evident from a reduced abundance of NaPi-IIa protein. Isolated perfused mouse proximal tubules further showed that the effect of dopamine is via luminal D_1 -like receptors. The effect of D_1 -like receptor agonists was blunted in the presence of an inhibitor of the cAMP-PKA pathway, but not by inhibitors of the PKC or the ERK1/2 pathway. Studies using a fluorescent-tagged NaPi-IIa in OK cells further confirmed the effect of dopamine. Thus our results demonstrate that dopamine induces internalization of NaPi-IIa via luminal D_1 -like receptors involving the cAMP-PKA pathway.

MATERIALS AND METHODS

Animals and kidney slice experiments

Experiments were conducted on 12- to 14-wk-old male C57BL/6J mice weighing ~25 g. Animals received tap water and were kept on a standard laboratory diet containing 0.8% P_i and 1.05% calcium (KLIBA). All experiments were approved by the Zürich Veterinäramt.

Kidney slice experiments were performed as described previously (2, 3, 6). Briefly, animals were anesthetized with a combination of ketamine (Narketan 10, Chassot, Belp, Switzerland) and xylazine (Rompun, Bayer, Leverkusen, Germany), injected intraperitoneally, and perfused through the left ventricle with 50 ml of warm (30°C) sucrose-phosphate buffer (140 mM sucrose and 140 mM NaH₂PO₄/NaH₂PO₄, pH 7.4) to remove all blood from the kidneys. Kidneys were rapidly harvested, adhering connective tissue and extrarenal vessels were removed, and thin (~1-mm) coronal slices were prepared. From each kidney, approximately seven slices could be prepared. Slices were transferred to 4 ml of prewarmed (37°C) Hanks' buffer (in mM: 110 NaCl, 5 KCl, 1.2 MgSO₄, 1.8 CaCl₂, 4 Na-acetate, 1 Na-citrate, 6 glucose, 6 L-alanine, 1 NaH₂PO₄, 3 Na₂HPO₄, and 25 NaHCO₃, pH 7.4, gassed with 5% CO₂-95% O₂) and allowed to adapt for 10 min at 37°C in a water bath before the start of the incubation. Slices were then left untreated (control) or incubated with PTH-(1–34) (100 nM), the selective D₁-like receptor agonists fenoldopam (10 μM) and SKF-38393 (10 μM), or the D₂-like receptor agonist quinpirole (1 μM) for 45 min. During the course of the experiments, all solutions were gassed with 5% CO₂-95% O₂, and pH was kept constant at 7.4 ± 0.1. Our laboratory previously demonstrated the viability of the kidney slices/proximal tubule cells during the incubation (6). The concentrations of activators and inhibitors had been tested in preliminary experiments or had been found to be effective in previous work (unpublished data; 6). After incubation, kidney slices were used for immunoblotting (see *Immunoblot*) or immunohistochemistry. For immunohistochemistry, slices were washed with fresh incubation buffer and transferred to a fixative solution consisting of 3% paraformaldehyde dissolved in 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mosmol/l with sucrose). After 4 h of fixation, the kidney slices were rinsed for 5 min with 0.1 M cacodylate buffer, mounted on small cork disks, frozen in liquid propane, and stored at –80°C until use.

Immunoblot

For immunoblotting, slices were transferred at the end of the incubation period to ice-cold buffer [in mM: 300 mannitol, 5 EGTA, 12 Tris (pH 7.1), and 0.1 PMSF], and BBMs were prepared as described previously using the Mg²⁺ precipitation technique (11). After measurement of the total protein concentration (Bio-Rad Protein Kit), BBM protein (10 μg) was solubilized in Laemmli sample buffer (23), and SDS-PAGE was performed on 10% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically from gels to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). After they were blocked with 5% milk powder in Tris-buffered saline-0.1% Tween at –20°C for 60 min, the blots were incubated with the primary antibodies [rabbit anti-NaPi-IIa raw serum (1:6,000) and mouse monoclonal antiactin (42 kDa; Sigma; 1:5,000)] for 2 h at room temperature or overnight at 4°C. After they were washed and blocked, blots were incubated

with the secondary antibodies [donkey anti-rabbit (1:10,000) and sheep anti-mouse (1:10,000), respectively, IgG conjugated with horseradish peroxidase (Amersham Life Sciences)] for 1 h at room temperature. Antibody binding was detected with the peroxidase/luminal enhancer kit (Pierce, Rockford, IL) with a camera and analyzed using AIDA Imaging software to calculate the NaPi-IIa-to-actin ratio.

Isolated perfused proximal tubules

In vitro microperfusion studies were performed on proximal straight tubules from C57BL/6J mice (20–25 g body wt), as described previously (5). Animals received tap water and were kept on a standard laboratory diet. Proximal tubule segments (~1 mm long) were dissected in Hanks' solution containing (in mM) 137 NaCl, 5 KCl, 0.8 MgSO₄, 0.33 Na₂HPO₄, 0.44 KH₂PO₄, 1 MgCl₂, 10 Tris·HCl, 0.25 CaCl₂, 2 glutamine, and 2 L-lactate at 4°C. Isolated tubules were transferred to a 1.2-ml temperature-controlled bath chamber. The tubules were perfused using concentric glass pipettes at 37°C. The perfusion and bathing solution was an ultrafiltrate-like solution containing (in mM) 115 NaCl, 25 NaHCO₃, 4.0 Na₂HPO₄, 10 Na-acetate, 1.8 mM CaCl₂, 1 MgSO₄, 5 KCl, 8.3 glucose, 5 alanine, 2 L-lactate, and 2 butyrate. This solution (pH 7.4) was gassed with 95% O₂-5% CO₂, and the osmolality was adjusted to 295 mosmol/kgH₂O. The pH and osmolality of the bathing solution were maintained constant by continuously changing the bath at a rate of 1 ml/min.

The tubules were perfused or bathed with 10 μM SKF-38393 (Sigma) and 10 μM quinpirole (Sigma) or ethanol vehicle. The tubules were fixed for 5–10 min in the bathing chamber with fixative, as described previously (5). The tubule was then released near the bottom of the bathing chamber, and the bathing chamber was removed to a dissection microscope for better visualization of the tubule. A drop of warm (37°C) 10% gelatin (type A 175, Bloom, Sigma) in PBS was placed on a microscope slide cooled to 4°C. After the gelatin hardened slightly, the tubule was transferred with an Eppendorf pipette to the gelatin. The excess fixative was removed from the drop of gelatin, and a second drop of cooled, but still molten, gelatin was placed on top of the tubule. After the slide was kept at 4°C for ~1 h, a cube of gelatin containing the tubule was stored in fixative at 4°C overnight, as described previously (5, 37). The gelatin droplets containing the tubules were washed for 2 h in PBS and cut into cubes before they were frozen.

Immunohistochemistry

Cryosections (3 μm thick) were mounted on chromalum-gelatin-coated glass slides, thawed, and stored in PBS until use. For immunofluorescence, sections were pretreated with 3% milk powder in PBS for 10 min and incubated overnight at 4°C with a rabbit anti-rat polyclonal antiserum against the NaPi-IIa protein (14) diluted 1:1,000 in 3% milk powder in PBS containing 0.3% Triton X-100 or with polyclonal antiserum against the NaSi-1 protein (27) diluted 1:500 in the same solution. Sections were then rinsed three times with PBS and covered for 45 min at 4°C with the secondary antibody (swine anti-rabbit IgG conjugated to fluorescein isothiocyanate; Dakopatts, Glostrup, Denmark) diluted 1:50 in PBS-milk powder. Double staining of β-actin filaments and NaPi-IIa was achieved by addition of rhodamine-phalloidin (Molecular Probes, Eugene, OR) at a dilution of 1:50 in the solution containing the secondary antibodies. Finally, the sections were rinsed three times with PBS,

plated on coverslips by using DAKO-Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo(2.2.2.)octane (Sigma) as a fading retardant, and studied with an epifluorescence microscope (Polyvar, Reichert-Jung). For control of the specificity of the NaPi-IIa antiserum, sections of isolated mouse proximal tubules and kidney slices were incubated with preimmune serum. Nonspecific binding to the tissue of the secondary antibodies was tested by omitting the primary antibody (not shown). All control incubations were clearly negative.

Cell culture and fluorescent confocal microscopy

OK cells [kind gift of J. Cole (12)] were cultured at 37°C in 95% air-5% CO₂ in high-glucose (450 mg/dl) DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100/g/ml). The full-length coding region of rat NaPi-IIa was cloned by PCR in-frame into the plasmid pEGFP (Clontech, Palo Alto, CA), resulting in a COOH-terminal fluorophore-tagged NaPi-IIa. For transient transfection, cells were grown to ~70% confluence on glass coverslips, and 1.5 mg of cDNA were introduced into each 60-mm plate using Lipofectamine (GIBCO, Carlsbad, CA). After removal of transfection reagents, cells were deprived of serum for 24 h and allowed to reach confluence.

NaPi-IIa-enhanced green fluorescent protein (EGFP) was visualized with fluorescent confocal microscopy. Confluent quiescent OK cells on glass coverslips were incubated with dopamine (10⁻⁵ M) or vehicle for 30 min. Cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, and then rinsed again with PBS. Cells were permeabilized using 0.1% Triton X-100 for 10 min at 4°C, rinsed, and then incubated with rhodamine red-X. Finally, cells were washed with PBS and mounted in antifading mounting medium. Confocal fluorescent images were visualized through a Zeiss ×100 objective lens using a Zeiss LSM-410 laser-scanning confocal microscope. Fluorescence of rhodamine red and green fluorescent protein (GFP) was detected using an excitation laser with wavelengths of 568 and 488 nm and an emission filter with a 590-nm long-pass filter and a 510- to 560-nm band pass.

All chemicals were obtained from Sigma unless stated otherwise. H-89 was obtained from Calbiochem.

Statistical analysis

Values are means ± SD and were tested for significance using ANOVA and the unpaired Student's *t*-test. *P* < 0.05 was considered to be statistically significant.

RESULTS

Effect of selective dopamine receptor agonists on distribution of the NaPi-IIa cotransporter in mouse kidney slices

Overviews of sections from control kidney slices showed that the NaPi-IIa protein was expressed in BBMs of proximal tubules throughout the cortex (Fig. 1A). As reported above, the intensity of the NaPi-IIa immunostaining was strongest in proximal convoluted tubules of juxtamedullary nephrons. After incubation of kidney slices with the selective D₁-like

receptor agonist fenoldopam (10 μ M) for 1 h, NaPi-IIa protein-specific immunofluorescence was scarcely detectable in the midcortical and superficial regions, whereas in proximal tubules of juxtamedullary nephrons, some apical expression of NaPi-IIa was still observed (Fig. 1B; $n = 5$). Similar effects were seen with another selective D₁-like receptor agonist, SKF-38393 (10 μ M, $n = 5$; Fig. 1C). In contrast, incubation with the selective D₂-like receptor agonist quinpirole (1 μ M) had no effect on NaPi-IIa immunoreactivity (Fig. 1D; $n = 5$). Double labeling of kidney slices with NaPi-IIa and β -actin as a marker of the BBM showed that both D₁-like agonists induced the disappearance of NaPi-IIa from the brush border and the appearance of NaPi-IIa-related immunostaining in subapical compartments (Fig. 1, E–H; $n = 5$). Treatment of kidney slices with fenoldopam or quinpirole had no effect on localization of the Na⁺/sulfate cotransporter NaSi (Fig. 2), demonstrating the specificity of the effect ($n = 5$ for each condition).

Immunoblots of BBM vesicles prepared from kidney slices confirmed the downregulatory effect of fenoldopam on NaPi-IIa protein abundance, with reduction by $38.1 \pm 9.6\%$ (mean $\pm$ SD). Quinpirole reduced NaPi-IIa protein abundance by $22.1 \pm 5.1\%$ (Fig. 3). The positive control, PTH (100 nM), reduced NaPi-IIa protein abundance by $26.0 \pm 1.5\%$ ($n = 3$ independent experiments).

Effect of D₁- and D₂-like receptor agonists on localization of NaPi-IIa cotransporter in isolated perfused proximal tubules

Experiments on isolated perfused proximal tubules were used to examine whether apical/luminal or basolateral receptors were mediating the downregulatory effect. After 20 min of perfusion of the isolated proximal tubules with the control solution, NaPi-IIa was expressed almost exclusively in the BBM (Fig. 4, Control). The same tubule sections were also stained for β -actin to ensure maintenance of the structural organization and integrity of the brush border. After 20 min of perfusion, polarity of the proximal tubule cells was well preserved under all tested conditions (Fig. 4, β -Actin), as described previously for similar experiments (5, 37).

The basolateral vs. luminal effects of the selective D₁-like receptor agonist SKF-38393 (100 μ M) on NaPi-IIa were also tested (Fig. 4, SKF-38393, bath and lumen). Luminal perfusion with 100 μ M SKF-38393 for 20 min provoked a complete disappearance of NaPi-IIa staining in the BBM. In contrast, basolateral superfusion with 100 μ M SKF-38393 for 20 min did not affect the expression of NaPi-IIa. The same experimental design was repeated with the D₂-like receptor agonist quinpirole (Fig. 4, Quinpirole, bath and lumen). Application of quinpirole did not induce internalization of NaPi-IIa, applied apically or basolaterally. These results obtained in isolated perfused proximal tubules clearly indicated the specificity of apical D₁-like receptors in the regulation of NaPi-IIa. All experiments were performed on at least four different proximal tubules from different animals.

Involvement of the PKA pathway in the D₁-like receptor-mediated downregulation of NaPi-IIa

PTH, a potent stimulus for the internalization and degradation of NaPi-IIa in the proximal tubule, has been shown to activate cAMP-PKA, phospholipase C-PKC, and MAPK

(ERK1/2) pathways (6, 30, 37). D₁-like receptors may couple in the proximal tubule to the cAMP-PKA pathway, and experiments in OK cells have implicated this pathway in the inhibitory effect of dopamine on Na⁺/P_i transport activity (25, 32). To further investigate which intracellular signaling pathways were involved in the D₁-like receptor-mediated NaPi-IIa internalization, kidney slices were incubated with fenoldopam in combination with specific inhibitors of several signaling cascades (Fig. 5; *n* = 4 for each condition). Incubation with the PKC inhibitor chelerythrine (1 μM) or the ERK1/2 MAPK inhibitor PD-098089 (10 μM) failed to prevent the fenoldopam-induced internalization of NaPi-IIa. However, incubation with the PKA inhibitor H-89 (10 μM) abolished the internalization of NaPi-IIa by fenoldopam (Fig. 5). Thus dopamine regulates NaPi-IIa localization and abundance through activation of the cAMP-PKA signaling pathway via apical D₁-like receptors.

Dopamine reduces apical membrane NaPi-IIa in OK cells

We used a third experimental system to confirm the effect of dopamine on NaPi-IIa. OK cells have been used extensively as a cell culture model to study hormonal regulation of proximal tubule transport. OK cells transfected with NaPi-IIa-eGFP showed exclusively apical membrane distribution with the typical punctuate staining pattern characteristic of a brush border protein (Fig. 6). After treatment with dopamine, the punctuate staining largely disappeared, and NaPi-IIa was seen primarily in intracellular pools (Fig. 6; *n* = 5). These findings in culture cells are compatible with those from kidney slices and isolated tubules.

DISCUSSION

NaPi-IIa is the major Na⁺/phosphate cotransporter in the BBM of the renal proximal tubule (28–30). The activity and expression of NaPi-IIa are tightly regulated by many factors, including several hormones. Among the hormones that have been shown to downregulate NaPi-IIa or to induce phosphaturia in vivo are PTH, dopamine, and adenosine or serotonin (17, 29, 30). The actions of PTH on NaPi-IIa are mediated by luminal and basolateral receptors, mediating the activation of phospholipase C-PKC- and cAMP-PKA-dependent pathways. In addition, cGMP and ERK1/2-MAPK kinase pathways have been described in cell culture models, isolated perfused tubules, and freshly isolated kidney slices (4, 6, 30, 37). Although the PTH-induced internalization of NaPi-IIa has been studied in some detail, little is known about the mechanisms by which hormones such as dopamine, serotonin, and adenosine induce phosphaturia. Therefore, we examined the effects of dopamine receptor agonists on the expression and localization of NaPi-IIa in ex vivo in vitro mouse kidney preparations and culture cells.

The phosphaturic effect of dopamine in vivo and in perfused kidneys is mimicked by selective D₁-like receptor agonists (10, 13, 17, 21). In agreement with these findings, we found that only D₁-like, but not D₂-like, receptor agonists induced internalization and a reduction of NaPi-IIa protein abundance. This was observed in isolated fresh kidney slices and isolated perfused proximal tubules. Furthermore, the isolated perfused tubule preparation allowed us to functionally localize these receptors to the luminal BBM. Addition of the D₁-like receptor agonist SKF-38393 to the bathing solution failed to induce NaPi-IIa internalization. At least four subtypes of dopamine receptors have been identified in the

kidney, and their distribution along the nephron has been characterized (26). In the proximal tubule, several subtypes have been described with apical (BBM) and basolateral localization (16, 26). Receptors have been pharmacologically and functionally characterized in the apical and basolateral membrane with D₁- and D₂-like features. Thus our finding that NaPi-IIa internalization occurs via an apical D₁-like receptor is in agreement with the previous localization of this receptor subtype. In contrast to the recent finding that the inhibitory effect of dopamine on NHE3 is exerted via apical and basolateral receptors (5), our results demonstrate that the effect of dopamine on NaPi-IIa is specifically mediated only by apical receptors.

The luminal concentrations of dopamine in the proximal tubule may be in the range 0.1–1 μ M, as estimated from urinary excretion in humans and rats (10, 20). Thus the concentrations used here are slightly above physiological concentrations. However, higher concentrations were necessary to allow for diffusion into the tissue slices and to compensate for continuous degradation.

Interestingly, inhibition of the ERK1/2 pathway failed to inhibit the internalization of NaPi-IIa induced by D₁-like receptor activation. We recently demonstrated that activation of PTH receptors or direct stimulation of the PKA pathway with 8-bromo-cAMP induces NaPi-IIa internalization in the same kidney slice preparation and that this effect was blunted by ERK1/2 inhibition (6). Thus it may be possible that activation of D₁-like receptors generates a PKA-mediated signal that induces NaPi-IIa internalization through a cascade that does not involve ERK1/2 activity.

In summary, we have identified a mechanism by which dopamine exerts its phosphaturic effects by inducing internalization of the major renal Na⁺/phosphate cotransporter NaPi-IIa. Using in vitro techniques, i.e., freshly isolated mouse kidney slices and perfused tubules, we demonstrated that luminal D₁-like receptors coupling to cAMP/PKA induce the internalization of NaPi-IIa, reduce its abundance in the BBM, and thus decrease its reabsorptive capacity. In addition, the inhibition of NaPi-IIa may also contribute to the dopamine-induced natriuresis in the proximal tubule. The preparation used for these experiments, i.e., ex vivo, allowed us to rule out confounding effects of systemic application of dopamine or its analogs and also to directly apply inhibitors of distinct signaling pathways without their systemic toxic effects. Furthermore, freshly isolated mouse kidney slices and proximal tubules circumvented potential loss or change of characteristics and features of proximal tubule cells kept in culture.

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Fig. 1.

Internalization of the NaPi-IIa cotransporter in mouse kidney slices after stimulation of D₁-like, but not D₂-like, receptors. *A–C*: immunohistochemical detection of the NaPi-IIa cotransporter in the renal cortex of control slices (*A*) and slices incubated with D₁-like agonists fenoldopam (10 μM; *B*) and SKF-38393 (10 μM; *C*). *D*: incubation with the D₂-like agonist quinpirole (1 μM) for 1 h had no effect on NaPi-IIa immunoreactivity. Original magnification: ×40. *E–H*: higher-magnification views of single proximal tubules stained for NaPi-IIa (green) and β-actin filaments (red). Scale bar, ~10 μm. *E*: a high degree of overlap (yellow) between NaPi-IIa and actin under control conditions, indicating localization in the brush border membrane (BBM). After treatment with fenoldopam (*F*) or SKF-38393 (*G*), NaPi-IIa immunoreactivity disappeared from the BBM and a distinct subapical signal was seen, demonstrating internalization. *H*: treatment with quinpirole did not alter BBM localization of NaPi-IIa (*H*).

Fig. 2.

No effect of dopamine receptor agonists on the renal Na⁺/sulfate cotransporter NaSi. Immunodetection of NaSi in mouse proximal tubules under control conditions (*top*) and after treatment with fenoldopam (*middle*) or quinpirole (*bottom*). Localization of NaSi in the BBM was not affected by treatments with fenoldopam or quinpirole. Scale bar, ~10 μ m.

Fig. 3.

Activation of D₁, but not D₂, receptors reduces NaPi-IIa abundance in the BBM. Mouse kidney slices were incubated with control solution (Co), PTH-(1–34) (100 nM), the D₁-like agonist fenoldopam (Fen, 20 μ M), or the D₂-like agonist quinpirole (Quin, 2 μ M) for 1 h, and BBMs were prepared. Protein (10 μ g/lane) was loaded, and membranes were stained for NaPi-IIa and actin to control for loading. Densitometry was performed, and values were normalized against actin, with control values set as 100%. $n = 3$ independent experiments. Values are means $\pm$ SD. Reduction in NaPi-IIa expression was significantly lower than

under control conditions. There was no significant difference between the 3 treatments.
Significant difference: * $P < 0.05$; ** $P < 0.001$.

Fig. 4. Effect of basolateral and luminal application of D₁ and D₂ agonists on expression of the NaPi-IIa cotransporter in isolated and perfused mouse proximal tubules. Immunodetection of the NaPi-IIa cotransporter (green) and β -actin (red) in isolated proximal tubules. Tubules were perfused, and the D₁-like agonist SKF-38393 or the D₂-like agonist quinpirole was added to the lumen or bath. Luminal administration of SKF-38393 induced removal of cotransporters without affecting β -actin staining. Basolateral application of SKF-38393 did not influence NaPi-IIa immunofluorescence. All downregulatory effects were specific for

the D₁-like receptor agonist, because application of the D₂-like receptor agonist quinpirole did not induce internalization of NaPi-IIa, applied apically or basolaterally. Scale bar, ~10 μ m.

Fig. 5.

D₁-like receptor agonist-induced NaPi-IIa internalization requires intact PKA. Freshly isolated mouse kidney slices were incubated with control solution or the dopamine D₁-like receptor agonist fenoldopam in the absence or presence of inhibitors of several signaling cascades for 1 h. Sections were stained for NaPi-IIa (green) and β-actin (red). Incubation with control solution (A) did not induce NaPi-IIa internalization, whereas incubation with fenoldopam did (B). Coincubation of fenoldopam with the PKA inhibitor H-89 (C) abolished internalization of NaPi-IIa. The PKC inhibitor chelerythrine (D) or the ERK1/2

MAPK kinase inhibitor PD-098089 (*E*) did not prevent fenoldopam-induced internalization.
Scale bar, 10 μ m.

Fig. 6.

Dopamine-induced internalization of NaPi-IIa in opossum kidney (OK) cells. OK cells were transiently transfected with NaPi-IIa with a COOH-terminal enhanced green fluorescent protein (EGFP) tag. At 48 h after transfection, vehicle or dopamine was added for 30 min. Cells were fixed, labeled with rhodamine, and visualized with laser confocal microscopy (red = actin; green = NaPi-IIa/EGFP). Two representative cells from vehicle- or dopamine-treated cells are shown from 2 independent experiments.