D3 Dopamine Receptor Directly Interacts With D1 Dopamine Receptor in Immortalized Renal Proximal Tubule Cells


Abstract—D3 receptors act synergistically with D1 receptors to inhibit sodium transport in renal proximal tubules; however, the mechanism by which this occurs is not known. Because dopamine receptor subtypes can regulate and interact with each other, we studied the interaction of D3 and D1 receptors in rat renal proximal tubule (RPT) cells. The D3 agonist PD128907 increased the immunoreactive expression of D1 receptors in a concentration- and time-dependent manner; these effects were blocked by the D3 antagonist U99194A. PD128907 also transiently (15 minutes) increased the amount of cell surface membrane D1 receptors. Laser confocal immunofluorescence microscopy showed that D1 receptor and D3 receptor colocalized in RPT cells more distinctly in Wistar-Kyoto rats than in spontaneously hypertensive rats (SHRs). In addition, D1 and D3 receptors could be communoprecipitated, and this interaction was increased after D1 receptor agonist stimulation for 24 hours in Wistar-Kyoto rats but not in SHRs. We propose that the synergistic effects of D3 and D1 receptors may be caused by a D3 receptor–mediated increase in total, as well as cell surface membrane D1 receptor expression, and direct D1 and D3 receptor interaction, both of which are impaired in SHRs. (Hypertension. 2006;47[part 2]:573-579.)

Key Words: receptors, dopamine ■ hypertension, essential ■ kidney ■ microscopy, confocal

Dopamine is an endogenous catecholamine that modulates many cellular activities, including behavior, hormone synthesis and release, blood pressure, and transmembrane ion transport.1–4 Dopamine receptors are classified into the D1- and D2-like subtypes based on their structure and pharmacology. D1-like receptors, comprised of D1 and D5 receptors, stimulate adenyl cyclase activity, whereas D2-like receptors, composed of D2, D3, and D4 receptors, inhibit adenyl cyclase activity and regulate/modulate the activity of several ion channels.1–4

The increase in sodium excretion after a sodium load is regulated, in part, by renal paracrine activation of D1-like receptors.1–4 However, D2-like receptors may act, synergistically, with D1-like receptors to increase urinary sodium excretion.5,6 Thus, we found that the increase in sodium excretion induced by Z-1046, a dopamine receptor agonist with the rank order potency D1 ≥ D2 > D3 > D4, was blocked by either a D1-like or D2-like receptor antagonist.5,6 Several studies have demonstrated that activation of D1-like receptors in renal proximal tubules (RPTs) decreases sodium reabsorption by inhibition of the activities of Na+/H+ exchanger type 3 (NHE3), Cl−/HCO3− exchanger, Na/Pi cotransporter, and Na+/HCO3− cotransporter in brush border membranes, and Na+/K+–ATPase activities in basolateral membranes.7–11 D2-like receptors may potentiate the inhibitory effect of D1-like receptors on Na-Pi cotransporter, NHE3, and Na+/K+–ATPase activities in RPTs.5,7,9,12

In the rat kidney, the major D1-like receptor in RPTs is the D1 receptor.1,3,13,14 The D2 receptor in the rat kidney appears to be located prejunctionally in dopaminergic nerves,15–17 whereas the D3 receptor is mainly expressed in collecting ducts18 and the S1 segment of the proximal tubule.19 However, both D2 and D3 receptors have been described in the opossum kidney cell, a proximal tubule cell line that has some distal tubular cell characteristics.19 The major D2-like receptor in RPTs is probably the D1 rather than the D2 receptor.1–4,20

Several studies have shown that G protein-coupled receptors (GPCRs), including dopamine receptors, can regulate other GPCRs by altering expression and/or via direct protein–protein interaction.21–24 The current studies were designed to test the hypothesis that the D3 receptor interacts with the D1 receptor. We also investigated the mechanism(s) of any D3 and D1 receptor interactions. To preclude contributions from receptors on nerve cells, we studied immortalized RPT cells that have characteristics similar to freshly obtained RPT brush border membranes and RPTs, at least with regard to D1 receptors and responses to G protein stimulation.25–27
Methods
Cell Culture
Immortalized RPT cells from microdissected S\textsubscript{i} segments of proximal tubules of 4- to 8-week-old Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs) were cultured at 37°C in 95% air/5% CO\textsubscript{2} atmosphere in DMEM/F-12 with transferrin (5 \mu g/mL), insulin (5 \mu g/mL), epidermal growth factor (10 ng/mL), dexamethasone (4 \mu g/mL), and FBS 5% on a 100-mm Petri dish.\textsuperscript{23–27} Cells were made quiescent by incubation for 2 hours in medium without FBS before the addition of drugs.

Immunoblotting of D\textsubscript{1} and D\textsubscript{3} Receptors
The antibodies are polyclonal and IgG-purified or affinity-purified antipeptides. The amino acid sequence of the D\textsubscript{1} receptor–immunizing peptide is 299-GSEEFTQPF-307 (Research Genetics).\textsuperscript{24–26,28} The amino acid sequence of the D\textsubscript{3} receptor–immunizing peptide is 288-QPPSPGQTHGGGLKY YSI C-306.\textsuperscript{6,23} The specificity of these antibodies has been reported.\textsuperscript{6,23–26,28}

The cells were lysed in a lysis buffer, sonicated, placed on ice for 1 hour, and centrifuged at 16,000g for 30 minutes. The supernatants were stored at −70°C until use. After measuring the protein concentrations, the supernatants were mixed with Laemmli sample buffer, boiled for 5 minutes, subjected to electrophoresis, and then transferred electrophoretically onto nitrocellulose membranes. The blots were probed with the D\textsubscript{3} receptor antibody (1:250) or the D\textsubscript{1} receptor antibody (1:1000) for 1 hour. The primary antibody binding was then probed by a peroxidase-conjugated goat anti-rabbit IgG antiserum. The signal was detected using chemiluminescence and developed on x-ray film. The density of the bands was quantified by densitometry using Quantiscan, as reported previously.\textsuperscript{23–26}

The amount of protein transferred onto the membranes was verified by Ponceau-S staining and immunoblotting for \alpha-actin.

Cell Surface D\textsubscript{1} Receptor Expression
Cultured RPT cells were starved in serum-free medium for 2 hours and then treated with the D\textsubscript{1} receptor agonist PD128907 (10^{-7} M) for varying periods (0, 15, and 30 minutes). Cell impermeable, non-cellular protein bands were visualized by enhanced chemiluminescence and then treated with peroxidase-conjugated streptavidin (1:5000 dilution, air/5% CO\textsubscript{2} atmosphere in DMEM/F-12 with transferrin (5 \mu g/mL) was added into the medium 20 minutes before the end of the drug treatment. The cells were washed 3 times (ice-cold PBS), lysed with a lysis buffer, sonicated, and placed on ice for 1 hour. The supernatants from the cell lysates were then immunoprecipitated with the anti-D\textsubscript{1} receptor antibody and then subjected to immunoblotting. The membrane sheets were blocked with 10% milk in wash buffer for 1 hour, washed with wash buffer 3 times, and incubated with peroxidase-conjugated streptavidin (1:5000 dilution; 30 minutes; Jackson ImmunoResearch Laboratory). The bionylated protein bands were visualized by enhanced chemiluminescence (Western blotting detection kit, Amersham). The intensity of the band represents D\textsubscript{1} receptor expression in the cell surface membrane.

Immunoprecipitation Studies
Immortalized RPT cells were incubated with vehicle or a D\textsubscript{3} receptor agonist, PD128907 (10^{-7} M), for 24 hours. The cells were lysed with an ice-cold lysis buffer for 1 hour and centrifuged at 16,000g for 30 minutes. The lysates (supernatant; 300 \mu g protein/mL) were then incubated with affinity-purified anti-D\textsubscript{3} receptor antibodies (1 \mu g/mL) at 4°C for 1 hour and protein-G agarose at 4°C for 2 hours. The immunoprecipitates were pelleted and washed 4 times with lysis buffer. After the sample buffer was added, the samples were boiled for 10 minutes and subjected to immunoblotting with the D\textsubscript{3} receptor antibody. To determine the specificity of the bands found on the immunoblots, preimmune serum of D\textsubscript{3} receptor antibody (negative control) and D\textsubscript{1} receptor antibodies (positive control) were used as the immunoprecipitants instead of the D\textsubscript{3} receptor antibodies.\textsuperscript{23–25}

Confocal Microscopy of Double-Stained RPT Cells
RPT cells, grown on coverslips, were fixed with 3% paraformaldehyde (30 minutes) and permeabilized with 0.1% Triton X-100 in PBS (15 minutes). Reactions with antibodies were performed as described previously.\textsuperscript{30} D\textsubscript{1} receptors were visualized using a monoclonal mouse anti-D\textsubscript{1} receptor antibody (1:600) followed by Alexa Fluor 568-goat anti-mouse IgG antibody (red; Molecular Probes). The D\textsubscript{3} receptor was visualized using an IgG affinity-purified rabbit anti-rat D\textsubscript{3} receptor antibody (1:300) followed by fluorescein isothiocyanate–conjugated goat anti-rabbit IgG antibody (green; Molecular Probes). Cells on coverslips were mounted with the ProLong Antifade kit (Molecular Probes). The immunofluorescence densities and images were acquired (Olympus AX70 laser confocal microscopy) at an excitation wavelength of 543 nm and 488 nm; emissions were detected using band pass filters of 578 to 623 nm and 505 to 525 nm, respectively. The colocalization of D\textsubscript{1} receptor and D\textsubscript{3} receptor was quantified, as described previously.\textsuperscript{30}

Materials
Rabbit anti-rat D\textsubscript{1} antibody was purchased from Alpha Diagnostic International (D3R12A, San Antonio, TX). Mouse anti-D\textsubscript{3} antibody was purchased from Zymed (32-0900, South San Francisco, CA). Rabbit anti-rat D\textsubscript{3} receptor antibody was produced against a synthetic oligopeptide of the rat D\textsubscript{3} receptor (amino acids 299 to 307; Research Genetics).\textsuperscript{23–26} PD128907 was purchased from Sigma. U99194A was from Research Biochemicals International. Peroxidase-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratory. Sulfosuccinimidyl-6-(biotinamido)hexanoate was purchased from Pierce. Other chemicals for various buffers were of the highest purity available and purchased either from Sigma or Gibco.

Statistical Analysis
The data are expressed as mean±SEM. Comparison within groups was made by repeated measures ANOVA (or paired \textit{t} test when only 2 groups were compared), and comparison among groups was made by factorial ANOVA with Duncan’s test (or \textit{t} test when only 2 groups were compared). A value of \(P<0.05\) was considered significant.

Results
Activation of the D\textsubscript{3} Receptor Increases D\textsubscript{1} Receptor Expression in RPT Cells From WKY Rats
The D\textsubscript{3} receptor agonist PD128907 increased D\textsubscript{1} receptor expression in a concentration-dependent manner (Figure 1A). The concentration causing a 50% increase in D\textsubscript{1} receptor expression was 6\times10^{-9} M (Figure 1A). The increase in D\textsubscript{1} receptor expression induced by PD128907 (10^{-7} M) was also time dependent; a significant increase was noted by 16 hours and lasted \(\geq\)30 hours (Figure 1B). The effect of PD128907 was exerted at the D\textsubscript{1} receptor, because a D\textsubscript{3} receptor antagonist, U99194A (10^{-5} M), which by itself had no effect on D\textsubscript{1} receptor expression [control=0.8±0.1 DU; PD128907=1.2±0.2 DU; and U99194A=0.7±0.1 DU], blocked the stimulatory effect of PD128907 (10^{-7} M) on D\textsubscript{1} receptor expression at 24 hours (PD128907+U99194A=0.7±0.2 DU; n=5/group; Figure 1C).

In RPT cells from SHRs, PD128907 (10^{-7} M) had no effect on D\textsubscript{1} receptor expression (WKY: control=1.0±0.2, PD128907=1.5±0.06; SHRs: control=0.9±0.1, PD128907=0.7±0.2 DU; n=8/group; Figure 1D).

D\textsubscript{1} Receptors Colocalize With D\textsubscript{3} Receptors in RPT Cells
In order determine whether D\textsubscript{1} and D\textsubscript{3} receptors can directly interact with each other, we studied the colocalization of D\textsubscript{1} and D\textsubscript{3} receptors in rat RPT cells using laser confocal microscopy and coimmunoprecipitation studies. As shown in Figure 2A and Figure 3, the D\textsubscript{1} and D\textsubscript{3} receptors colocalized...
laser confocal microscopy) and coimmunoprecipitated in RPT cells from WKY rats. The 45-kDa band (Figure 3), representing the coimmunoprecipitated D3 and D1 receptors, was increased by a 24-hour treatment with the D3 receptor agonist PD128907 (10^{-7}M) in RPT cells from WKY rats but had no effect in SHRs (WKY: control = 21±3; PD128907 = 35±4; SHR: control = 22±4, PD128907 = 18±3 DU; *P<0.05; n=8; Figure 3). The basal cell surface colocalization of D1 receptor with D3 receptor is much greater in RPT cells from WKY (47±3%, n=5) than in RPT cells from SHRs (12±1 n=5; *P<0.001; Figure 2A and 2B). The results of the confocal images cannot be equated with the immunoprecipitation data, because the latter used whole cells, whereas the quantification of colocalization could only be performed for cell surface expression.

**Activation of the D3 Receptor Increases Cell Surface Membrane D1 Receptors in RPT Cells of WKY But Not SHRs**

Because our previous short-term studies^6,32 have shown a synergistic interaction between D3 and D1 receptors, we determined whether D3 receptor stimulation affects the cellular localization of D1 receptors. As shown in Figure 4, in WKY RPT cells, the D3 receptor agonist PD128907 (10^{-7}M) increased the amount of D1 receptors in cell surface membranes at 15 minutes and returned to baseline at 30 minutes.
In contrast, in SHR RPT cells, PD128907 did not increase cell surface membrane expression of D1 receptors and actually decreased it at 30 minutes. The basal level of cell surface membrane D1 receptors was also greater in WKY than in SHR cells (WKY: control = 18±4 DU, 15 minutes = 27±3 DU, 30 minutes = 18±3 DU; SHR: control = 6±1 DU, 15 minutes = 7±2 DU, 30 minutes = 2±0.7 DU; n = 14; P < 0.05).

Discussion

The effect of stimulation of D2-like receptors, independent of D1-like receptors, on sodium excretion has ranged from antinatriuresis, no effect, to natriuresis. Although bromocriptine, a D2-like agonist with similar selectivity for all D2-like receptors, has not been found to affect sodium excretion in vivo,33 it has been reported to increase Na⁺⁻K⁺-ATPase activity in RPTs in vitro.34 Some studies have found that the D2-like antagonist haloperidol had natriuretic effects.35,36 However, haloperidol does not distinguish among the D2-like receptors and can also bind to D1-like receptors in RPTs.37 The intrarenal infusion of another D2-like receptor antagonist, YM-09151, in chronically instrumented conscious dogs on a moderate sodium diet also increases sodium excretion,38 whereas the infusion of the D2-like agonist quinpirole, with a 35-fold selectivity on D3 receptor over the D2 receptor, results in a decrease in urine flow and sodium excretion.39 These studies suggest an antinatriuretic effect of D2-like receptors. However, in vitro studies have suggested that D2-like receptors, in concert with D1-like receptors, could synergistically act to decrease Na⁺⁻K⁺-ATPase and NHE3 exchanger activities in RPTs and brain striatal cells and inhibit sodium-phosphate cotransport in opossum kidney cells.5–7,9,12,40

D1- and D2-like receptors synergistically increase sodium excretion in WKY rats.5,12 However, those in vivo studies are limited, because proximal tubular effects could not be distinguished from distal tubular effects, and those in vivo studies did not determine the specific D1- and D2-like receptor subtypes that synergistically interact. As mentioned in the introduction, in the rat kidney, the major D2-like receptor in RPTs is the D4 receptor.14 We now report that the D1 and D3 receptors colocalize and interact in rat RPT cells. A D3
receptor agonist increases the coimmunoprecipitation of the D1 and D3 receptor. We propose the D1-like and D2-like receptors that synergize to influence renal function are the D1 and D3 receptor. We have provided evidence that the D1 receptor regulates D3 receptor expression. First, a D3 receptor agonist, PD128907, increased D3 receptor expression in a time- and concentration-dependent manner. Second, a D3 receptor antagonist alone had no effect on D1 receptor expression, but it completely blocked the effect of the D1 receptor agonist. The stimulatory effect of a D3 receptor agonist on D1 receptor expression was selective, because the same agonist decreased AT1 receptor expression, and technical problems in the analysis (differences in loading and transferring of proteins for the Western blots) could be excluded. We did not determine the mechanism by which the D3 receptor increases D1 receptor protein expression in these studies. However, our previous study showed that stimulation of the D3 receptor increased D1 receptor protein expression in RPT cells from WKY rats but decreased it in cells from SHRs. We also found that, in RPT cells, activation of the D1 receptor had no effect on D1 receptor mRNA levels in WKY but decreased it in SHR cells, indicating that the D1-like receptor upregulation of D3 receptor protein in WKY may be secondary to posttranscriptional or posttranslational (eg, decreased protein degradation) mechanisms, whereas in SHR cells, D3-like receptor downregulation of protein expression may occur at the transcriptional or posttranscriptional level (C. Zeng, Z. Yang, P.A. Jose, unpublished data, 2005). Based on these results, we presume that the regulation of the receptor expression occurs via similar mechanisms. However, the effect of D1 receptor stimulation, by PD128907, on D1 receptor mRNA needs to be studied. We also did not study the mechanism by which D3 receptor agonist stimulation increased D1 and D3 receptor coimmunoprecipitation. This could be because of the increase in D1 and D3 receptor expression or increased interaction via some adapter protein. The later remains speculative at this time.

In our previous in vivo study, Z1046, through D1/D2-like receptor synergism, increased sodium excretion in 80 minutes. The period was too short to be explained by increased D1 and D3 receptor expression. Because the activity of GPCRs is, in part, dependent on their localization on cell surface membranes, we also investigated the effect of D1 receptor stimulation on cell surface membrane expression of D1 receptors. Previous studies have shown that D1 receptors can be recruited to the cell surface membrane from the cytosol within minutes after D1 receptor stimulation. We now report that D1 receptor agonist stimulation can increase cell surface membrane expression of D1 receptors. We suggest that a D1 receptor–mediated increase in cell surface membrane expression of D1 receptors, rather than an increase in D1 receptor expression in the whole cell, is the mechanism for the synergism between D1 and D3 receptors to acutely increase sodium excretion.

GPCR kinase 4 (GRK4) plays an important role in the desensitization of the human D1 receptors in RPTs. However, the first 20 minutes of homologous desensitization of the human D1 receptor are GRK independent, the mechanism of which remains to be determined. In the early and late stages of desensitization, sucrose, which prevents endocytosis, has no effect on total GRK expression but prevents the desensitization of the D1 receptor response. These data indicate that the desensitization of the human D1 receptor in renal RPT cells appears to involve the formation of endocytic vesicles and GRK-dependent and -independent mechanisms. Figure 4 shows that stimulation of the D1 receptor decreases D1 receptor expression at 30 minutes but not at 15 minutes in RPT cells from SHRs. This is consistent with the D1 receptor desensitization time frame. We assume that activation of the D3 receptor increases GRK4 activity, which, in turn, induces D3 receptor endocytosis in SHR cells. In another study, we found that stimulation of the D1 receptor activates GRK4 activity in human RPT cells.

Basal D1 receptor expression in surface membranes of RPT cells is decreased in SHRs relative to WKY rats. Furthermore, D1 receptor stimulation failed to increase D1 and D3 receptor expression in SHRs. Our previous studies also showed that the D1 receptor–mediated stimulation on D1 receptor expression is impaired in SHR RPT cells. We also found that the costimulation of D1-like and D3 receptors led to additive vasorelaxation in WKY rats but not in SHRs. Luippold et al reported that both expression and function of the renal D1 receptor are impaired in salt-sensitive Dahl rats as compared with salt-resistant Dahl rats. In contrast, these investigators did not find a defective response to the intravenous infusion of a D1 receptor agonist [R(+)-7-hydroxy-dipropylaminotetralin] in SHRs. However, the nonrenal systemic effects of D1 receptor stimulation may have obfuscated any potential differences between WKY and SHRs. The studies of Luippold et al in SHRs were also not performed in salt-loaded rats; moderate salt loading enhances the natriuretic effects of dopaminergic drugs. Indeed, we have preliminary data showing that the intrarenal arterial infusion of a D1 receptor agonist, PD128907, the ligand used in the

![Figure 4](image_url)
current studies, increased sodium excretion in salt-loaded WKY rats but not SHRs.49 We have reported that R(+)-7-hydroxy-dipropylamidotetralin can inhibit both NHE1 and NHE3 activity in RPT cells from 4- to 8-week-old WKY rats and SHRs.50,51 In RPTs from 12-week-old rats, the ability of R(+)-7-hydroxy-dipropylamidotetralin to inhibit NHE activity is greater in WKY rats and than in SHRs (L.D. Asico, P.A. Jose, unpublished studies, 2004). We suggest that in adult (12-week-old) SHRs, D3 receptors can inhibit NHE activity, NHE3 to a lesser extent, because it is expressed in luminal and subluminal membranes of rat RPT cells where GRK4 is also expressed, and NHE1 to a greater extent, because it is expressed in basolateral membranes. GRK4 is not expressed in basolateral membranes of rat RPT cells (Z. Wang, P.A. Jose, unpublished data, 2004). The inhibitory effect of D3 receptors on NHE3 activity is impaired in SHRs in any age.1,3,5,22 Thus, our current and previous data strongly suggest that deficiency in D3 receptors and D3/D1 interaction is present in spontaneous hypertension.

In summary, we have demonstrated that D3 receptors positively regulate the expression of D1 receptors in rat RPT cells. Furthermore, D3 and D1 receptors immunoprecipitate in RPT cells, and D1 receptor agonist stimulation enhances the interaction between these two GPCRs. In the RPT cells from SHRs, this interaction between D1 and D3 receptors is impaired.

**Perspectives**

Dopamine receptors are classified into 2 groups, D1-like (D1 and D3) and D2-like receptor (D2, D3, and D4) subtypes based on their structure and pharmacology.1-3 In RPTs, D1, D3, D4, and D5 receptors are expressed.13,14,16,20 Previous studies have shown that stimulation of the D1-like or D3 receptor induces diuresis and natriuresis, which are impaired in SHRs.1,3,6,49,53 D3 and D4 receptors are also found in RPTs; however, whether those 2 receptors control sodium reabsorption in RPT cells is not certain. Similar to the D1 receptor, stimulation of the D3 receptor increases cAMP production50; D1 receptor–deficient (D1−/−) mice develop hypertension, which is aggravated by sodium load.54 Intraperitoneal administration of the AT1 receptor antagonist losartan (20 mg/kg per day for 8 days) normalized blood pressure in pentobarbital D5 receptor–deficient (D5−/−) mice,55 but minimally affected blood pressure of D5−/− littermates (L.D. Asico, Z. Yang, C. Zeng, P.A. Jose, unpublished data, 2004). Our previous studies show interactions among D1, D3, and D5 receptors in RPT cells;32,41,42 stimulation of renal D1-like and D3-like receptors synergistically increases sodium excretion in WKY rats.5,6 We hypothesize that D1, D3, D4, and D5 receptors interact (in RPTs or elsewhere in the nephron) among or between themselves, and/or other GPCRs, such as angiotensin and endothelin receptors, to regulate sodium excretion.5,7,12,24,32,40,41,55,56 The ultimate effect of dopamine is the sum of the interactions of those dopamine receptor subtypes and other GPCRs that may depend on the state of sodium balance. These interactions are impaired in SHRs. This hypothesis needs to be confirmed by future study.

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