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ORIGINAL ARTICLE

D_3 dopamine receptor regulation of D_5 receptor expression and function in renal proximal tubule cells

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Dopamine receptor, via D_1-like and D_2-like receptors, increases sodium excretion in kidney. We have reported positive interactions between D_3 and D_1 receptors in renal proximal tubule (RPT) cells. These reports, however do not preclude that there may be also interaction between D_3 and D_5 receptors, because of the lack of selective D_1 and D_5 receptor agonists or antagonists. We hypothesize that D_3 receptors can regulate D_5 receptors, and that D_3 receptor regulation of D_5 receptors in RPTs is impaired in spontaneously hypertensive rats (SHRs). It showed that a D_3 receptor agonist, PD128907, by the activation of protein kinase C activity, increased the expression of D_5 receptors in a concentration- and time-dependent manner in RPT cells from Wistar-Kyoto (WKY) rats. The stimulatory effect of the D_3 receptor on D_5 receptor expression was impaired in RPT cells from SHRs. The effect of D_3 receptor on D_5 receptor is functionally relevant; stimulation of D_5 receptor decreases Na^+–K^+ adenosine triphosphatase (ATPase) activity in WKY cells. Pretreatment with D_3 receptor agonist for 24 h enhances the D_5 receptor expression and D_5 receptor-mediated inhibitory effect on Na^+–K^+ ATPase activity in WKY cells, but decreases them in SHR cells. The effect of D_3 receptor on D_5 receptor expression and function was also confirmed in the D_5 receptor-transfected HEK293 cells. It indicates that activation of D_3 receptor increases D_5 receptor expression and function. Altered regulation of D_3 receptor on D_5 receptors may have a role in the pathogenesis of hypertension.

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INTRODUCTION

Essential hypertension is one of the most common risk factors of cardiovascular disease in developed and developing countries, affecting about 25% of the middle-aged adult population.[1,2] Essential hypertension is heterogeneous and its expression is influenced by genetic and environmental factors.[2–5] The long-term regulation of blood pressure rests on renal and non-renal mechanisms, and abnormalities in the renal regulation of ion transport, intrinsic and extrinsic to the kidney, have been proposed to cause essential hypertension.[4–6] Several studies have reported that an impaired renal dopaminergic system may contribute to the pathogenesis of hypertension.[7,8]

Dopamine receptors expressed in mammals belong to the α group of the rhodopsin family of G protein-coupled receptors. The five mammalian dopamine receptor subtypes, identified by molecular cloning, differ in their primary structures, and have distinct affinities for dopamine receptor agonists and antagonists. The D_1-like receptors, comprising D_1 and D_5 receptor subtypes, couple to the stimulatory G proteins G_s and activate adenylyl cyclases. The D_2-like receptors, comprising D_2, D_3 and D_4 receptor subtypes, couple to the inhibitory G proteins G_i and inhibit adenylyl cyclases and modulate ion channels.[7,8]

There are increasing evidences for a direct interaction between D_1-like and D_2-like receptors in the kidney. *In vitro* studies have shown that a D_2-like receptor, in concert with a D_1-like receptor, synergistically decreases Na^+–K^+ ATPase, sodium–phosphate cotransporter and sodium–hydrogen exchanger activities in renal proximal tubule (RPT) and other cells.[8–12] In rats, during conditions of normal sodium load, and especially with increased sodium load, D_1- and D_2-like receptors synergistically interact to increase sodium excretion.[13–15] We have reported positive interactions between D_3 and D_1 receptors in RPT cells; stimulation of one receptor increases the expression and function of the other receptor. In contrast, in hypertensive states, the D_3 and D_1 receptor interaction is impaired.[16] These reports, however do not preclude that there may be also interaction between D_3 and D_5 receptors. Indeed, the reported interactions between D_2-like and D_1-like receptors *in vivo* did not distinguish the effects exerted specifically by the D_1 or D_5 receptor, because of the lack of selective D_1 and D_5 receptor agonists or antagonists. Because both D_1 and D_5

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receptors belong to the D_1-like receptor family,[8,13] we presume that there is also an interaction between D_3 and D_5 receptors in RPT cells. In order to test the above hypotheses, we studied D_3 and D_5 receptor interaction in immortalized RPT cells from Wistar-Kyoto (WKY) rats and in D_5 receptor-transfected HEK293 cells. To determine whether studied D_3 and D_5 receptor interaction is impaired in hypertension, similar studies were performed in spontaneously hypertensive rats (SHRs). These RPT cells behave similarly to freshly obtained RPT cells, at least with regard to dopamine receptors and responses to G protein stimulation.[17–20]

METHODS

Cell culture

Immortalized RPT cells from WKY and SHRs were cultured at 37 °C in 95% air and 5% CO_2 atmosphere in Dulbecco's modified Eagle medium/F-12 culture media, as previously described.[16–20] Those RPT cells were from Ulrich Hopfer's laboratory in Case Western Reserve School of Medicine (Cleveland, OH, USA). HEK293 cells were transfected with the human D_5 receptor complementary DNA (cDNA) fused to a V5-His tag at the C-terminus or the empty vector (pcDNA6/V5-His) (36), which served as a control. The cells (80% confluence) were extracted in ice-cold lysis buffer (phosphate-buffered saline with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} ethylene glycol tetraacetic acid (EGTA), 1 mmol l^{-1} phenylmethylsulfonyl fluoride, 10 μg ml^{-1} aprotinin and 10 μg ml^{-1} leupeptin), sonicated, kept on ice for 1 h and centrifuged at 16 000 *g* for 30 min. The supernatants were stored at −70 °C until use for immunoblotting.

Immunoblotting

Rat RPT cells were treated with vehicle (dH_2O), a D_3 receptor agonist (PD128907) (Sigma, St Louis, MO, USA)[21] or a D_3 receptor antagonist (U99194A, Sigma)[22] at the indicated concentrations and times. Immunoblotting was performed as previously reported,[20,23] except that the transblots were probed with the D_5 receptor (1 : 500; Research Genetics, Huntsville, AL, USA). The amino-acid sequence of the peptide for the rabbit anti-human D_5 receptor antibody corresponds to the third intracellular loop of the D_5 receptor. The receptor densities were normalized by α-actin.

All immunoblot bands in one group (receptor of interest or actin) were given a value of 100%. The density of each sample was calculated as a fraction of 100%. The ordinate indicates the ratio of the density of the protein of interest as a fraction of 100% and the density of actin as a fraction of 100%.

Antisense oligonucleotides

Antisense–sense oligodeoxynucleotide (ODN) against rat D_1 receptor messenger RNA (mRNA) and its control, sense ODN, were synthesized and purified with reverse-phase high-performance liquid chromatography as 21-mer phosphorothioate-modified ODNs (antisense: 5′-GGT AGA AGT GTT AGG AGC CAT-3′, sense: 5′-ATG GCT CCT AAC ACT TCT ACC-3′) from nucleotides 60 to 80 of the rat D_1 receptor cDNA. The designed sequences showed no homology with other known mammalian sequences deposited in the Genbank database (GenBank accession no. M35077), as screened using the BLAST program.[24]

The effects of 50 nM of antisense ODN were compared with sense controls. Briefly, cells were grown in 6-well plates until 60% confluence, and 50 nM antisense or sense ODN were mixed with 6 μl of oligofectamine in Optimem medium (Invitrogen, Life Technologies, Shanghai City, China) and incubated for 24 h, then switched to growth medium and incubated for another 24 h. The cells were collected and processed for reverse transcriptase (RT)-PCR for the D_1 receptor to check whether the antisense works well or not.

Na^+–K^+ ATPase activity assay

Na^+–K^+ ATPase activity was determined as the rate of inorganic phosphate released in the presence or absence of ouabain.[24–26] To prepare membranes for Na^+–K^+ ATPase activity assay, RPT cells were cultured in 21 cm^2 plastic culture dishes, washed twice with 5 ml chilled phosphate-free Modified Krebs buffer (118 mM NaCl, 4 mM KCl, 27.2 mM $NaHCO_3$, 1.2 mM $MgCl_2.6H_2O$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 0.25 mM $CaCl_2.2H_2O$) and centrifuged at 3000 *g* for 10 min. The cells were then placed on ice and lysed in 2 ml of lysis buffer (1 mM $NaHCO_3$, 2 mM $CaCl_2$ and 5 mM $MgCl_2$). Cell lysates were centrifuged at 3000 *g* for 2 min to remove intact cells, debris and nuclei. The resulting supernatant was suspended in an equal volume of 1 M sodium iodide, and the mixture was centrifuged at 48 000 *g* for 25 min. The pellet (membrane fraction) was washed two times and suspended in 10 mM Tris and 1 mM EDTA (pH 7.4). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to 1 mg ml^{-1}. The membranes were stored at −70 °C until further use. To measure Na^+–K^+ ATPase activity, 100 μl aliquots of membrane fraction were added to a 800-μl reaction mixture (75 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 6 mM sodium azide, 1 mM Na_4EGTA, 37.5 mM imidazole, 75 mM Tris HCl and 30 mM histidine; pH 7.4) with or without 1 mM ouabain (final volume=1 ml) and preincubated for 5 min in a water bath at 37 °C. Reactions were initiated by adding Tris ATP (4 mM) and terminated after 15 min of incubation at 37 °C by adding 50 μl of 50% trichloracetate. For determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixtures containing ouabain. To quantify the amount of phosphate produced, 1 ml of coloring reagent (10% ammonium molybdate in 10 N sulfuric acid + ferrous sulfate) was added to the reaction mixture. The mixture was then combined thoroughly and centrifuged at 3000 *g* for 10 min. Formation of phosphomolybdate was determined spectrophotometrically at 740 nm, against a standard curve prepared from K_2HPO_4. Na^+–K^+ ATPase activity was estimated as the difference between total and ouabain-insensitive ATPase activity, and results are expressed as μmol phosphate released per mg protein per hour.

To eliminate the effect of proteases on the results, we added protease inhibitor (1 mM phenylmethylsulfonyl fluoride, 10 mg ml^{-1} each leupeptin and aprotinin) in all solutions in this experiment.[27]

Reverse transcriptase-PCR

A total of 2–3 μg of total RNA extracted from RPT cells was used to synthesize cDNA and served as a template for amplification of D_1, D_3 receptor and β-actin as an endogenous standard.[28] For β-actin, the forward primer was 5′-GTGGGTATGGGTCAGAAGGA-3′ and the reverse primer was 5′-AGCGCG TAACCCTCATAGAT-3′. The amplification was performed with the following conditions: 35 cycles of denaturation at 94 °C for 2 min, annealing for 30 s at 52.5 °C and extension for 45 s at 72 °C. For D_1 receptor, the forward primer was 5′-ACTCTGCCCTACTACGAATAA-3′ and the reverse primer was 5′-CTCCTGCTGTAAGGCTCAAT-3′. The amplification was performed with the following conditions: 40 cycles of denaturation at 94 °C for 2 min, annealing for 30 s at 60 °C and extension for 45 s at 72 °C. For D_3 receptor, the forward primer was 5′-GGCTGCCCTTCTTCTTGAC-3′ and the reverse primer was 5′-CAGCAAGACAGGATCTTGAGG-3′. The amplification was performed with the following conditions: 40 cycles of denaturation at 94 °C for 2 min, annealing for 30 s at 60 °C and extension for 45 s at 72 °C.

Quantitative RT-PCR

For real-time quantitative RT-PCR analysis, cDNA was synthesized from 0.5 μg. of total RNA with a cDNA synthesis kit (High Capacity RNA-to-cDNA Kit; Takala, Tokyo, Japan) for quantitative RT-PCR. In the thermal cycle, 1 μl cDNA was used per 25 μl final reaction volume. PCRs were carried out with the Brilliant SYBR Green QPCR Master Mix kit (Takala) in a total volume of 25 μl. Primers were designed using DNA-Star software (DNA Star Co., Madison, WI, USA), and sequences are listed in Table 1. A BLAST search of GenBank was performed on the primer sequences to ensure specificity. β-actin served as a housekeeping/reference gene for normalization. Amplification profiles for PCR were optimized for primer sets. The amplification profile for D_5 receptor and α-actin used on the BIO-RAD CFX96 (Bio-Rad Laboratories) was 94 °C for

Table 1 Synthetic oligonucleotide primers for qRT-PCR

Primer	*Forward*	*Reverse*
D5	CTCCTGACTCTCCTAATC	TACGATGAAGATGTTGGT
β-actin	CCCTGAAGTACCCCATTGAA	CTTTTCACGGTTGGCCTTAG

Note: All sequences are from rat and listed as 5′–3′.

3 min followed by 40 cycles of 94 °C per 10 s and 60 °C per 40 s. Quantitative RT-PCR experiments were repeated for three times.[29]

Protein kinase C (PKC) activity assay

PKC activity was measured by using a PKC kinase activity assay Kit (Stressgen, Ann Arbor, MI, USA). The lysed cells were suspended in 50 μl of the kinase assay dilution buffer and loaded on 96-well plates coated with PKC substrate peptide. The PKC assay was initiated by the addition of 10 μl of ATP (diluted 1 mg ml^{-1}) to each well at 30 °C and assayed as per the manufacturer's instructions, measuring incorporation of phosphate into the substrate peptide at 50 min. The wells were then washed twice with antibody dilution buffer, and 40 μl of phosphospecific substrate antibodies were added to each well and incubated for 1 h. Each well was subsequently washed three times for 10 min with wash buffer and a 1 : 1000 dilution of anti-rabbit IgG horseradish peroxidase-conjugated antibody preparation in dilution buffer, and incubated for 30 min. The wells were washed three times, and 60 μl of tetramethylbenzidine substrate (Stressgen) was added and incubated in the wells for 45 min. The horseradish peroxidase reaction was quenched by addition of 20 μl of acid stop solution, and absorbance at 450 nm was measured. The reaction was found to be linear with protein concentrations between the range of 0 and 100 μg, and time periods between 15 and 90 min.[30,31]

Determination of the second messenger(s) involved in the regulation of D_3 on D_5 receptor expression in RPT cells from WKY rats

To determine the second messenger(s) involved in the regulation by D_3 of D_5 receptor expression in RPT cells from WKY rats, several inhibitors or agonists were used: PKC inhibitor (PKC inhibitor 19–31, 10^{-6} M), protein kinase A (PKA) inhibitor (PKA inhibitor 14–22, 10^{-6} M), PKC activator (phorbol 12-myristate 13-acetate, 10^{-7} M), PKA activator (Sp-cAMP-S, 10^{-7} M), calcium channel blocker (nicardipine, 10^{-6} M) and calcium channel agonist (BAY-K8644, 10^{-6} M).

PKC inhibitor 19–31, phorbol 12-myristate 13-acetate, Sp-cAMP-S, nicardipine and BAY-K8644 were purchased from Sigma; PKA inhibitor 14–22 was purchased from Calbiochem (Darmstadt, Germany).

Statistical analysis

The data are expressed as mean ± s.e.m. Comparison within groups was made by repeated measures analysis of variance with Duncan's test (or paired *t*-test when only two groups were compared); comparison among groups was made by factorial analysis of variance with Duncan's test. A value of $P<0.05$ was considered significant.

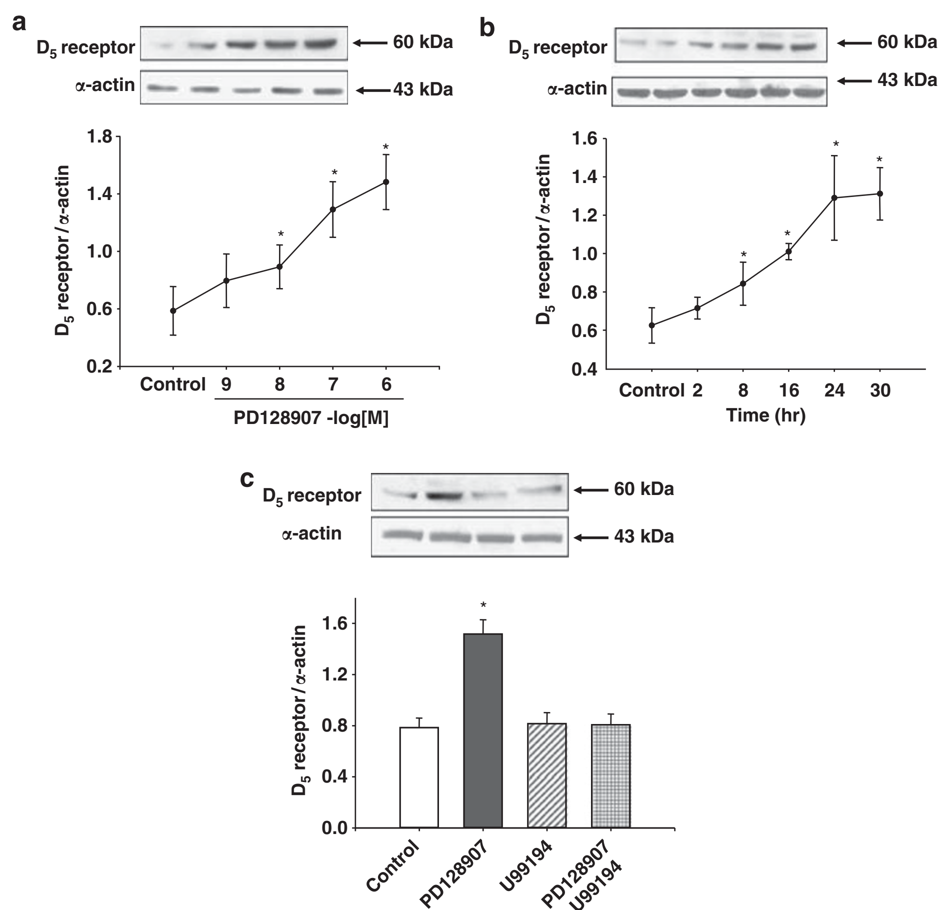

Figure 1 Effect of a D_3 receptor agonist, PD128907, on D_5 receptor expression in WKY RPT cells. (**a**) Concentration response of D_5 receptor protein expression in WKY cells treated with different concentrations of PD128907 for 24 h. Results are expressed as the ratio of D_5 receptor and α-actin densities (n=6, *$P<0.05$ *vs.* Control (C), ANOVA (analysis of variance), Duncan's test). (**b**) Time course of D_5 receptor protein expression in WKY cells treated with PD128907 (10^{-7} M). Results are expressed as the ratio of D_5 receptor and α-actin densities (n=4, *$P<0.05$ *vs.* Control (C), ANOVA, Duncan's test). (**c**) Effect of a D_3 receptor agonist (PD128907) and a D_3 receptor antagonist (U99194A) on D_5 receptor expression in WKY cells. The cells were incubated with the indicated reagents (PD128907, 10^{-7} M; U99194A, 10^{-6} M) for 24 h. Results are expressed as the ratio of D_5 receptor and α-actin densities (n=7, *$P<0.05$ *vs.* others, ANOVA, Duncan's test). A full color version of this figure is available at the *Hypertension Research* journal online.

RESUTLS

D_3 receptors increase D_5 receptor expression in RPT cells from WKY rats, but decrease it in SHRs

A D_3 receptor agonist, PD128907, increased D_5 receptor expression in a concentration- and time-dependent manner in WKY RPT cells. The stimulatory effect was evident at 10^{-8} M, noted as early as 8 h, and maintained for at least 30 h (Figures 1a and b).

The specificity of PD128907 as a D_3 receptor agonist was determined using the D_3 receptor antagonist, U99194A, in WKY RPT cells. Consistent with the results shown in Figures 1a and b, PD128907 (10^{-7} M per 24 h) increased D_5 receptor expression. U99194A (10^{-6} M), by itself, had no effect on D_5 receptor expression, but reversed the stimulatory effect of PD128907 on D_5 receptor expression (Figure 1c).

Opposite to the stimulatory effect of PD128907 on D_5 receptor expression in WKY cells, stimulation of D_3 receptor inhibited the D_5 receptor expression in SHR cells, which was also in a concentration- and time-dependent manner (Figures 2a and b), was also blocked by U99194A (Figure 2c).

To investigate the mechanisms of D_3 receptor upregulation of D_5 receptor expression in WKY cells, we checked the D_5 receptor protein degradation and mRNA levels after stimulation with D_3 receptor agonist. To study potential posttranscriptional mechanisms, we examined protein expression in the presence of 20 μg ml^{-1} cycloheximide to inhibit *de novo* protein synthesis.[32] At the indicated time, steady-state levels of D_5 receptor were determined by immunoblotting. In vehicle-treated WKY cells, D_5 receptor levels were rapidly decreased during the time course of 4 h. PD128907 treatment prevented cellular D_5 receptor protein depletion (Figure 3a). However, in SHR cells, D_3 receptor also inhibited the degradation of D_5 receptor, there were no significant difference between WKY and SHR cells (Figure 3b), indicating that the posttranscriptional mechanism was not involved into the differential regulation of D_3 receptor on D_5 receptor in WKY and SHR cells. Our further study showed that stimulation of D_3 receptor with PD128907 (10^{-7} M per 24 h) increases D_5 receptor mRNA expression (Figure 4).

PKC is involved into the regulation of D_3 receptor on D_5 receptor expression in WKY RPT cells

To investigate a mechanism for the D_3 receptor-induced upregulation of D_5 receptor, the RPT cells from WKY were treated with different agonists or antagonists. Treatment with the PKC inhibitor 19–31 (10^{-6} M), which by itself had no effect on D_5 receptor expression, blocked the stimulatory effect of D_3 receptor on D_5 receptor expression in RPT cells from WKY rats (Figures 5), indicating that PKC was involved in the signal transduction pathway activated by D_3 receptor. We also evaluated the involvement of other key cell signaling proteins with the use of a PKA inhibitor (PKA inhibitor

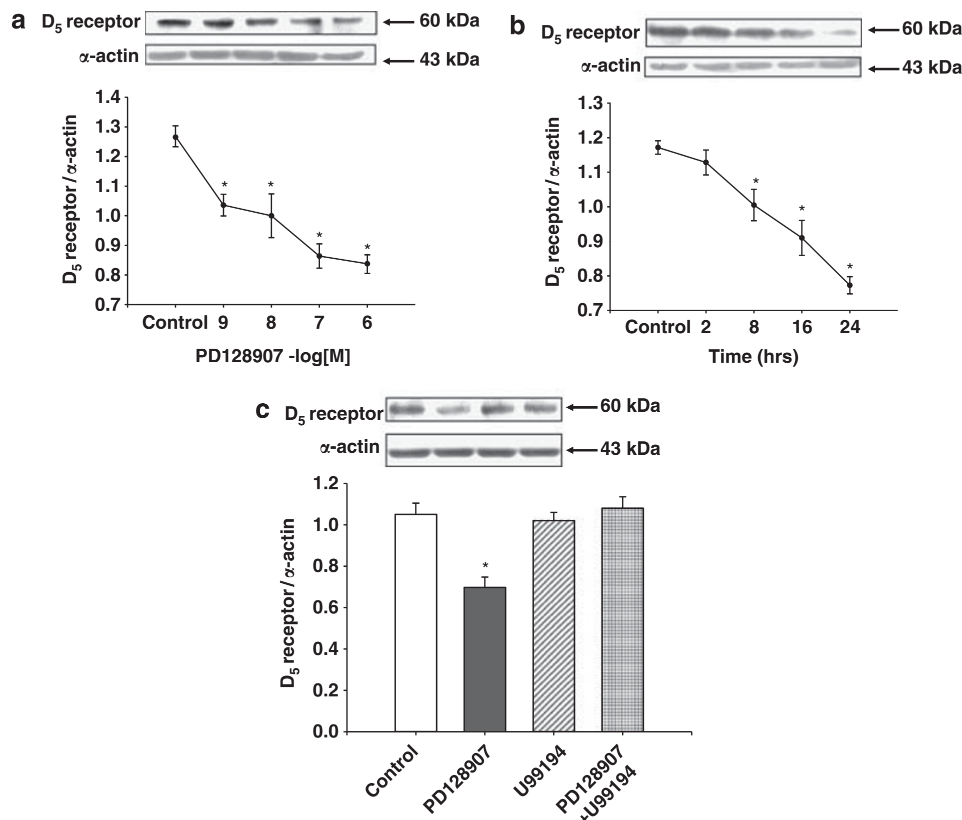

Figure 2 Effect of a D_3 receptor agonist, PD128907, on D_5 receptor expression in SHR RPT cells. (**a**) Concentration response of D_5 receptor protein expression in SHR cells treated with different concentrations of PD128907 for 24 h. Results are expressed as the ratio of D_5 receptor and α-actin densities (n=5, *P<0.05 *vs.* Control (C), ANOVA, Duncan's test). (**b**) Time course of D_5 receptor protein expression in SHR cells treated with PD128907 (10^{-7} M). Results are expressed as the ratio of D_5 receptor and α-actin densities (n=6, *P<0.05 *vs.* Control (C), ANOVA, Duncan's test). (**c**) Effect of a D_3 receptor agonist (PD128907) and a D_3 receptor antagonist (U99194A) on D_5 receptor expression in SHR cells. The cells were incubated with the indicated reagents (PD128907, 10^{-7} M; U99194A, 10^{-6} M) for 24 h. Results are expressed as the ratio of D_5 receptor and α-actin densities (n=7, *P<0.05 *vs.* others, ANOVA, Duncan's test). A full color version of this figure is available at the *Hypertension Research* journal online.

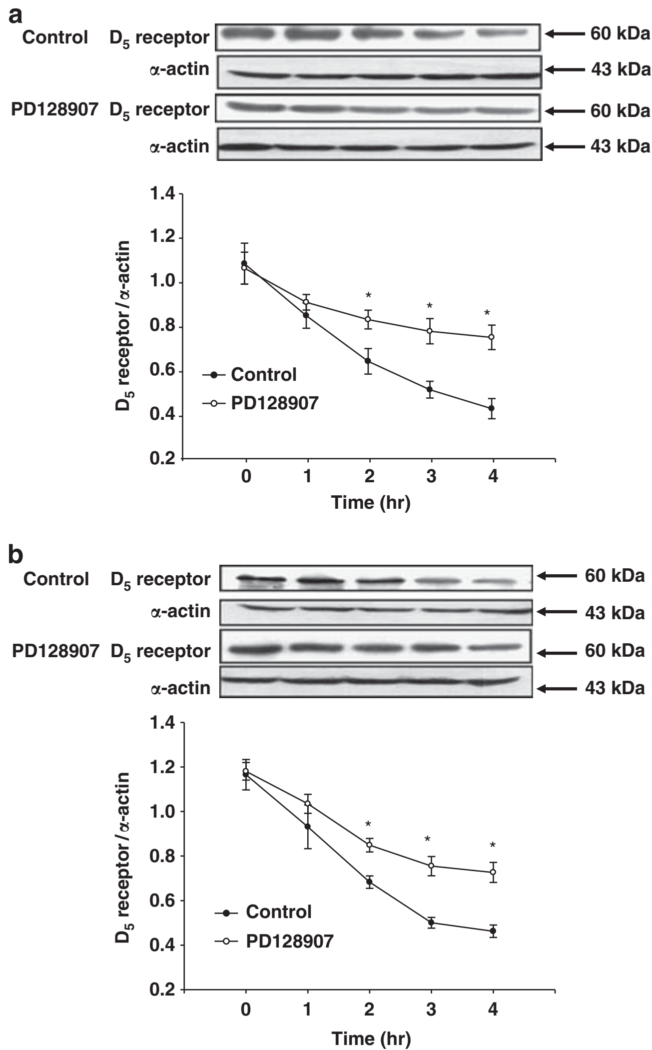

Figure 3 Effect of D_3 receptor on D_5 receptor protein degradation in RPT cells. WKY cells (**a**) or SHR cells (**b**) were pretreated with the D_3 receptor agonist, PD128907 (10^{-7} M) or vehicle for indicated times in the presence of cycloheximide (20 μg ml^{-1}), an inhibitor of *de-novo* protein synthesis. After the indicated time periods, D_5 receptor protein expression was analyzed by immunoblotting. Results are expressed as the ratio of D_5 receptor and α-actin densities (n=5, *$P<0.05$ *vs.* control, ANOVA, Duncan's test).

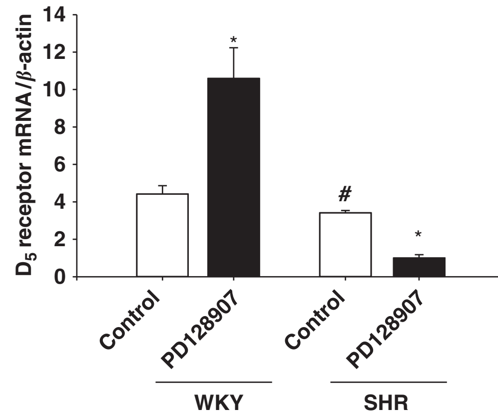

Figure 4 Effect of D_3 receptor on D_5 receptor mRNA expression in WKY and SHR cells. RPT cells from WKY or SHRs were treated with the D_3 receptor agonist, PD128907 (10^{-7} M) or vehicle for 24 h. The D_5 receptor mRNA expression was determined by RT-PCR. Results are expressed as the ratio of D_5 receptor and β-actin densities (n=7, *$P<0.05$ *vs.* control, $^{\#}P<0.01$ *vs.* WKY control, ANOVA, Duncan's test).

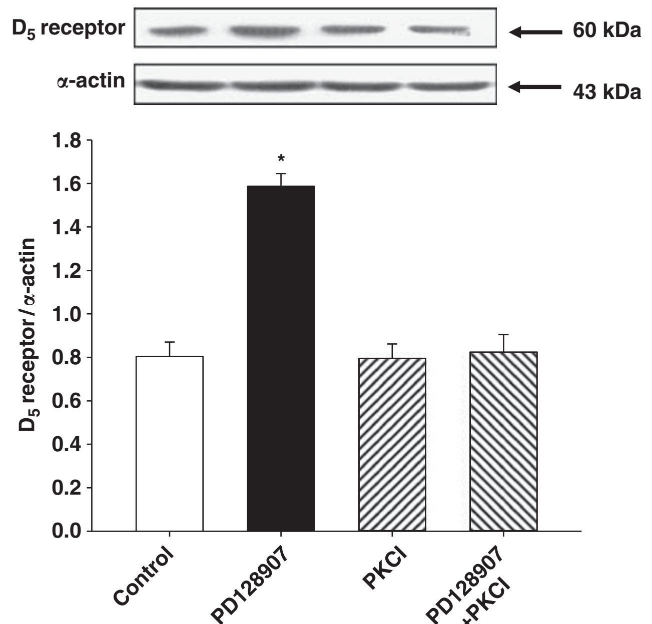

Figure 5 Role of PKC in the regulation of D_3 receptor on D_5 receptor expression in WKY RPT cells. The RPT cells were incubated with the indicated reagents (PD128907, 10^{-7} M; PKC inhibitor 19–31 (PKCI), 10^{-6} M) for 24 h. Results are expressed as the ratio of D_5 receptor to α-actin densities (n=9, *$P<0.05$ *vs.* others, ANOVA, Duncan's test). A full color version of this figure is available at the *Hypertension Research* journal online.

14–22, 10^{-6} M), PKC activator (phorbol 12-myristate 13-acetate, 10^{-7} M), PKA activator (Sp-cAMP-S, 10^{-7} M), calcium channel blocker (nicardipine, 10^{-6} M) and calcium channel agonist (BAY-K8644, 10^{-6} M). None of these reagents was able to block the stimulatory effect of D_3 receptor on D_5 receptor expression (data not shown).

Our further experiment also found that, consistent with the results in Figure 5, stimulation of RPT cells with D_3 receptor agonist, PD128907 (10^{-7} M per 15 min), increased PKC activity in WKY cells, which was partially blocked by D_3 receptor antagonist, U99194A (10^{-6} M per 15 min) (Figure 6).

Pretreatment with PD128907 increases the D_5 receptor-mediated inhibitory effect on Na^+–K^+ ATPase activity in WKY RPT cells, not in SHR cells

To investigate the physiological significance of D_3/D_5 receptor interaction, we used antisense oligonucleotides to reduce the expression of either D_1 receptor in both WKY and SHR cells. RPT cells were incubated with D_1 receptor antisense or sense propyne/phosphorothioate oligonucleotides for 48 h, and D_1 receptor was quantified by RT-PCR. D_1 receptor expression was decreased by the D_1 receptor antisense but not by sense oligonucleotides in WKY and SHR cells; the inhibition of D_1 receptor antisense on D_1 receptor mRNA expression had no significant difference (Figures 7a and b).

To investigate the physiological significance of D_3/D_5 receptor interaction, the effect of D_3 and/or D_5 receptors on Na^+–K^+

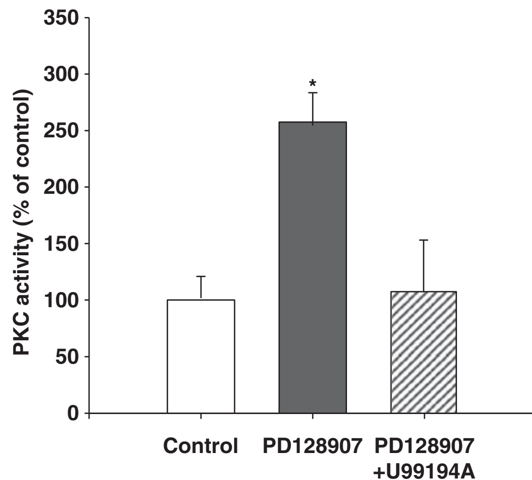

Figure 6 Effect of D_3 receptor on PKC activity in WKY RPT cells. The RPT cells were incubated with the indicated reagents (PD128907, 10^{-7} M; U99194A, 10^{-6} M) for 15 min. Results are expressed as the percent of control (n=13, *$P<0.05$ *vs.* others, ANOVA, Duncan's test). A full color version of this figure is available at the *Hypertension Research* journal online.

ATPase activity was determined in WKY and SHR cells after inhibition of D_1 receptor expression by D_1 receptor antisense, as observed in Figure 7a. Stimulation of D_5 receptors by fenoldopam (10^{-7} M per 15 min) decreased Na^+–K^+ ATPase activity in WKY cells, which could be blocked by the D_1-like receptor antagonist, SCH23390 (10^{-7} M per 15 min) (Figures 8a and b). Pretreatment with PD128907 (10^{-7} M) for 24 h augmented the inhibitory effect of fenoldopam (10^{-7} M per 15 min) on Na^+–K^+ ATPase activity in WKY cells, but decreased it in SHR cells (Figure 8c), which was consistent with the results in Figures 1 and 2, because stimulation of D_3 receptor increased D_5 receptor expression in WKY cells, but decreased in SHR cells.

D_3 receptors increase D_5 receptor expression and function in D_5 receptor-transfected HEK293 cells

Before transfection, we determine the D_3 and D_1 receptor expressions in HEK293 cells, we found specific D_3 receptor ($\sim$45 kDa) bands in D_5-HEK293 cells and WKY cells (as positive control) (Figure 9a); the 45-kDa band was no longer visible when the antibodies were pre-adsorbed with the immunizing peptide. The D_3 receptor mRNA, not D_1 receptor mRNA, was also found in HEK293 cells, determined by RT-PCR (Figures 9b and c).

Consistent with the results in WKY cells, a D_3 receptor agonist, PD128907 (10^{-7} M per 24 h), increased D_5 receptor expression in D_5 receptor-transfected HEK293 cells (control=0.8 ± 0.06, PD128907=1.5 ± 0.1 DU; n=4). Stimulation of D_5 receptors by fenoldopam (10^{-7} M per 15 min) decreased Na^+–K^+ ATPase activity, which could be blocked by the D_1-like receptor antagonist, SCH23390 (10^{-7} M per 15 min) (control=0.41 ± 0.02, fenoldopam=0.31 ± 0.016; SCH23390=0.42 ± 0.032, fenoldopam+SCH23390=0.41 ± 0.025 μmol phosphate released per mg protein per hour; n=6, $P<0.05$). Pretreatment with PD128907 (10^{-7} M) for 24 h augmented the inhibitory effect of fenoldopam (10^{-7} M per 15 min) on Na^+–K^+ ATPase activity in D_5 receptor-transfected HEK293 cells (Figure 10). The cells prereated with PD128907 for 24 h were washed three times (15 min per wash) with serum-free culture medium to remove all the added PD128907, kept in serum-free culture medium for 2 h and then treated with vehicle or fenoldopam for 15 min.

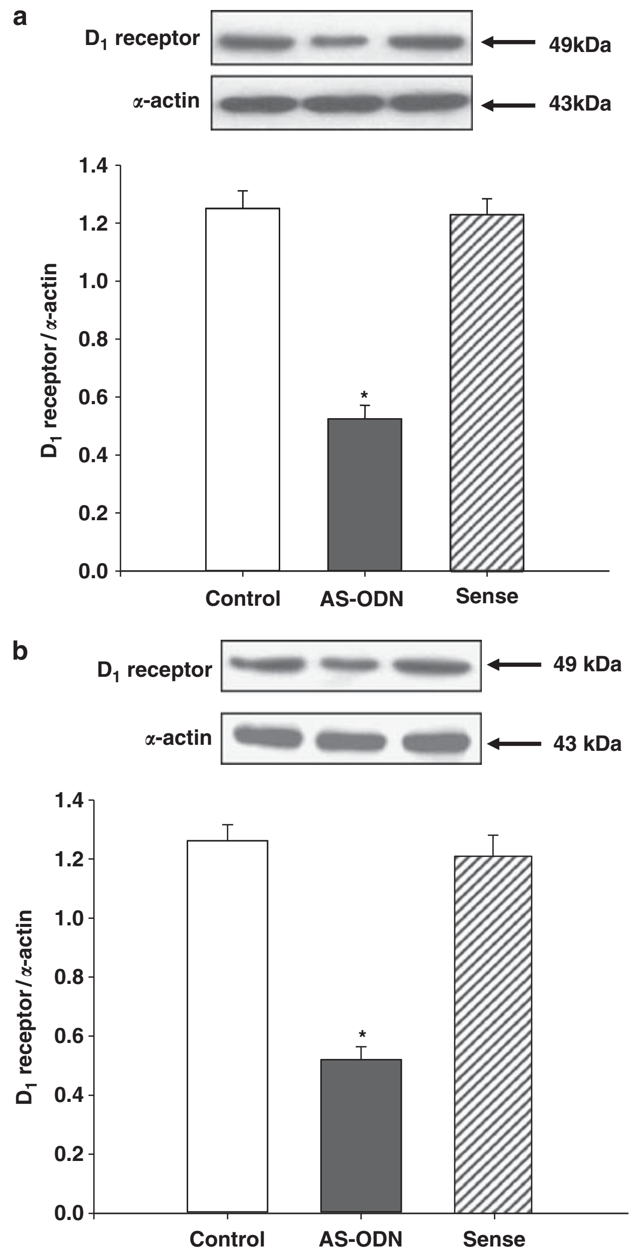

Figure 7 Effect of D_1 receptor antisense oligodeoxynucleotide on D_1 receptor expression in WKY (**a**) and SHR (**b**) RPT cells. 50 nM antisense (AS-ODN) or sense were mixed with 6 μl of oligofectamine in Optimem medium (Invitrogen Life Technologies) and incubated for 24 h, then switched to growth medium and incubated for another 24 h. The cells were collected and processed for immunoblotting for the D_1 receptor. Results are expressed as the ratio of D_1 receptor and β-actin densities (n=6-8, *$P<0.05$ *vs.* others, ANOVA, Duncan's test). A full color version of this figure is available at the *Hypertension Research* journal online.

DISCUSSION

D_1-like receptors induce diuresis and natriuresis in WKY rats.[7,8] Owing to the lack of selective D_1 and D_5 receptor agonists or antagonists,[33] the relative contribution of D_1 and D_5 receptors to the natriuretic effect caused by D_1-like receptor stimulation is not known. We have presumed that both D_1 and D_5 receptors are involved because both receptors increase cyclic adenosine monophosohate production and cyclic adenosine monophosohate mediates the D_1-like receptor-mediated inhibition of ion transport.[33]

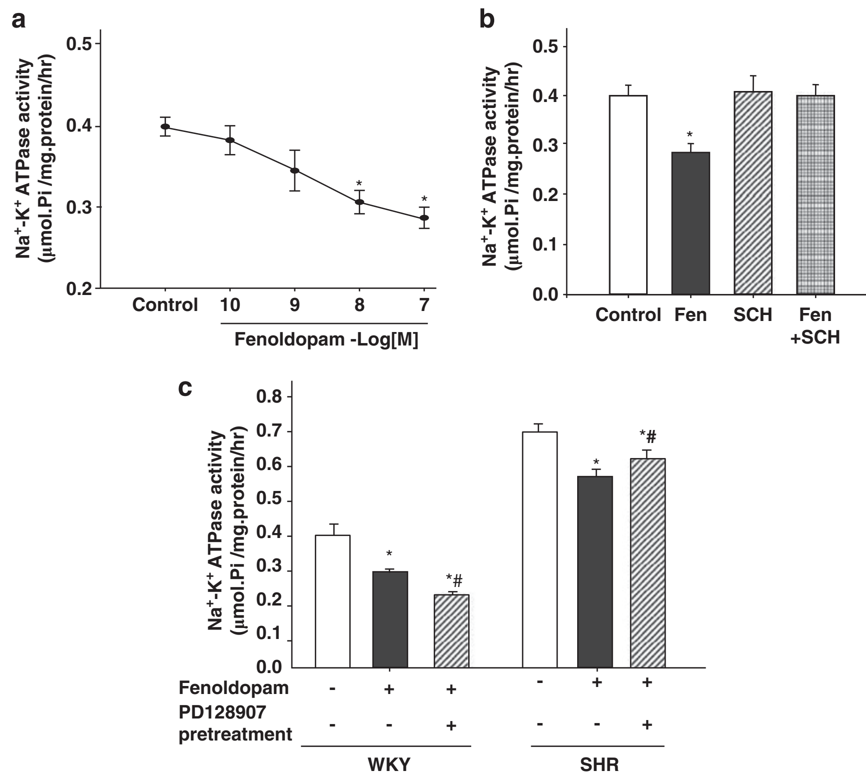

Figure 8 Effect of D_5 or D_3 receptor on Na^+–K^+ ATPase activity in RPT cells incubated with D_1 receptor antisense. (**a**) Effect of D_5 receptor on Na^+–K^+ ATPase activity in WKY RPT cells. RPT cells were treated with fenoldopam (10^{-10} M–10^{-7} M) for 15 min. Results are expressed as μmol phosphate released per mg protein per hour (*$P<0.05$ *vs.* control, $n=6$, ANOVA, Duncan's test). (**b**) Effect of a D_1-like receptor agonist (fenoldopam) and a D_1-like receptor antagonist (SCH23390) on Na^+–K^+ ATPase activity in WKY RPT cells. The cells were incubated with the indicated reagents (fenoldopam, 10^{-7} M; SCH23390, 10^{-7} M) for 15 min. Results are expressed as μmol phosphate released per mg protein per hour ($n=6$, *$P<0.05$ *vs.* others, ANOVA, Duncan's test). (**c**) Effect of pretreatment with a D_3 receptor agonist PD128907 on the inhibitory effect of the D_5 receptor on Na^+–K^+ ATPase activity in WKY and SHR RPT cells. The cells were pretreated with PD128907 (10^{-7} M) or vehicle (dH_2O) for 24 h. After PD128907 pretreatment, the cells were washed three times (15 min per wash) with serum-free culture medium to remove all the added PD128907, kept in serum-free culture medium for 2 h and then treated with fenoldopam (10^{-7} M) for 15 min. Results are expressed as μmol phosphate released per mg protein per hour (*$P<0.05$ *vs.* control, $^{\#}P<0.05$ *vs.* fenoldopam, $n=10$, ANOVA, Duncan's test). A full color version of this figure is available at the *Hypertension Research* journal online.

The D_5 receptor has generated significant interest because of its relatively high affinity for dopamine compared with the other dopamine receptors.[34] Moreover, the D_5 receptor can be activated in the absence or presence of low concentrations of endogenous agonist. In D_5 receptor null ($D_5^{-/-}$) mice, a high salt diet further increases blood pressure, suggesting that the renal D_5 receptor has an important role in the control of blood pressure by regulating renal sodium chloride transport.[28,35] In the present study, we found that activation of D_5 receptor inhibits Na^+–K^+ ATPase activity in D_5 receptor-transfected HEK293 cells.

As aforementioned, there is a synergistic effect between the D_1-like and the D_2-like receptors in the regulation of renal function. In normotensive rats, stimulation of renal D_1- and D_2-like receptors produces natriuresis that is greater than that observed with D_1-like receptors alone.[13,14] In concert with a D_1-like receptor agonist, a D_2-like receptor agonist acts synergistically to inhibit Na^+–K^+ ATPase and sodium–hydrogen exchanger activity in RPT cells and sodium–phosphate cotransporter activity in opossum kidney cells.[8–12] The underlying mechanisms were not completely understood. As one of major signals of dopamine receptor, stimulation of D_1-like receptor increases whereas D_2-like receptor decreases cyclic adenosine monophosohate production, which is impossible to explain the synergistical interaction between those two dopamine receptor sub-families. As the major D_2-like receptor subtype in RPT cells, D_3 receptor has direct or indirect with others. Our previous study found an interaction between D_1 and D_3 receptors in RPT cells from WKY rats; activation of either receptor increases each other's expression[16] whereas in the hypertensive states the stimulatory effect of D_1 receptor on D_3 receptor is lost. Besides of the interaction between D_3 and D_1 receptor, D_3 receptor also could regulate the other D_1-like receptor (D_5). In the present study, we found that activation of the D_3 receptor increases D_5 receptor expression in WKY cells. In contrast, in SHR cells not only is the stimulatory effect of D_3 receptor on D_5 receptor lost but also an inhibitory effect is actually observed. The effect of D_3 receptor on D_5 receptor is functionally relevant; pretreatment with D_3 receptor for 24 h enhances the inhibitory effect of fenoldopam on Na^+–K^+ ATPase activity in WKY cells, but decreases it in SHR cells. It is possible that a desensitized D_3 receptor is partly responsible for the lower basal levels of D_5 receptors in SHR cells than in WKY cells; the D_3 receptor is constitutively active.[36] The lower expression of D_5 receptors in SHR RPT cells,[23] and the aberrant interaction between D_3 and D_5 receptors may participate in the abnormal renal sodium handling in essential hypertension.

In summary, we have demonstrated that D_3 receptors regulate the expression and function of D_5 receptors in immortalized rat RPT cells. Altered regulation of D_3 receptor on D_5 receptors may have a role in the pathogenesis of hypertension.

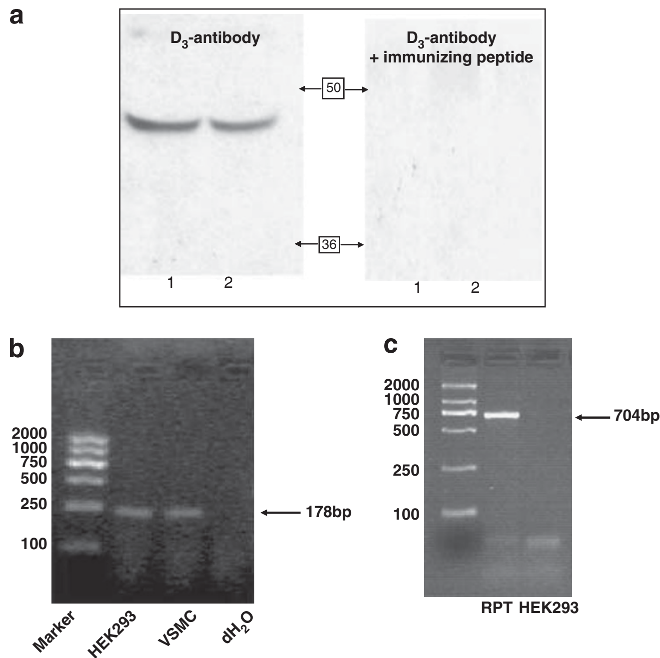

Figure 9 D_3 and D_1 receptor expressions in HEK293 cells. (**a**) Cell lysate proteins (100 μg) from HEK293 cells (lane 1) and WKY RPT cells (lane 2) were subjected to immunoblotting with anti-D_3 receptor antibody (1:250). In HEK293 cells and WKY cells, the 45 kDa band was no longer visible when the antibody was pre-adsorbed with the immunizing peptide (1:20 *w/w* incubation for 12 h). The molecular sizes are given. (**b**) D_3 receptor mRNA expression in HEK293 cells determined by RT-PCR. Vascular smooth muscle cells (VSMC) were taken as positive control and dH_2O without sample was taken as negative control. (**c**) D_1 receptor mRNA expression in HEK293 cells determined by RT-PCR. RPT cells from WKY rats was taken as positive control.

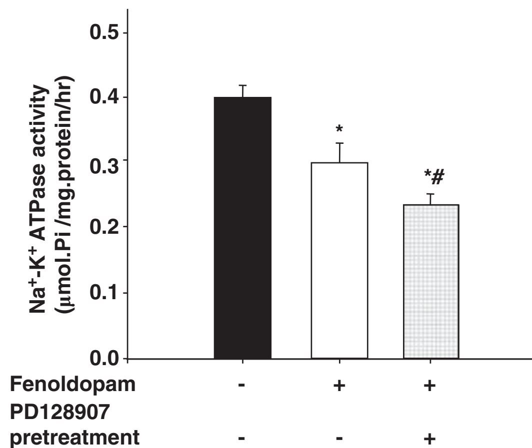

Figure 10 Effect of pretreatment with a D_3 receptor agonist, PD128907 on the inhibitory effect of the D_5 receptor on Na^+–K^+ ATPase activity in D_5 receptor-expressing HEK293 cells. The cells were pretreated with PD128907 (10^{-7} M) or vehicle (dH_2O) for 24 h. After washing for three times (15 min per wash) with serum-free culture medium to remove all the added PD128907, kept in serum-free culture medium for 2 h and then treated with the D_1-like receptor agonist, fenoldopam (10^{-7} M), for 15 min. Results are expressed as μmol phosphate released per mg protein per hour ($^*P<0.05$ *vs.* control, $^\#P<0.05$ *vs.* fenoldopam, $n=10$, ANOVA, Duncan's test).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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